The Periplasmic PDZ Domain–Containing Protein Prc Modulates Full Virulence, Envelops Stress Responses, and Directly Interacts with Dipeptidyl Peptidase of *Xanthomonas oryzae* pv. *oryzae*

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PDZ domain–containing proteases, also known as HtrA family proteases, play important roles in bacterial cells by modulating disease pathogenesis and cell-envelope stress responses. These proteases have diverse functions through proteolysis- and nonproteolysis-dependent modes. Here, we report that the genome of the causative agent of rice bacterial blight, *Xanthomonas oryzae* pv. *oryzae*, encodes seven PDZ domain–containing proteins. Systematic inactivation of their encoding genes revealed that *PXO_01122* and *PXO_04290* (*prc*) are involved in virulence. *prc* encodes a putative HtrA family protease that localizes in the bacterial periplasm. Mutation of *prc* also resulted in susceptibility to multiple environmental stresses, including H₂O₂, sodium dodecylsulfate, and osmolarity stresses. Comparative subproteomic analyses showed that the amounts of 34 periplasmic proteins were lower in the *prc* mutant than in wild-type. These proteins were associated with proteolysis, biosynthesis of macromolecules, carbohydrate or energy metabolism, signal transduction, and protein translocation or folding. We provide in vivo and in vitro evidence demonstrating that Prc stabilizes and directly binds to one of these proteins, DppP, a dipeptidyl peptidase contributing to full virulence. Taken together, our results suggest that Prc contributes to bacterial virulence by acting as a periplasmic modulator of cell-envelope stress responses.

When growing in free-living conditions or invading into host organisms, pathogenic bacteria are subjected to diverse physical or chemical stresses generated by environmental factors and hosts' immune responses. These stresses include changes in osmolarity, ion concentrations, pH, temperature, oxidants, and antimicrobial chemicals (Coleman et al. 2006). Such adverse conditions cause various types of cellular damage that threaten bacterial survival. Among them, stress-elicited

protein misfolding, mislocating, and fragmenting are fatal injuries, because they directly interfere with normal cellular structure and physiological processes (Ehrmann and Clausen 2004). To maintain cellular homeostasis, bacteria are equipped with molecular machineries to control protein quality, such as chaperones and folding catalysts that refold and relocate malfunctioning proteins (Miot and Betton 2004). In addition, proteins that will not be reused are degraded by proteolytic enzymes to prevent their harmful accumulation. In gram-negative bacteria, the degradation usually occurs within the periplasmic space that contains various proteases; therefore, periplasmic space is a critical place for protein quality control and signaling processes during bacterial pathogenesis (Merdanovic et al. 2011).

Among the periplasmic proteases, serine proteases that contain a PDZ (postsynaptic density of 95 kDa discs large and zonula occludens 1) domain, also known as HtrA (high temperature requirement A) family proteases, play important roles during envelope stress responses and bacterial pathogenesis (Clausen et al. 2002). All of these proteins have one or more PDZ domains with a peptidase domain. Besides degrading misfolded, mislocated, and fragmented proteins (Clausen et al. 2011), this type of protease has regulatory functions (Iwanczyk et al. 2007; Kalyoncu et al. 2010; Lee et al. 2008). DegP, the first HtrA protease studied in *Escherichia coli*, is a well-defined example of this multifunctionality (Ades 2008): DegP binds to hydrophobic C-termini of mislocated or misfolded protein aggregates with its PDZ domain and cuts the substrates by a two-step degradation process, yielding products with defined sizes (Krojer et al. 2008). In addition, in *E. coli* grown under stress conditions, DegP captures and degrades a periplasmic adaptor, CpxP, together with the misfolded PapG and PapE proteins (MacRitchie et al. 2008) and, then, activates the CpxR-CpxA two-component signal transduction system that controls the bacterial stress response (Isaac et al. 2005). Third, DegP acts as a chaperone to assist in refolding the periplasmic α -amylase MalS and prevent its aggregation (Iwanczyk et al. 2007; Spiess et al. 1999). All of these functions are associated with the cell-envelope stresses and bacterial survival within the host (Huston 2010; Ingmer and Brondsted 2009; Meltzer et al. 2009). PDZ domain–containing proteins (PDZ proteins) have also been identified in plants and animals, in which they control diverse physiological processes, such as photosystem assembly (Huesgen et al. 2005), tumor suppression (Pang et al. 2009),

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stress responses of human fibroblasts (Gray et al. 2000), and apoptosis (Galande et al. 2001). Therefore, this kind of protein is among the most important signaling factors in both prokaryotic and eukaryotic cells.

Almost all bacteria belonging to the genus *Xanthomonas* are plant pathogens. These opportunistic pathogens are gram-negative, rod-shaped, and aerobic. After successful infection, the bacteria cause disease symptoms such as wilting, necrosis, cankers, spots, and blight in plant leaves, stems, and fruits, resulting in serious yield losses. In recent years, along with the rapid development of functional and comparative genomics, remarkable progress has been made in identifying novel virulence factors of *Xanthomonas* spp. and their regulators (Ryan et al. 2011). These include type III secretion system-dependent effectors (Song and Yang 2010), diverse pathogen-associated molecular patterns (Rong et al. 2010), two-component signal transduction systems (Wang et al. 2010), and regulatory RNAs (Vorholter 2013). However, there has been less investigation into how these bacteria survive under stress conditions in the environment and in the host. Such studies could undoubtedly provide insights into bacterial pathogenesis (Subramoni and Sonti 2005; Yang et al. 2007). To our knowledge, PDZ protein-mediated signal transduction and stress responses have never been studied in phytopathogenic bacteria.

The aim of this study was to investigate the role of PDZ proteins in the virulence of *X. oryzae* pv. *oryzae* PXO99, the causal agent of bacterial blight of rice. We systematically inactivated all seven genes encoding putative PDZ proteins. Plant inoculation revealed that the mutation of two genes, *PXO_01122* and *PXO_04290* (*prc*), attenuated the virulence of the bacterium. Subsequent genetic and biochemical analyses showed that Prc localizes in the bacterial periplasm and that it is a putative serine protease belonging to the HtrA family. Our experiments also showed that Prc contributes to multiple cell-envelope stress responses in *X. oryzae* pv. *oryzae*. Comparative subproteomic analysis on the periplasmic proteins that constitute subproteomes of bacterial strains, based on two-dimensional electrophoresis (2-DE) together with mass spectrometry techniques, revealed 34 periplasmic proteins whose abundance levels were decreased in the absence of Prc. Among them, Prc physically interacts with and stabilizes a virulence-associated dipeptidyl peptidase, DppP, during salt stress treatment. Identification of the virulence-associated PDZ proteins in *X. oryzae* pv. *oryzae* could advance research on their regulatory roles during bacterial pathogenesis.

RESULTS

X. oryzae pv. *oryzae* PXO99 encodes seven PDZ proteins.

To determine how many PDZ proteins are present in *X. oryzae* pv. *oryzae* PXO99, the whole putative proteome of this bacterium was used to search the Pfam database using the HMMER2 algorithm (Finn et al. 2010), in which the cutoff E value was set to 1.0. The search retrieved seven proteins: PXO_00005, PXO_01122, PXO_01202, PXO_01713, PXO_03424, PXO_03547, and PXO_04290. These proteins included well-defined PDZ domains (E values range from 1.3×10^{-13} to 0.18×10^{-13}), implying that *X. oryzae* pv. *oryzae* PXO99 contains seven PDZ proteins. In addition, PXO_01316 contained a low-matched PDZ domain (E value = 0.96). We included this protein in the present study, even though it is likely to be a short chain dehydrogenase rather than a PDZ protein. The general properties and the predicted domain organizations of the eight putative proteins are shown in Figure 1. Among them, PXO_01122, PXO_01713, and PXO_03424 each contained tandem, C-terminal PDZ domains. Six of the PDZ proteins contained peptidase domains (Fig. 1), indicating that they are HtrA family proteases involved in proteolysis. PXO_01202 was initially annotated as a serine protease (Salzberg et al. 2008), but it is a short protein (125 aa) with no other motifs except the PDZ domain, suggesting that it is not a protease.

Attenuation of bacterial virulence by inactivation of *prc* and *PXO_01122*.

To investigate the biological functions of PDZ protein-encoding genes, especially their roles in bacterial virulence and the cell-envelope stress response, we used an insertional inactivation method to knock out each of the eight genes (including *PXO_01316*) in the wild-type (WT) strain of *X. oryzae* pv. *oryzae* PXO99 by pK18mob suicide vector integration. We successfully constructed insertional mutants for all eight genes, and verified their mutations by Southern blotting in which the correct mutant contains two incomplete copies of the targeted gene (Supplementary Fig. S1). These mutants were then inoculated onto 8-week-old plants of a susceptible rice cultivar (*Oryza sativa* cv. IR24), with the WT strain (PXO99) as the positive control and sterile water as the negative control (Fig. 2A; Supplementary Fig. S2A). Among these insertional mutants, the virulence levels of mutants DLT1122 and DLT4290 were significantly lower than that of the WT strain. The average lesion length after infection with DLT1122

Fig. 1. Schematic view of domain organizations of PDZ domain-containing proteins encoded by *Xanthomonas oryzae* pv. *oryzae* PXO99. Secondary structures of proteins were predicted by searching the Pfam 26.0 database (the cut-off E value ≤ 1.0). TPR_3 = tetratricopeptide repeat (Pfam accession number PF07720); peptidase_M61 = M61 glycyl aminopeptidase family (PF05299); peptidase_M50 = peptidase family M50 (PF02163); PDZ = PDZ domain (also known as DHR or GLGF domain, PF13180); PDZ-L = low-matched PDZ domain; adh_short = short-chain dehydrogenase domain (Pfam accession number PF00106); trypsin = trypsin domain (PF00089); peptidase_S41 = peptidase family S41 (PF03572); DUF = domain of unknown function 3340 (PF11818). Length of each domain is shown above.

and DLT4290 was 16.8 ± 2.60 and 20.3 ± 4.90 cm, respectively, significantly less than that of the WT strain (32.4 ± 2.40 cm, $P < 0.05$). This result indicated that these two genes were required for the full virulence of *X. oryzae* pv. *oryzae* (Fig. 2A). The production of extracellular enzymes, including amylase and cellulase, was also compared between these mutants and the WT strain, but there were no significant differences (Supplementary Fig. S3A and B), suggesting that PDZ proteins are not associated with the activity of these virulence-associated extracellular enzymes. No remarkable difference was detected when these mutants grew in the rich PS medium (10 g of peptone, 10 g of sucrose, and 1 g of glutamic acid per liter of H₂O, pH 7.0). In the minimal medium MMX (minimal medium for *X. campestris*), although DLT0005, DLT1122, DLT1202, DLT1713, and DLT3424 exhibited decreased growth rates as compared with the WT strain, none of them failed to grow, suggesting inactivation of these genes did not lead to auxotrophy.

Next, we focused on the function of *prc* (*PXO_04290*), because our previous study had identified a Tn5 transposon insertion in its ortholog (*XC_0714*) in *X. campestris* pv. *campestris* 8004 that resulted in a remarkable attenuation of virulence (Qian et al. 2005). This gene appears to be conserved and important in the virulence of *Xanthomonas* spp., even though its role in virulence remains unclear. The *prc* gene encodes a

putative ATP-independent, tail-specific serine protease. It consists of three domains (Fig. 1): an N-terminal PDZ domain (230 to 314 aa), a central peptidase domain (358 to 541 aa) with putative serine protease activity, and a C-terminal, function-unknown DUF3340 domain (543 to 698 aa). A possible cleavage position of a signal peptide was predicted to be located between 20 and 21 aa, and the first 18 aa of the N-terminus was predicted to be a transmembrane helix. A BlastP search revealed that Prc of *X. oryzae* pv. *oryzae* is significantly similar to the Prc protease (also known as TSP) of *E. coli* and *Pseudomonas aeruginosa*, with positive values of 55 and 59% (E value $< 1 \times 10^{-30}$), respectively. Prc of *E. coli* processes the carboxy-terminal region of penicillin-binding protein 3, which is a lipoprotein with peptidoglycan-synthesizing activity (Hara et al. 1991). In addition, Prc protease of *E. coli* also degrades incomplete polypeptide chains specifically tagged with SsrA RNA (Keiler et al. 1996).

Since *prc* was predicted to be a monocistronic gene by VIMSS (Huang et al. 2011), we constructed a genetic complementary strain (C4290) in which the full-length *prc* sequence was under the control of a *plac* promoter and provided in trans by broad-host, recombinant vector pHM1. A virulence assay showed that the pathogenic phenotype of C4290 was partially restored (Fig. 2B). The average lesion length after C4290 infection increased to 25.6 ± 3.40 cm, significantly longer than that

Fig. 2. Virulence assay of bacterial strains of *Xanthomonas oryzae* pv. *oryzae* PXO99. **A**, Average lesion lengths in rice plants inoculated with insertional mutants of PDZ protein-encoding genes. **B**, Genetic complementation partially restored the virulence of *prc* mutants. CK = DLT4290 containing a pHM1 vector. **C**, In planta growth assay of bacterial strains. Bacterial strains were inoculated onto rice leaves at approximately 10^4 CFU/ml. Leaves were harvested at indicated times after inoculation. Each data point is the average bacterial population from six inoculated leaves. **D**, Inactivation of *dppP* (*PXO_03743*) attenuated bacterial virulence. CK = DLT3743 containing a pHM1 vector. In A, B, and D, susceptible rice (*Oryza sativa*) cultivar IR24 was used as host. Lesion lengths ($n > 24$) were measured at 14 days after inoculation. Vertical bars show standard deviation. Asterisks indicate significant differences compared with the wild-type (WT) strain (Student's *t*-test, $P < 0.05$). WT strain *X. oryzae* pv. *oryzae* PXO99 was used as positive control, and sterile water was used as negative control. In A through D, vertical bars represent standard deviations.

of the DLT4290 mutant (22.8 ± 4.25 cm, $P < 0.05$). This confirmed the role of *prc* in *X. oryzae* pv. *oryzae* full virulence. In addition, an in planta growth assay showed that inactivation of *prc* resulted in impaired bacterial growth (approximately 30- to 300-times lower than that of WT from 9 to 15 days after inoculation [DAI]). Although genetic complementation partially restored the growth of the DLT4290 mutant before 9 DAI, for an unknown reason, the bacterial population of the complemented strain was similar to that of the mutants from 12 to 15 DAI (Fig. 2C).

Sensitivity of the *prc* mutant to multiple envelope stresses.

Growth deficiency in response to multiple stresses is one possible reason for virulence attenuation by mutants of PDZ domain-encoding genes (Walsh et al. 2003). To investigate whether *prc* of *X. oryzae* pv. *oryzae* is responsible for stress responses, we conducted several envelope stress assays after determining the appropriate conditions by preliminary testing. When bacterial strains were grown on PS plates, the growth of the WT strain and the DLT4290 mutant were similar (Fig. 3A). However, on PS plates containing 428 mM NaCl, the growth of mutant DLT4290 was completely arrested (Fig. 3B). This growth deficiency was restored by genetic complementation (Fig. 3B). A growth-curve assay confirmed that the growth of DLT4290 under high salt-stress conditions was much slower than that of the WT strain (Fig. 3C and D). Genetic complementation partially restored this growth deficiency under salt stress (Fig. 3D). In addition, compared with the WT strain, growth of the DLT4290 mutant was strongly suppressed by 1% H_2O_2 and by 5% sodium dodecyl sulfate (SDS) (Fig. 3E

and F). The minimal inhibitory concentrations of these two chemicals for the DLT4290 mutant were 0.000625 and 0.00625%, respectively, as determined by the standard double-dilution method. These values were substantially lower than those for the WT strain (0.0025 and 0.0125%, respectively; Fig. 3E and F). Genetic complementation restored these deficiencies, and bacterial growths were similar to the WT strain level (Fig. 3E and F). Taken together, these results demonstrate that *prc* is required in bacterial responses to detergent, osmolarity, and superoxide stresses, so that it likely controls the general cell envelope stress responses.

Prc expression level changed along with bacterial growth and under stress treatments.

To investigate whether Prc expression was changed during bacterial growth and the stress response, initially, we measured its transcript levels by Northern blot analysis but failed to detect any positive signals (data not shown). Consequently, we used real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to determine its transcriptional level in the WT strain. The relative transcriptional level of *prc* gradually increased from 1.0 to 1.48 during bacterial growth (Fig. 4A). The increase was especially evident after the optical density at 600 nm (OD_{600}) value reached 0.6 (approximately 8×10^8 CFU/ml). To determine the amount of Prc protein, we constructed a recombinant strain (Prc-His₆) in which a His₆ epitope tag coding sequence was inserted in-frame at the 3' end of the *prc* gene and was introduced into the chromosome of *X. oryzae* pv. *oryzae* PXO99 by the double cross-over method. The growths of this strain were unaffected either in PS or in PS-

Fig. 3. Prc is required for multiple envelope stresses. **A**, Colony morphology of bacterial strains grown on PS (10 g of peptone, 10 g of sucrose, and 1 g of glutamic acid per liter of H_2O , pH 7.0) plates. Inoculating concentrations of bacterial cells (CFU/ml) are indicated. **B**, Colony morphology of bacterial strains grown on PS+NaCl (428 mM) plates. In both A and B, bacteria were cultured at 28°C for 72 h. CK = DLT4290 containing a pHM1 vector. **C**, Growth curves of bacterial strains in PS medium. **D**, Growth curves of bacterial strains in PS+NaCl medium. Each data point is the average of four samples. Vertical bars show standard deviation. **E**, Sensitivity of *prc* mutant to H_2O_2 and **F**, sodium dodecyl sulfate (SDS) stresses, respectively. H_2O_2 and SDS inhibition of bacterial growth. Filter paper disks ($\Phi = 0.6$ mm) containing 5 μ l 1% H_2O_2 or 5% SDS were used to suppress bacterial growth. Minimal inhibition concentration (MIC) values of H_2O_2 and SDS for different bacterial strains were determined by a standard serial-dilution method.

NaCl media (Supplementary Fig. S4). Western blotting also showed that the protein level of Prc along bacterial growth increased gradually (Fig. 4B). The expression kinetics of Prc was also evaluated when the bacterium was subjected to high-NaCl stress shock. After the NaCl stress treatment, the relative transcriptional level of *prc* in the WT strain slightly decreased from 1.0 to 0.7 (within 30 min) but then returned to the pre-stress treatment level by 60 min (Fig. 4C). Prc protein level of the Prc-His₆ strain decreased slightly after 10 min of NaCl treatment but also was restored at 60 min posttreatment (Fig. 4D), which is in accordance with the result of qRT-PCR (Fig. 4C). Collectively, these evidences indicated that, although both the mRNA and protein levels of Prc changed slightly in response to bacterial growth phases and stress treatment, the extent of the changes varies within a relatively narrow spectrum and it is not an on and off process.

Prc is a periplasmic protein and its truncated forms did not have endopeptidase activity in vitro.

Subcellular localizations of the seven PDZ proteins were predicted by pSORT-B (Gardy et al. 2003). The putative subcellular locations of PXO_01122, PXO_03547, and PXO_04290 were in the cytoplasmic membrane, whereas PXO_01713 was predicted to be a periplasmic protein. The theoretical localizations of the other four proteins (PXO_00005, PXO_01202, PXO_01316, and PXO_03424) were uncertain because of ambiguous prediction results. In *E. coli*, however, most of the proteins with similar domain compositions, including Prc (TSP) (Keiler et al. 1995), DegP (Bass et al. 1996), and DegQ (Waller and Sauer 1996), are periplasmic proteins. To experimentally confirm the subcellular localization of Prc, we again used a recombinant strain (Prc-His₆) in which Prc was tagged with a C-terminal His₆ epitope for determining its localization. Proteins from subcellular fractions of the periplasm, membrane, and cytoplasm were obtained by ultracentrifugation, and then, the Prc protein was detected by Western blot using His₆ monoclonal antibody. Histidine kinase (SreS) and HPPK (6-hydroxy-

methyl-7,8-dihydropterin pyrophosphokinase) were used as positive controls for determining the localizations of membrane-bound and cytoplasmic proteins, respectively. Alkaline phosphatase activity was assayed simultaneously and was used as an indicator for periplasmic proteins, because alkaline phos-

Fig. 5. Prc is localized in periplasmic space. **A**, Western blot analysis of Prc in different cellular fractions. Prc-His₆ = recombinant PXO99 strain containing a C-terminal His₆-tagged Prc. TCP = total cellular proteins. Anti-His₆ was used to detect the Prc-His₆ recombinant protein. Anti-SreS and anti-HPPK were used to detect membrane-bound histidine kinase SreS and cytoplasmic HPPK, respectively. The wild-type strain of *Xanthomonas oryzae* pv. *oryzae* PXO99 was used as negative control. **B**, Alkaline phosphatase activity of different cellular fractions of the *X. oryzae* pv. *oryzae* PXO99 Prc-His₆ strain. Equal amounts of protein (100 μg) were used to detect alkaline phosphatase activity, which serves as a marker for periplasmic proteins. Each data point is the average of triplicate analyses. Vertical bars show standard deviation.

Fig. 4. The expression of Prc responds to growth phases and NaCl stress treatment. **A**, Quantitative reverse transcription-polymerase chain reaction analysis (qRT-PCR) of *prc* transcript mRNA levels along with bacterial growth. Total RNA was isolated from the wild-type strain of *Xanthomonas oryzae* pv. *oryzae* PXO99 grown in PS medium (10 g of peptone, 10 g of sucrose, and 1 g of glutamic acid per liter of H₂O, pH 7.0). Samples were collected when the optical density at 600 nm reached the indicated values. cDNA amplified from transfer-messenger RNA as template was used as internal standard. Experiments were conducted in biological and technical triplicate. Results from one representative experiment are shown, and vertical bars show standard deviation. **B**, Protein levels of Prc during bacterial growth. The recombinant strain (Prc-His₆) producing C-terminal His₆-tagged Prc was used. Prc-His₆ levels were detected with Western blot using an anti-His₆ monoclonal antibody. The RNA polymerase αsubunit was detected by a polyclonal antiserum and used as loading control. The intensity of the band representing Prc-His₆ was measured by Image J software and is shown below the bands. Experiments were repeated three times, and results from one representative experiment were shown. **C**, qRT-PCR of *prc* transcript mRNA levels during the high-salt stress response, using total RNA from *X. oryzae* pv. *oryzae* PXO99 grown in PS medium containing 428 mM NaCl. Experiments were conducted as described in A and samples were collected at indicated times after stress treatment. **D**, Protein levels of Prc during the high salt-stress response were determined as described in B.

phosphatases are active only in this part of the gram-negative bacterial cell envelope (Michaelis et al. 1983). Prc-His₆ was located in the periplasmic fraction (Fig. 5A), and there was no positive signal in the membrane and cytoplasmic fractions. The alkaline phosphatase activity was strongest in the periplasmic fraction (Fig. 5B). This result supports the idea that Prc is a periplasmic protein.

To verify whether Prc has peptidase activity *in vitro*, we used *E. coli* and *Bacillus subtilis* expression systems to express the mature Prc protein without the N-terminal signal peptide sequence but repeated efforts failed. Eventually, we expressed and purified two truncated, C-terminal His₆-tagged Prc proteins, one lacking the 125-aa N-terminal sequence but containing intact PEP, PDZ, and DUF (domain of unknown function) domains (PrcΔ125) and the other containing intact PEP and PDZ domains (PEP + PDZ). In addition, we mutated the putative active site (S467A) of each of the two recombinant proteins and obtained their purified, soluble products. However, an endo-peptidase activity assay showed that all these recombinant

proteins cannot degrade β-casein or bovine serum albumin, and there is no enzymatic difference between the truncated protein and its point mutated form (Supplementary Fig. S5). This result suggests that, under our experimental conditions, these Prc recombinant proteins did not have peptidase activity *in vitro*.

Comparative subproteomic analysis between WT and the *prc* mutant.

Identification of physiological substrate for protease involved in cell signaling is a major challenge in the study area (Turk et al. 2012). Because Prc localizes in the bacterial periplasmic space, we expected that it would form complexes with protein substrates and affect subproteome in this cellular fraction. To compare the differences in periplasmic proteins between the *prc* mutant and the WT strain, bacterial strains were subjected to high-NaCl stress (428 mM) for 10 min, and their periplasmic proteins were extracted, were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and were detected by silver

Table 1. Periplasmic proteins differently expressed in the *prc* mutant as compared with the wild-type strain of *Xanthomonas oryzae* pv. *oryzae*

Spot ID	Protein code ^a	Annotation or description	Length (aa)	Score ^b	Theoretical ^c	Observed ^c	WT/DLT ratio ^d
Amino acid, lipid metabolism, and resistance to antibiotics							
407	PXO_00346	Argininosuccinate lyase	431	182	46.5	45.0	∞
254 ^e	PXO_00684	Alkaline phosphatase	630	126	67.4	68.0	∞
301	PXO_00727	Histidine ammonia-lyase	513	96	53.6	54.0	∞
465	PXO_02610	3-Isopropylmalate dehydrogenase	357	135	38.3	38.5	2.15
1059	PXO_03805	Acyl-CoA dehydrogenase	384	83	41.5	42.0	∞
341	PXO_04833	β-lactamase	529	541	58.4	56.0	∞
438 ^e	PXO_03494	Glycerophosphoryl diester phosphodiesterase	366	132	39.5	40.0	∞
Carbohydrate and energy metabolism							
353	PXO_00608	Glucose-6-phosphate 1-dehydrogenase	476	110	52.9	54.0	∞
1101	PXO_01402	Exopolysaccharide xanthan biosynthesis pyruvyltransferase GumL	264	34	89.5	29.0	13.23
439	PXO_02116	Phosphoglycerate kinase	391	41	40.9	42.0	∞
288	PXO_02430	Rhamnogalacturonase B	533	292	57.2	55.0	∞
482	PXO_03160	Sugar nucleotide epimerase/dehydratase	358	33	38.4	38.0	∞
463	PXO_03296	Alcohol dehydrogenase	352	193	38.2	40.0	∞
616	PXO_03749	Oxidoreductase	286	111	49.2	32.0	∞
Ribosome, protein biosynthesis, and refolding							
494	PXO_01131	Elongation factor Ts	292	245	31.2	37.5	∞
732	PXO_04532	50S ribosomal protein L10	177	88	38.3	20.0	∞
1027	PXO_03703	Chaperonin GroEL	546	44	57.2	57.0	∞
Proteolysis							
1068	PXO_00179	Serine carboxypeptidase	490	20	53.8	32.0	∞
487	PXO_01713	Periplasmic protease	528	40	54.2	40.0	∞
156	PXO_02761	Peptidyl-dipeptidase	722	143	80.1	73.0	∞
313	PXO_02856	Peptidase	575	85	62.1	53.0	∞
124 ^e	PXO_03743	Dipeptidyl peptidase	786	344	87.8	75.0	∞
154	PXO_04132	Prolyl oligopeptidase	697	207	77.2	73.5	2.83
742	PXO_04290	Tail-specific protease	719	125	79.2	21.0	∞
Signal transduction							
568	PXO_01872	Thioredoxin	285	140	31.2	35.0	∞
264	PXO_00993	Transcriptional regulator	494	43	54.5	56.0	2.37
Flagellar, lipoprotein, and secretion system							
409	PXO_04699	ImpA-like protein	357	80	39.4	47.0	∞
571	PXO_04718	Lipoprotein	282	305	31.5	32.0	4.19
417	PXO_06165	Flagellin	390	80	39.5	46.0	∞
Unknown function							
514	PXO_01147	Hypothetical protein	336	138	37.5	37.0	2.06
653	PXO_03477	Hypothetical protein	397	20	43.3	30.0	2.77
1083	PXO_04100	GatB/YqeY motif protein	148	88	16.0	18.0	∞
410	PXO_04309	Hypothetical protein	394	220	43.5	48.0	∞
273	PXO_04546	Hypothetical protein	551	200	60.4	58.0	∞

^a Names and codes of identified proteins are according to genomic annotation of *X. oryzae* pv. *oryzae* PXO99.

^b Scores are based on Mowse search. Scores greater than 49 are significant ($P < 0.05$).

^c Values represent molecular mass (kDa).

^d WT = wild type; DLT = insertional inactivation mutant.

^e Fragments of these proteins were identified from different sites of the gels.

staining. The general patterns of the DLT4290 mutant and *X. oryzae* pv. *oryzae* PXO99 were remarkably different (Supplementary Fig. S6A), and this difference was largely recovered in the complemented strain C4290.

To identify which periplasmic proteins associate with Prc, we performed a comparative subproteomic analysis based on 2-DE technique together with mass spectrometry to detect possible differences between strains. A total of 34 periplasmic proteins exhibited significant differences (>2.0-fold) between the DLT4290 mutant and the WT strain. Interestingly, all of them were downregulated in the *prc* mutant. These proteins were classified into seven categories based on their function (Table 1). Besides the categorized proteins, an alkaline protein, a lipoprotein, a response regulator belonging to the two-component signal transduction system, a flagellin, a type IV secretion system protein, and a GatB/YqeY protein were also identified. The Prc protein itself was identified from the protein sample from the WT strain, which verified that Prc is a bona fide periplasmic protein. Among these proteins, seven proteases or peptidases (including Prc itself) were revealed in the subproteomic analysis, suggesting that a cooperative relationship exists between them and Prc during regulation. Although most of the above-identified proteins were putatively located in periplasm, some are well-defined cytoplasmic proteins (e.g., the response regulator protein and ribosomal subunit L10). These proteins may have been mislocated into the periplasm during the high-salt treatment.

Since the proteomic differences may be caused by transcriptional, posttranscriptional, and translational level of regulations, we conducted semiquantitative RT-PCR analyses to determine if the downregulation of 15 selected coding genes was detectable at the transcriptional level. Among these genes, *PXO_01131* and *PXO_03743* showed slightly decreased transcript levels in the *prc* mutant under high salt-stress conditions

(Fig. 6A), and their transcript levels were restored in the complemented strain. Only the transcription level of *PXO_03494* was upregulated in the *prc* mutant, which was contrary to the result of 2-DE analysis, and the reason remains to be determined. Apart from the above three genes, the transcription levels of the other 12 genes were generally unaffected, suggesting their differences in protein amounts were caused by posttranscriptional or translational regulation.

In Table 1, a number of proteins modulating biosynthesis of exopolysaccharides (EPS) and flagellar assembly were identified, such as GumL (PXO_01402), G6PD (PXO_00608), and flagellin (PXO_06165), suggesting that *prc* mutation also affects the related phenotypes. The level of EPS production (Supplementary Fig. S7A) and the ability of flagella-dependent swimming motility of the *prc* mutant (3.47 ± 0.41 mg/ml and 1.4 ± 0.23 cm, respectively) were significantly lower than those of the WT strain (5.40 ± 0.16 mg/ml and 1.8 ± 0.22 cm, respectively, $P < 0.05$). These phenotypic alterations were suppressed by genetic complementation. The results suggest that Prc regulates bacterial EPS production and swimming motility by affecting the expression level of these proteins.

Prc directly interacts with virulence-associated PXO_03743 (DppP) and modulates its stability under stress condition.

Detailed analyses of the 2-DE results indicated that three proteins (PXO_00684, PXO_03743, and PXO_03494) were present in more than one region on the gel (Table 1). This might be caused by Prc-mediated proteolysis, autoprotoleolysis, protein modification, or artificial defects. To explore the possible interactions between these proteins and Prc, we constructed three pHM1 recombinant vectors in which a His₆ tag was fused in-frame with the C-terminal of the sequences of the above three genes. After transformation into the WT strain and the *prc* mu-

Fig. 6. Verification of protein expressions identified by two-dimensional electrophoresis analysis. **A**, Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses of the transcriptional levels of Prc-related periplasmic proteins. RT-PCR amplification was conducted using cDNAs synthesized from total RNA of bacterial strains. PCR cycles differed (22 to 35 cycles) depending on genes. Amplifications using bacterial DNA and cDNA of transfer-messenger RNA as templates were used as positive control and loading control, respectively. PS indicates bacterial strains grown in the PS medium (10 g of peptone, 10 g of sucrose, and 1 g of glutamic acid per liter of H₂O, pH 7.0), and PS + NaCl indicates high salt (428 mM NaCl, 10 min) treatment. The experiment was repeated three times. **B**, Inactivation of *prc* affects stability of DppP (PXO_03743) after salt-stress treatment. **C**, Inactivation of *prc* did not affect the amount of PXO_03494. In B and C, C-terminal His₆-tagged DppP (B) and PXO_03494 (C) was overexpressed in the wild-type (WT) strain (PXO99) and the *prc* mutant (DLT4290), respectively. After challenge with PS+NaCl medium, periplasmic proteins were extracted and DppP or PXO_03494 was detected by Western blot using anti-His₆. Periplasmic proteins from the WT strain and the *prc* mutant (no His₆-tagged Prc) were used as negative controls. Each lane contains 30 μg of periplasmic proteins. Sodium dodecyl sulfate-polyacrylamide electrophoresis gels containing equal amounts of proteins were stained with Coomassie bright blue (CBB) and were used as loading controls. Min indicates the time after stress treatment. Each experiment was repeated three times.

tant to overexpress these proteins, the periplasmic fraction was extracted from each cell line and the amounts of the His₆-tagged proteins were detected by Western blot. The protein amounts of periplasmic PXO_03743, which is a putative dipeptidyl peptidase, were stable in the WT strain under the stress condition (Fig. 6B). However, in the *prc* mutant, the level of PXO_03743 was remarkably lower than that of the WT strain before NaCl treatment (Fig. 6B, lane 6). In addition, this protein was unstable in the absence of Prc, showing two additional nearly 32-kDa bands representing its degraded fragments containing C-terminal His₆ tag (Fig. 6B, lanes 6 to 9). For PXO_03494 (a glycerophosphoryl diester phosphodiesterase), protein levels remained stable between the WT strain and *prc* mutant, regardless of NaCl treatment (Fig. 6C, lanes 2 to 9). For PXO_00684 (an alkaline phosphatase), no signal representing PXO_00684-His₆ was observed in either the genetic backgrounds of the WT strain or the *prc* mutant (data not shown). Collectively, these results indicate that the stability of PXO_03743, rather than its degradation, is subject to the control of Prc during bacterial stress response. Accordingly, we named PXO_03743 DppP (dipeptidyl peptidase depended on Prc).

To investigate whether Prc and DppP bind directly in vivo, we used coimmunoprecipitation (Co-IP) to check the possible interaction between them. Anti-His₆ monoclonal antibody was used to immunoprecipitate DppP-His₆ from the periplasmic proteins extracted from PXO99-OX3743H and DLT4290-OX3743H strains, in which DppP-His₆ was overexpressed as

mentioned above, and then a polyclonal antiserum of Prc was used to detect if there is coimmunoprecipitated Prc. This Co-IP assay confirmed that Prc binds to DppP in vivo before and after salt-stress treatment (Fig. 7A). In the absence of Prc (strain DLT4290-OX3743H), there is no Prc signal being detected. In addition, it seems that salt-stress stimulation decreased the Prc-DppP binding affinity, since the intensity of the signal was lower than that with no stimulation (Fig. 7A).

To determine the affinity of Prc-DppP interaction and their binding kinetics in vitro, the recombinant Prc (minus N-terminal 125 aa) and DppP (full length), respectively, were expressed in the *E. coli* BL21(DE3) strain with pET30a vectors (Fig. 7B). After purification by affinity chromatography, the proteins were over 95% pure; surface plasmon resonance (SPR) was then employed to quantify the real-time parameters of Prc-DppP interaction. Prc proteins were trapped on a sensor chip, CM5, by the amine coupling method and increasing concentrations (from 126.3 nM to 10 μ M) of DppP were injected to flow over Prc at a rate of 30 μ l/min for 4 min. Prc and DppP exhibited typical binding curves (Fig. 7C); at 25°C, Prc-DppP interaction displayed high affinity with a combination of a fast association rate and a slow dissociation rate. The observed equilibrium dissociation constant rate (K_D) between Prc-DppP interactions is 1.51 nM (Fig. 7C), and this strongly supports that Prc-DppP interacts in a high affinity manner in vitro.

DppP is a dipeptidyl peptidase that has an N-terminal DPP IV domain and a C-terminal peptidase domain. It was predicted to be localized in periplasmic or outer membrane by PSORT-B prediction. DppP contains a typical N-terminal signal peptide whose putative splicing site is between the 15th and 16th amino acid residues. However, the biological function of this protein was never experimentally studied. To this end, we inactivated *dppP* by vector integration and characterized the virulent capability of the mutant (DLT3743). As compared with the WT strain, the virulence level of *dppP* mutant decreased significantly (Fig. 2D). The average lesion length of this mutant was 13.0 ± 1.96 cm, lower than that of the WT strain (15.2 ± 1.71 cm, $P < 0.05$). Genetic complementation (strain C3743) restored this deficiency to the WT level (15.9 ± 2.11 , $P < 0.05$). This result suggests that *dppP* is also required for the full virulence of *X. oryzae* pv. *oryzae*.

DISCUSSION

PDZ proteins, especially HtrA family proteases, are associated with virulence of a number of bacterial pathogen. For example, inactivation of the HtrA-like proteins in *Salmonella typhimurium* (Johnson et al. 1991), *Listeria monocytogenes* (Wilson et al. 2006), *Bacillus anthracis* (Chitlaru et al. 2011), *Mycobacterium tuberculosis* (Mohamedmohaideen et al. 2008), *Helicobacter pylori* (Hoy et al. 2010), and *Burkholderia cenocepacia* (Flannagan et al. 2007) resulted in attenuation of virulence. Consequently, these proteins are ideal targets for development of novel antibiotics (White et al. 2010). In the present study, we predicted that the genome of the phytopathogenic bacterium *X. oryzae* pv. *oryzae* PXO99 encodes at least seven PDZ proteins. Among them, six proteins are predicted to be typical HtrA family proteases (Fig. 1). Using genetic mutagenesis, we found that inactivation of *PXO_01122* and *prc* (*PXO_04290*), both of which are HtrA family proteases, caused deficiencies in full bacterial virulence (Fig. 2A). In addition, the growth of the *prc* mutant decreased markedly in response to multiple cell-envelope stresses (Fig. 3). Further analyses revealed that Prc is localized in the periplasm (Fig. 5). Using a comparative subproteomic method, we found that 34 periplasmic proteins with various biological functions showed decreased levels in the *prc* mutant compared with their respective levels

Fig. 7. Prc directly binds to DppP (PXO_03743) in vivo and in vitro. **A**, Coimmunoprecipitation (Co-IP) assay of Prc-DppP binding events. DppP-His₆ was overexpressed in the wild-type (PXO99-OX3743H) and *prc* mutant (DLT4290-OX3743H) backgrounds, respectively. Before and after NaCl stress (428 mM), periplasmic fraction was extracted and anti-His₆ antibody was used to immunoprecipitate DppP-His₆. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8%) separation, polyclonal antiserum of Prc was used to detect Prc. IB = immunoblotting, CBB = Coomassie bright blue staining of SDS-PAGE gel, which is used as the control of periplasmic protein amount. **B**, Purities of Prc and DppP recombinant proteins. Prc and DppP proteins (10 μ g) were loaded on the SDS-PAGE gel, which was stained by CBB. **C**, In vitro binding affinity of Prc-DppP interaction. Surface plasmon resonance was used to estimate Prc-DppP interaction kinetics. Prc was trapped on a sensor CM5 chip and various concentrations of DppP were injected at a flow rate of 30 μ l/min at 25°C. Data were analyzed using a model for a single set of identical binding sites. Binding kinetics of Prc-DppP interaction. k_a = association rate constant; k_d = dissociation rate constant; K_A = equilibrium association rate constant; K_D = equilibrium dissociation rate constant. The parameters were calculated using BIAevaluation software (version 4.1).

in the WT strain (Table 1). Among them, in vivo and in vitro evidence revealed that Prc directly binds to DppP, a virulence-associated dipeptidyl peptidase (Fig. 7A and C), and controls its stability during salt stress response (Fig. 6B). These results suggest that Prc is involved in full virulence by controlling general bacterial stress responses when grown in plant tissues. To our knowledge, this is the first report on the functions of HtrA family proteins and identification of their binding substrates in phytopathogenic bacteria.

Various studies have revealed that HtrA family proteases contribute to bacterial virulence through versatile pathways. These proteases participate in stress responses by affecting the survival of the bacterium in host tissues (Flanagan et al. 2007). HtrA proteases are also involved in secretion or activation of other virulence determinants (Ibrahim et al. 2004). In some pathogens, HtrA are effectors that directly regulate host immune responses (Hoy et al. 2010; Lad et al. 2007). To identify the possible periplasmic factors that might be directly or indirectly affected by the Prc in *X. oryzae* pv. *oryzae*, we conducted a comparative subproteomic analysis to determine which periplasmic proteins showed significant differences between the *prc* mutant and the WT strain. Among those proteins, GumL is a putative polysaccharide pyruvyl transferase involved in extracellular polysaccharides biosynthesis (Vorholter et al. 2008), and flagellin, a component of the bacterial flagellum that is also a pathogen-associated molecular pattern recognized by the FLS2 receptor kinase of the host plant (Chinchilla et al. 2006), was identified. The EPS production and swimming motility of the *prc* mutant were decreased accordingly. Since extracellular polysaccharides and flagellin are experimentally confirmed virulence factors, Prc may play a role in virulence by regulating the expression of these factors. In addition, several typical stress-response proteins were identified, including a thioredoxin, an oxidoreductase, and a lipoprotein. We can speculate that changes in their expressions attenuated the ability of the bacterium to react to high salt stress. We also identified proteins associated with the synthesis and degradation of macromolecules such as those with functions in amino acid metabolism, fatty acid synthesis, and energy metabolism. Changes in the levels of these proteins may decrease the ability of the bacterium to survive and proliferate in the host plant, as revealed by genome-scale analyses (Qian et al. 2005; Ryan et al. 2011). Taken together, our results suggest that Prc is a regulator that regulates multiple physiological cascades associated with bacterial stress responses and virulence.

One intriguing result from the comparative subproteomic analysis is that all of the identified proteins were downregulated in periplasmic samples of the *prc* mutant. In fact, 27 of the identified proteins were completely absent from the *prc* mutant (Table 1). This result was unexpected, because we initially anticipated that Prc-associated protein substrates would be cut by Prc and, therefore, their levels would be lower in the WT strain and higher in the mutant. There are several possible explanations for this phenomenon: First, bacterial cells may secrete responsive proteins into the periplasmic space when they detect envelope stresses. If Prc is located upstream of this stress-response process, inactivation of *prc* will disrupt the signaling cascade and result in decreased levels of proteins. If this hypothesis is correct, then Prc seems to be mainly involved in cell signaling during the stress response, rather than cutting malfunctioning proteins directly. Second, the HtrA family proteases have chaperone activity or play direct roles in the bacterial secretion system, to aid the secretion and transport of proteins. For example, DegP of *Bordetella pertussis* is also a chaperone that guides the membrane protein filamentous hemagglutinin to the cell surface in the periplasm (Baud et al. 2009). If Prc also has these biochemical activities, its mutation would

block protein accumulation in the periplasm. Third, previous studies have demonstrated that several HtrA family proteases, such as DepS of *E. coli* (Zeth 2004), tend to degrade outer membrane proteins, which usually have high molecular masses. However, a limitation of the 2-DE method is that the large molecular-mass proteins aggregate at the upper region of the SDS-PAGE gel. Consequently, they are usually poorly resolved and are, therefore, difficult to identify.

Notably, nearly a quarter of the proteins identified (Table 1) were peptidases involved in proteolysis, indicating there is functional cooperation between these peptidases and Prc. Among them, Prc directly binds to a virulence-associated dipeptidyl peptidase DppP with high affinity and stabilizes this protein during the salt-stress response (Fig. 7A and C). In the absence of Prc, DppP was degraded into at least two fragments with different sizes (Fig. 6B), supporting the notion that the role of Prc is more like a chaperone protecting proteins rather than a peptidase degrading misfolded proteins when bacteria are grown under stress conditions. This postulation is reinforced by the fact that the transcription level of *prc* is very low with insufficient amounts to efficiently degrade accumulated malfunctioning proteins. In addition, truncated Prc did not exhibit endopeptidase activity in vitro, albeit such truncated, recombinant HtrA family proteases (including intact PDZ and peptidase domains) usually retained the ability to degrade protein substrates (Coleman et al. 2013). However, the regulatory relationship between the two peptidases, Prc and DppP, remains unclear. For example, is the enzymatic activity of Prc tightly regulated? What is the role of Prc peptidase activity in stabilizing DppP, which is also a peptidase? Is there an additional protease involved in forming a protein complex of Prc-DppP to degrade DppP during stress response and pathogenesis? Further genetic and biochemical investigations are needed to answer these questions.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

The bacterial strains and plasmids used in this study are described in Supplementary Table S1. *E. coli* was cultured at 37°C in Luria-Bertani medium. All *X. oryzae* pv. *oryzae* strains were cultured at 28°C in PS medium with appropriate antibiotics added. Competent cells of *X. oryzae* pv. *oryzae* were prepared by culturing the bacterium in 210 medium (yeast extract, 4 g; casein enzymatic hydrolysate, 8 g; sucrose, 5 g; K₂HPO₄, 3 g; and MgSO₄ · 7H₂O, 0.3 g per liter, pH 7.0) and were washed with ice-cold glycerol (10%), as described previously (Qian et al. 2008). Antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; spectinomycin, 100 µg/ml; and streptomycin, 200 µg/ml.

Construction of bacterial recombinant strains.

Basic molecular and genetic techniques were performed as described (Maniatis et al. 1989). Insertion inactivation was achieved by homologous, single-crossover recombination using the pK18mob suicide vector, as described in our previous work (Qian et al. 2008). The primer sequences for amplifying the eight PDZ-domain containing gene fragments during construction of the insertional mutants are listed in Supplementary Table S2. The constructed suicide vectors were transformed into *X. oryzae* pv. *oryzae* competent cells by electroporation (2.0 kV/cm, 25 µF, 200 Ω) using a GenePulser Xcell system (Bio-Rad, Hercules, CA, U.S.A.). Single-crossover mutants were screened on PS plates containing 50 µg of kanamycin per milliliter and were confirmed by PCR or Southern blot. To construct the epitope-tagged strain, we used a double cross-over method based on the suicide vector pK18mobsacB,

as described in a previous study (Schafer et al. 1994). To construct complemented strains, the full-length *prc* sequence was amplified and ligated into the broad host-range plasmid pHM1 (Innes et al. 1988). The recombinant vector was extracted and was subsequently electroporated into competent cells of *X. oryzae* pv. *oryzae*.

Phenotypic characterizations.

Plant inoculation and in planta growth assays were performed as described previously (Hu et al. 2007). Briefly, strains of *X. oryzae* pv. *oryzae* were cultured to approximately 10^6 CFU/ml and were inoculated onto susceptible, 8-week-old rice (*O. sativa* cv. IR24) plants by clipping the leaf tips with presterilized scissors dipped into the bacterial cultures. Lesion lengths were measured at 14 DAI. For the in planta growth assay, bacterial populations of every strain were determined in six inoculated leaves every 72 h. Each leaf was harvested and ground in PS medium. The leaf homogenate was serially diluted and plated onto PS plates. Bacterial numbers were counted after incubation at 28°C for 48 h. Each assay was independently repeated three times, and bacterial populations of each strain were calculated as the average values from six inoculated leaves at every time point. The methods for the extracellular enzyme assay and measuring the production of extracellular polysaccharides are according to previous studies (Lu et al. 2009; Qian et al. 2008). Bacterial swimming motility assay was conducted by inoculating strains onto a semisolid PS plate (0.15% agar), and measuring the diameters of growth zone after 36 h.

RT-PCR and Western blot.

Total RNA was extracted from bacterial strains at the exponential growth phase ($OD_{600} = 0.8 \pm 0.05$) using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's instructions. RNA concentrations were determined by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, U.S.A.). Semiquantitative RT-PCR and real-time RT-PCR analyses were used to quantify the mRNA level. Bacterial total RNA was treated with RNase-free DNase (Ambion, Austin, TX, U.S.A.) to remove contaminating DNA, and then, first-strand cDNA was synthesized with random primers (Promega, Madison, WI, U.S.A.) using Superscript III reverse transcriptase (Invitrogen). Real-time qRT-PCR was conducted using Maxima SYBR Green (Fermentas, Glen Burnie, MD, U.S.A.) with a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.). The transcript level of *tmrA* was detected as the internal control.

Western blot procedures were as described (Maniatis et al. 1989). The RNA polymerase α subunit was detected and used as the internal control.

Subcellular cell fraction.

The procedures to prepare total cell proteins and periplasmic and soluble cytoplasmic fractions were as described in the pET System Manual, 11th edition (Novagen, Madison, WI, U.S.A.). To isolate the membrane fraction, bacterial cells were harvested, resuspended, and sonicated in low-salt buffer (100 mM sodium phosphate, pH 7.0, 10% glycerol, 5 mM EDTA, 10 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF]). After removing the unbroken cells and debris by two rounds of low-speed centrifugation ($8,000 \times g$, 10 min at 4°C), the membrane fraction was collected by ultracentrifugation ($150,000 \times g$, 60 min at 4°C). The membrane fraction was then washed twice with high-salt buffer (20 mM sodium phosphate, pH 7.0, 2 M KCl, 10% glycerol, 5 mM EDTA, 5 mM DTT, and 1 mM PMSF). The membrane was resuspended in storage buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol) at -80°C before use.

Protein expression, purification, and Co-IP.

The pET30a vector was used to express C-terminal His₆-tagged Prc and DppP proteins. Recombinant vectors were transformed into the *E. coli* BL21 (DE3) strain. Protein expression and purification protocols were as described in the manufacturer's manual. In brief, *E. coli* strains were grown to $OD_{600} = 0.6$ and were induced with 0.5 mM isopropyl- β -D-thiogalactoside for 18 h at 16°C. The bacteria were collected by centrifugation and were treated with lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF). The following buffers were used to purify the proteins with Ni-NTA beads (Novagen): Ni-NTA-binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.0, 10 mM imidazole, pH 8.0), Ni-NTA-washing buffer (300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.0, 40 mM imidazole, pH 8.0), and Ni-NTA-elution buffer (300 mM NaCl, 250 mM imidazole, 50 mM sodium phosphate buffer, pH 7.0). Purified His₆-tagged proteins were dissolved in storage buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 50 mM NaCl, and 5% glycerol).

Co-IP detecting Prc-DppP binding was performed with a 1/1,000 dilution of a mouse His₆ monoclonal antibody raised against DppP-His₆. Periplasmic proteins (500 μ g), 1 μ g antibody, together with 30 μ l of 50% slurry protein A/G agarose beads (Abmart, Shanghai, P.R. China) were incubated overnight. The resulting complexes were washed twice with PBS (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, 0.24 g of KH₂O₄ per liter of H₂O, pH 7.4), followed by resuspending the pellet with 80 μ l of 1 $\times$ SDS loading buffer. After boiling for 8 min at 100°C, the samples were centrifuged and the supernatants were electrophoresed on 8% SDS-PAGE gels. Immunoprecipitation was performed with an anti-Prc rabbit polyclonal antiserum constructed by our lab to detect the co-immunoprecipitated Prc protein, and the immunoblotting with anti-His₆ antibody to check the concentrated DppP-His₆ protein.

Protease activity assay and alkaline phosphatase assay.

For the alkaline phosphatase assay, 100 μ g of each subcellular fraction was added to test tubes. Triplicate analyses were conducted for each fraction. The sample in each tube was completed to a volume of 2.4 ml with Tris buffer (0.05 M Tris-HCl, pH 8.0, 1 mM MgCl₂) and was then incubated for 3 min at 37°C. Then, 0.1 ml 0.5% p-nitrophenyl-phosphate was quickly added to each tube. The reaction was timed from this point. After 3 min and 10 min incubation, a portion (500 μ l) of the reaction mixture was removed and was added to a tube containing 100 μ l of 1 M potassium phosphate (pH 8.0) to stop the reaction. The absorbance of the solution at 410 nm was determined spectrophotometrically, and the alkaline phosphatase activity was calculated as standard definition.

Comparative subproteomic analysis of periplasmic proteins.

Bacterial strains were grown in PS medium to $OD_{600} = 0.8 \pm 0.05$, the cells were then harvested and treated with PS+NaCl (428 mM) for 10 min. Bacterial cells were harvested by centrifugation at 3,500 rpm for 20 min at 4°C. The osmolarity-shock method was used to extract the periplasmic fraction, as described elsewhere (Ausubel et al. 1989). Periplasmic proteins were dissolved in PBS buffer, and a 2-DE clean-up kit (GE Healthcare, Piscataway, NJ, U.S.A.) was used to purify the proteins, according to the product manual. The pellet was resuspended in isoelectric focusing (IEF) buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 2% (vol/vol) immobilized pH gradient (IPG) buffer, pH 4 to 7). The protein concentration was determined using a 2-DE Quant kit (GE Healthcare).

The procedures for first-dimension IEF and 2-DE were as described previously (Zhao et al. 2010). To achieve the best resolution, 250 µg periplasmic protein extracted from bacterial strains was loaded onto Immobiline DryStrips (pH 4 to 7; GE Healthcare) with rehydration solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 18.18 mM DTT, and 1% (vol/vol) IPG buffer (pH 4 to 7) for gel rehydration. IEF was performed using an Ettan IPGphor III system (GE Healthcare) according to the following protocol: step and hold at 100 V for 3 h, 300 V for 1 h, 500 V for 1 h, gradient to 1,000 V for 1 h, gradient to 4,000 V for 1 h, gradient to 8,000 V for 12 h, and finally, step and hold at 1,000 V for 5 h. After equilibration, the protein samples were separated on 12.5% SDS-PAGE gels for 2-DE, which was performed using an Ettan DALTsix system (GE Healthcare). Gels were run at 15°C and at a constant current in two steps, 1 W/gel for 1 h, and then, 8 W/gel for 5 h. Finally, 2-DE gels were stained with colloidal Coomassie stain. Each sample pair was analyzed three times. The gels were scanned at 300-dpi resolution using an ImageScanner III scanner, and data was analyzed using ImageMaster 2D Platinum 6.0 software (GE Healthcare).

Protein spots were detected in automatic analysis mode, and those showing significant differences (P value < 0.05) were selected and excised manually from gels. The excised portions of the gel were subjected to in-gel digestion, using trypsin for subsequent protein identification with the Autoflex II matrix-assisted laser desorption-ionization time of flight mass spectrometry (MS) system or the Thermo nanoLTQ Orbitrap XL system. Details of the procedures for in-gel digestion and MS analysis were given in a previous work (Zhao et al. 2010).

Surface plasmon resonance.

The binding kinetics between Prc-DppP were estimated in vitro by SPR, using Biacore 3000 (GE healthcare). PrcΔ125 purified protein was immobilized on a CM5 sensor chip using standard amino-coupling chemistry. The binding experiments were executed by injection of purified DppP protein (126.3 nM to 10 µM) in PBS buffer with 0.005% Tween-20 buffer, which was also used as reference control to subtract the nonspecific binding. The surface was regenerated between binding cycles by injection of 5 mM NaOH at a rate of 30 µl/min for 20 s. Evaluation of the binding parameters were carried out by the BIAevaluation Software (version 4.1).

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