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The *clpX* gene plays an important role in bacterial attachment, stress tolerance, and virulence in *Xanthomonas campestris* pv. *campestris*

Hsueh-Hsia Lo[1] · Chao-Tsai Liao[1] · Chih-En Li[1] · Ying-Chuan Chiang[1] · Yi-Min Hsiao[1]

Abstract

Xanthomonas campestris pv. *campestris* is a bacterial pathogen and the causal agent of black rot in crucifers. In this study, a *clpX* mutant was obtained by EZ-Tn*5* transposon mutagenesis of the *X. campestris* pv. *campestris*. The *clpX* gene was annotated to encode ClpX, the ATP-binding subunit of ATP-dependent Clp protease. The *clpX* mutant exhibited reduced bacterial attachment, extracellular enzyme production and virulence. Mutation of *clpX* also resulted in increased sensitivity to a myriad of stresses, including heat, puromycin, and sodium dodecyl sulfate. These altered phenotypes of the *clpX* mutant could be restored to wild-type levels by *in trans* expression of the intact *clpX* gene. Proteomic analysis revealed that the expression of 211 proteins differed not less than twofold between the wild-type and mutant strains. Cluster of orthologous group analysis revealed that these proteins are mainly involved in metabolism, cell wall biogenesis, chaperone, and signal transduction. The reverse transcription quantitative real-time polymerase chain reaction analysis demonstrated that the expression of genes encoding attachment-related proteins, extracellular enzymes, and virulence-associated proteins was reduced after *clpX* mutation. The results in this study contribute to the functional understanding of the role of *clpX* in *Xanthomonas* for the first time, and extend new insights into the function of *clpX* in bacteria.

Keywords Biofilm formation · Environmental adaptation · Pathogenicity

Introduction

Phytopathogenic bacteria of the *Xanthomonas* genus cause severe diseases in approximately 400 host plants, including a wide variety of economically important crops (Ryan et al. 2011). *Xanthomonas campestris*, the most dominant species within the *Xanthomonas* genus, has at least 141 pathovars capable of infecting a wide range of plants with agronomical interest (He and Zhang 2008). *X. campestris* pv. *campestris*, the causal agent of black rot disease, infects cruciferous plants and is responsible for major yield and quality losses in

✉ Yi-Min Hsiao
ymhsiao@ctust.edu.tw

1   Department of Medical Laboratory Science
     and Biotechnology, Central Taiwan University of Science
     and Technology, Taichung 406, Taiwan

Brassica crops, such as broccoli, cabbage, cauliflower, and kale (He and Zhang 2008; Vicente and Holub 2013).

X. campestris pv. *campestris* is known to produce a range of factors such as exopolysaccharides and extracellular enzymes (cellulase, mannanase, pectinase, and protease), which collectively have an important role in pathogenesis (Chan and Goodwin 1999; Dow et al. 2003). The exopolysaccharides can cause tissue necrosis and leaf wilting by obstructing the water flow in xylem vessels (He and Zhang 2008; Buttner and Bonas 2010), and the extracellular enzymes can degrade plant cell components and play a role in facilitating plant tissue maceration (Chan and Goodwin 1999). In addition, experimental evidences indicate that biofilm formation and bacterial adhesion are associated with the virulence of *X. campestris* pv. *campestris*; as shown for genes encoding extracellular mannanase (*manA*), isocitrate dehydrogenase (*icd2*), major outer-membrane protein (*mopB*), tail-specific protease (*prc*), and UTP-glucose-1-phosphate uridylyltransferase (*galU*), mutation in these genes has an impact in biofilm formation or bacterial attachment and leads to an attenuation

of bacterial virulence (Dow et al. 2003; Chen et al. 2010; Liao et al. 2014, 2016; Chiang et al. 2017).

To identify novel genes related to *X. campestris* pv. *campestris* biofilm formation, a previously constructed mutant library of *X. campestris* pv. *campestris* strain 17 (hereinafter, Xcc17) (Liao et al. 2016) was screened in a biofilm formation assay. Here, one mutant designated as J67 exhibiting a reduced ability in bacterial attachment was characterized. The mutant strain J67 was found to have a transposon inserted in the locus_tag AAW18_RS04855 in the recently completed genome sequence of Xcc17 (GenBank accession no. NZ_CP011946) (Liu et al. 2015), which was annotated to encode ClpX, the ATP-binding subunit of ATP-dependent Clp protease.

The ATP-dependent Clp protease is widely conserved within the bacterial domain and has a multitude of functions in bacteria, such as protein quality control, stress tolerance, and virulence factor expression (Malik and Brotz-Oesterhelt 2017). Clp protease consists of two functional units with separate activities: ATPase-active chaperone rings and a proteolytic core (Kress et al. 2009; Schmidt et al. 2009). The ATPase component is responsible for substrate recognition, unfolding, and translocation into the proteolytic component (Gottesman 2003; Kress et al. 2009; Schmidt et al. 2009). The proteolytic component possesses multiple active sites of serine or threonine-type, which enable protein hydrolysis (Kress et al. 2009). The existence of two different proteolytic cores (ClpP and ClpQ) and multiple different chaperone rings (ClpA, ClpC, ClpE, ClpX, and ClpY) results in an inventory of several ATP-dependent Clp proteases (Kress et al. 2009). In *Escherichia coli*, various ATP-dependent Clp proteases exist, including ClpAP, ClpXP, and ClpYQ (also called HslUV) (Porankiewicz et al. 1999; Gottesman 2003; Kress et al. 2009; Schmidt et al. 2009). The ClpP is classified as a serine-type protease and associates with either ClpA or ClpX, whereas the ClpQ (HslV) has a threonine active site and associates with ClpY (HslU) (Porankiewicz et al. 1999). An adaptor protein, such as ClpS, may also be associated with ClpA and influences the ClpAP complex (Erbse et al. 2006). ClpXP is the most ubiquitous of Clp proteases as it is found in almost all bacteria (Kress et al. 2009). ClpYQ exists alongside ClpAP in most proteobacteria and is also found in certain Gram-positive bacteria (Kress et al. 2009). The ClpP protease core can also interact with ClpC and ClpE, which is found in Gram-positive bacteria (Kress et al. 2009). In the fully sequenced genomes of several *X. campestris* pv. *campestris* strains, such as ATCC33913 (da Silva et al. 2002), 8004 (Qian et al. 2005) as well as Xcc17 (Liu et al. 2015), several putative Clp protein encoding genes (such as *clpA*, *clpX*, *clpY*, *clpP*, and *clpQ*) have been annotated, whereas

none of them have been studied. Here, the *X. campestris* pv. *campestris clpX* gene was characterized.

Materials and methods

Bacterial strains, media and growth conditions

Escherichia coli strain DH5α ECOS™ 101 (Yeastern Biotech, Taiwan) was used as cloning host. *X. campestris* pv. *campestris* strain Xcc17 is a virulent wild-type strain isolated from the leaves of the infected plants in Taiwan (Yang and Tseng 1988). J67, a derivative of Xcc17, was a *clpX* mutant constructed in this study. *E. coli* strains were cultured in Luria–Bertani (LB) medium (Sambrook et al. 1989) at 37 °C. *X. campestris* pv. *campestris* strains were grown in LB medium or XOLN medium (Fu and Tseng 1990) at 28 °C. Glucose or glycerol were supplemented (2%) as necessary. Liquid cultures were grown with shaking at 180 rpm. Solid media contained 1.5% agar. Ampicillin (50 μg/mL), kanamycin (50 μg/mL), and tetracycline (15 μg/mL) were supplemented as necessary.

Recombinant DNA techniques

Bacterial genomic and plasmid DNAs were extracted by the Wizard® Genomic DNA Purification Kit (Promega) and the Gene-Spin™ Miniprep Purification Kit (Protech), respectively. Polymerase chain reaction (PCR) was done as previously described (Hsiao et al. 2005). Restriction endonuclease digestion, DNA ligation, agarose gel electrophoresis, and transformation were performed by standard protocols as described previously (Sambrook et al. 1989).

Generation of transposon mutants and determination of transposon insertion site

X. campestris pv. *campestris* transposon mutants were generated using EZ-Tn5™ < R6Kγ*ori*/KAN-2 > Tnp Transposome Kit (Epicentre) as described by Liao et al. (2016). The transposon insertion site was determined by "rescue cloning", as described by Liu et al. (2018).

Construction of plasmid for complementation

To construct the plasmid for complementation of the *clpX* mutant, the fragment encompassing the upstream 52-bp region, the entire coding region and 6 bp downstream of the *clpX* stop codon in Xcc17 were amplified by PCR with primers clpX-CF (5′-<u>AAGCTT</u>CTCGAAATCGAACCC CCGTGC-3′, *Hin*dIII restriction site underlined) and clpX-CR (5′-<u>TCTAGA</u>GGCCTCAGTCGCCAGACGC-3′, *Xba*I restriction site underlined). The product was cloned into

cloning vector yT&A (Yeastern, Taiwan). After sequence confirmation, the fragment containing *clpX* (1345-bp) was excised with *Hin*dIII and *Xba*I, then ligated into broad-host-range vector pRK415 (Keen et al. 1988) to generate pRK-clpX. For complementation, pRKclpX was transferred into the *clpX* mutant J67 by electroporation (2.5 kV, 25 μF, 200 Ω), yielding the complementary strain J67c. For comparison, Xcc17 and J67 carrying the empty vector pRK415 were generated in parallel and were designated as Xcc17v and J67v, respectively.

Bacterial attachment analysis, extracellular enzyme production assay, and virulence test

Bacterial attachment was analyzed by examining the ability of cells to adhere to the 96-well polystyrene microtiter plates (Nunc) and was quantified by staining of the attached cells with crystal violet, as the previously described method (Liao et al. 2016). Quantitative analysis of adhesion was also examined on glass surface as described previously (Ryan et al. 2007) with some modifications. Briefly, bacteria from overnight cultures were diluted to an OD_{550} of 0.35 in XOLN medium containing 2% glucose. Then, 1.0 mL of cultures prepared as aforementioned was added in borosilicate glass tubes (10 cm × 1.3 cm, Kimble) and incubated at 28 °C for 24 h without shaking. After incubation, the medium and unattached cells were removed carefully. Then, surface-attached bacteria were washed with 1.5 mL distilled water (three times) and stained with 1.5 mL crystal violet (0.1%) for 5 min at room temperature. The free dye was removed and the tube was washed three times with distilled water. The dye that was incorporated into the attached cells was solubilized with 2.0 mL ethanol (95%) and quantified by spectrophotometry at 595 nm. Bacterial adhesion to leaves surface was conducted as described previously (Hsiao et al. 2012). The experiments were carried out at least three times.

Extracellular enzyme production was evaluated by inoculating bacteria strains on substrate-supplementary agar plates and was detected by monitoring the appearance of hydrolytic zones around bacterial colonies according to previously described methods (Hsiao et al. 2011) with minor modifications. Briefly, 3 μL of diluted overnight culture ($OD_{550} = 1$) was deposited onto the surface of XOLN plates containing the appropriate substrates. The supplemented substrates were carboxymethyl cellulose (0.5%, for cellulase), locus bean gum (0.2%, for mannanase), sodium polypectate (0.2%, for pectinase), and skim milk (1%, for protease). Activity of extracellular enzymes was assayed after incubation at 28 °C for 2 days as described previously (Hsiao et al. 2011). Each experiment was done at least three times.

Bacterial virulence was assayed using the scissors-clipping method as described (Liao et al. 2016). The lesion length was measured and the disease symptom was photographed 14 days after inoculation. Three independent experiments, each with six replicates, were done.

Stress tolerance assay

For temperature tolerance assay, bacteria were grown overnight and diluted with fresh LB medium to a starting density of $OD_{550} = 0.5$ (5×10^8 cells/mL), and then followed a tenfold dilution. Five microliters of resulting cultures (5×10^8 to 5×10^5 cells/mL) were spotted on LB plates and incubated at either 28 °C or 37 °C for 3 days. To evaluate the tolerance of bacteria to H_2O_2, polymyxin B, puromycin, and sodium dodecyl sulfate (SDS), disk diffusion assays were performed. Bacterial strains were grown in LB medium until the OD_{550} of the cultures reached about 0.6 (mid-exponential phase). The mid-exponential-phase cultures were collected and suspended in fresh LB broth to yield an $OD_{550} = 1$. One milliliter of resulting cultures was spread on LB plate and a disc (6 mm) containing 10 μl of H_2O_2 (3%), polymyxin B (5 mg/mL), puromycin (5 mg/mL), and SDS (5%) was placed on top. The plates were incubated at 28 °C for 3 days and the growth-inhibition zones surrounding the discs were measured. All assays were performed at least three times.

RNA extraction, reverse transcription (RT), and quantitative real-time PCR (qPCR)

Bacteria were cultivated in XOLN–glycerol medium to mid-exponential phase ($OD_{550} = 0.6$) and the cells were collected for RNA extraction and RT-qPCR analysis as previously described by Liao et al. (2019). Briefly, total RNA was extracted from cells of Xcc17 and J67 using the RNeasy Mini Kit (Qiagen), subjected to DNase I treatment using the RNase-Free DNase Set (Qiagen), and reverse-transcribed to cDNA using the iScript™ cDNA Synthesis Kit (BIO-RAD). Subsequent qPCR was then performed in a CFX96 Real Time PCR system (BIO-RAD) using the iQ™ SYBR® Green Supermix (BIO-RAD). Each assay was carried out in triplicate with *16S rRNA* gene as a reference gene. Primer sequences of the tested target genes are listed in Table S1. The fold change for transcript was calculated by the $2^{-\Delta\Delta Ct}$ method.

Cell extract preparation and gel electrophoresis

X. campestris pv. *campestris* strains were cultivated in XOLN–glycerol medium. After reaching an OD_{550} of approximately 0.6 (mid-exponential phase), the cells were harvested by centrifugation at 10,000*g* for 5 min at 4 °C. The cell pellets were washed with 10 mM Tris–HCl (pH 7.5) three times. After washing, proteins from the pelleted cells were prepared using ExtractPRO™ Protein Extraction Reagent (Visual Protein, Taiwan), according to the

manufacturer's instructions. The protein concentration was estimated using the Bradford method. Equal amounts of protein (15 µg) were loaded and resolved on 12.5% SDS-PAGE. After electrophoresis, the gel was stained with the VisPRO 5 min Protein Stain Kit (Visual Protein). After staining, the gel was washed with distilled water and stored at 4 °C until processing for in-gel digestion.

In-gel digestion and peptide preparation

In-gel digestion was performed according to a method described previously (Liao et al. 2019). Briefly, the gel was incubated with sequencing-grade trypsin (6.25 ng/µL) overnight at 25 ºC after reduction with dithiothreitol (10 mM) for 1 h at 56 ºC and alkylation with iodoacetamide (25 mM) in the dark for 45 min at room temperature. The tryptic peptides were then extracted and concentrated as the previously described method (Liao et al. 2019).

Mass spectrometric analysis, mass data searching, protein identification and quantification

Tryptic peptides obtained as described above were separated by nano liquid chromatography (nLC) using the Ultimate 3000 nLC system (Dionex) and then analyzed by tandem mass spectrometry (MS/MS) through the LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) as described previously (Liao et al. 2019). BioWorks 3.3 (Thermo Fisher Scientific) was used to interpret the acquired MS/MS spectra. Database search was carried out against the *X. campestris* pv. *campestris* strain 8004 database from UniProt (4252 sequences; 1 445 974 residues). The search parameters were: (1) 10 ppm for the precursor mass tolerance (2) 0.6 Da for fragment mass tolerance (3) variable modification: oxidation of methionine (4) fixed modification: carbamidomethylation of cysteine (5) allowing two missed cleavage sites, and (6) the minimum number of tryptic termini was 1. Scaffold (versions 1.07 and 2.01) (Proteome software) was used to validate peptide and protein identification. Peptides with a probability greater than 95% and proteins with a greater than 99% probability were accepted. Protein quantification was estimated by spectral counting as described previously (Liao et al. 2019) and proteins with a 2.0-fold change between wild type Xcc17 and *clpX* mutant J67 was considered as differentially expressed proteins. The cluster of orthologous group (COG) analysis of the identified proteins was conducted using the *Xanthomonas* Genome Browser (https://xgb.leibniz-fli.de/cgi/index.pl) against *X. campestris* pv. *campestris* strain 8004.

Statistical analysis

Statistical analysis was performed by two-tailed Student's *t* test using the Microsoft Excel statistical package. For each quantification experiment, at least three times were carried out and *p* value less than 0.05 was considered statistically significant.

Results and discussion

The *clpX* gene is involved in bacterial attachment and extracellular enzyme production

Through bulk screening of the *X. campestris* pv. *campestris* mutant library generated previously (Liao et al. 2016; Liu et al. 2018) for bacterial attachment, the J67 mutant that had a reduced ability to form biofilms in the polystyrene 96-well microtiter plates was obtained. To identify the transposon–inserted gene, the transposon flanking region was rescued by the rescue cloning method as described by the manufacture (Epicentre) and sequenced by primer R6KAN-2 RP-1 (Liu et al. 2018). A BLAST search using the rescued DNA sequence against the Xcc17 genome (GenBank accession no. NZ_CP011946) revealed that the transposon insertion was mapped to a site within the locus_tag AAW18_RS04855 in the genome sequence of Xcc17, which predicts to encode ClpX, the ATP-binding subunit of ATP-dependent Clp protease. The *X. campestris* pv. *campestris* *clpX* gene encodes a protein consisting of 428 amino acid residues and has a theoretical molecular mass of 47,136 Da with a predicted p*I* of 5.44 (calculated using the Compute pI/MW tool from ExPASy; https://www.expasy.org/). The transposon insertion was localized at a distance 383 bp from the site of ATG translation start of *clpX* gene.

Limited information is available about the role of ClpX in bacterial attachment. In *Streptococcus mutans*, the *clpX* mutant strain revealed less biofilm mass in the presence of glucose, whereas the biofilm formation was enhanced when sucrose was provided (Kajfasz et al. 2009). In *Burkholderia cenocepacia*, no considerable differences in biofilm formation were observed between the *clpX* mutant and the parental strain (Veselova et al. 2016). In our preliminary analysis, the mutant strain J67 showed a reduced attachment on polystyrene plates compared with the parental strain Xcc17 (data not shown). To determine whether the reduced ability to form biofilms in J67 was specifically attributable to ClpX, a complemented strain named J67c was generated by introducing pRKclpX (with *clpX* gene cloned into pRK415) into the transposon mutant J67. The parental strain Xcc17 and the transposon mutant J67 were also transformed with empty vector pRK415 (named Xcc17v and J67v, respectively) to control for any effects of the vector itself. Biofilm formation
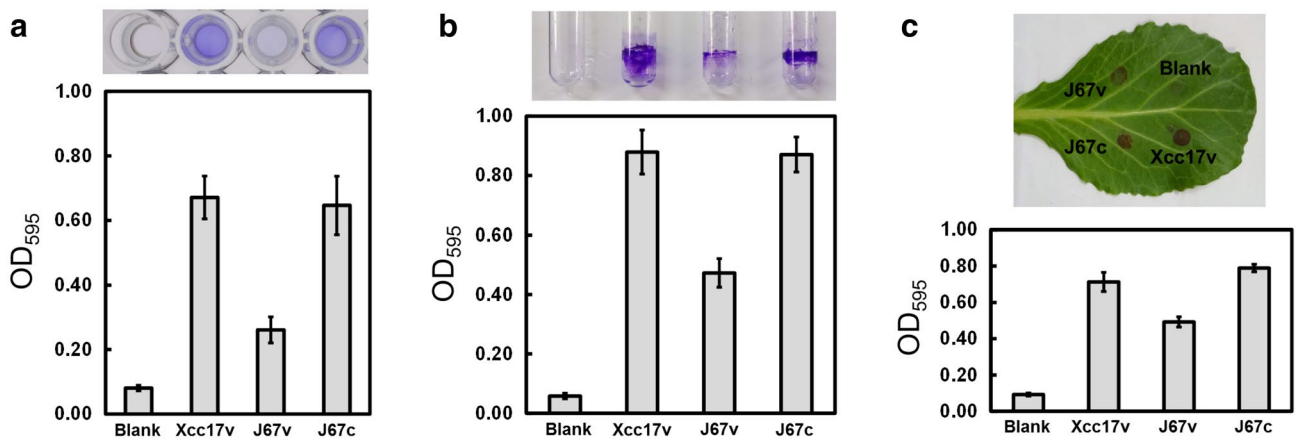
Fig. 1 Effects of mutation of *clpX* on cell attachment to polystyrene plates (**a**), glass tubes (**b**), and cabbage leaf surfaces (**c**) in *X. campestris* pv. *campestris*. Strains to be tested were grown overnight, cells were washed and diluted using fresh XOLN medium supplemented with glucose, and were assayed as described in the "Material and methods" section. Xcc17v: wild-type strain Xcc17 carrying empty vector pRK415; J67v: *clpX* mutant J67 carrying pRK415; J67c: complemented *clpX* mutant; Blank: XOLN medium supplemented with glucose without inoculation of bacteria. Values presented are the mean ± standard deviation (*n* = 3)

was assayed on polystyrene microtiter plate (Fig. 1a), borosilicate glass tube (Fig. 1b), and leaf surface (Fig. 1c). It was revealed that introduction of *clpX* mutant J67 with pRKclpX but not pRK415 restores attachment ability to those of the parent strain. These results indicated that the reduced bacterial attachment of J67 is due to a mutation in the *clpX* gene.

To get more understanding of ClpX in *X. campestris* pv. *campestris*, the production of a range of virulence factors including exopolysaccharide and extracellular enzymes were determined to assess whether ClpX influences the synthesis of these virulence determinants. The results revealed that the exopolysaccharide yields produced by the mutant were similar to those of the wild type (data not shown). However, the extracellular enzymes produced by the mutant were lower than the wild type. As depicted in Fig. 2, the clear zones of hydrolytic activity surrounding J67v colonies grown on plates supplemented with carboxymethyl cellulose, locust bean gum, sodium polypectate, and skim milk, were smaller than those observed for the Xcc17v and J67c. These results indicated that the *clpX* gene is required for the ability to synthesize extracellular enzymes (including cellulase, mannanase, pectinase, and protease).
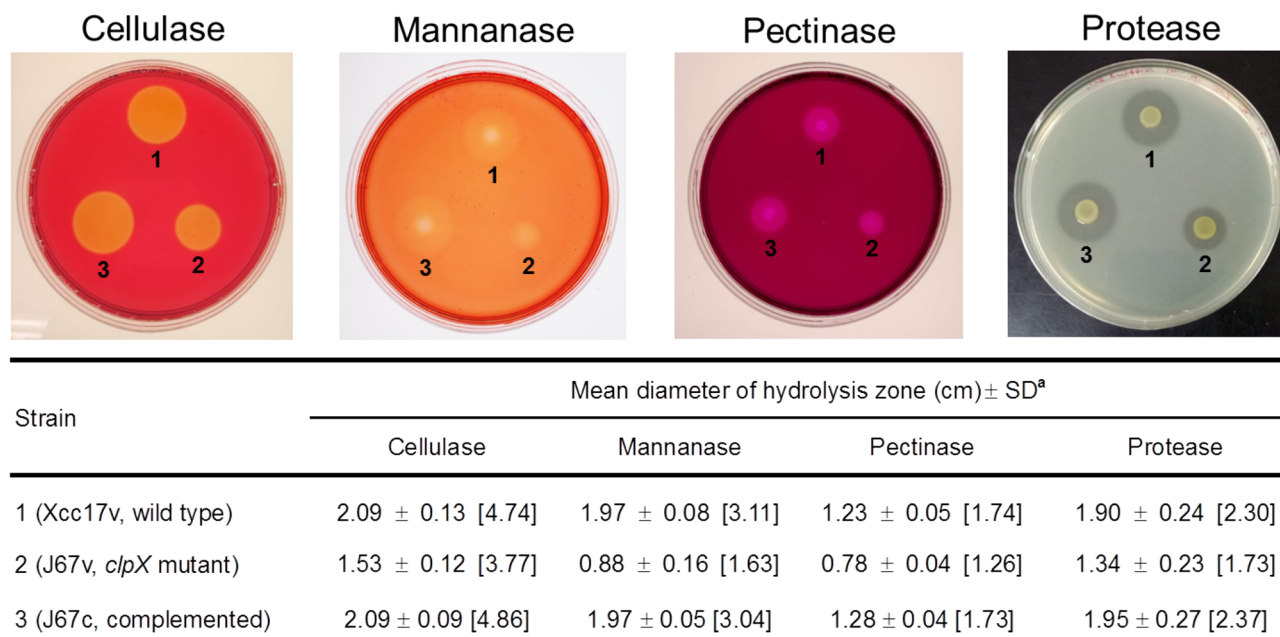
The observations showing reduced bacterial attachment (Fig. 1) and extracellular enzyme production (Fig. 2) in *clpX* mutant prompted us to test whether mutation of *clpX* affected the expression of genes associated with bacterial attachment and extracellular enzyme synthesis. To test the possibility, the expression of four attachment-related genes (*galE*, *galU*, *ha*, and *yapH*), along with four genes encoding extracellular enzymes (*engA*, *manA*, *pelA*, and *prt1*), was compared in strains Xcc17 and J67. Consistent with the phenotypic observations, the expressions of these tested genes were all reduced in the mutant J67 compared to the wild-type Xcc17 (Table 1), analyzed by RT-qPCR. These results indicated that a mutation in *clpX* affects the transcription of genes involved in bacterial attachment and extracellular enzymes. As the RT-qPCR results revealed that mutation in *clpX* reduced the expression of these genes, it is implying that ClpX might affect the expression of these genes at transcriptional level and that the reduced bacterial attachment and extracellular enzyme production of the *clpX* mutant may be attributable to the reduced expression of these genes.

ClpX has been implicated in expression of extracellular virulence factors in several organisms. In *Staphylococcus aureus*, the amount of both α-hemolysin and β-hemolysin as well as the activity of extracellular protease are decreased in the *clpX* mutant, and ClpX affects α-hemolysin production by reducing the transcription of the α-hemolysin encoding gene *hla* (Frees et al. 2003). In *Dickeya dadantii*, inactivation of the *clpX* led to reduce the production of pectate lyase (Li et al. 2010).

The *clpX* gene is essential for bacterial virulence

As ClpX was found to be involved in biofilm formation and virulence factor synthesis, experiment was conducted to evaluate whether ClpX plays a role in pathogenesis. In the pathogenicity test, the *clpX* mutant showed decreased virulence to the host plant cabbage. The lesion lengths caused by Xcc17 (wild type) and J67c (complementary strain) 14 days after inoculation were approximately 1.67 cm and 1.75 cm, respectively, whereas the lesion lengths caused by J67 (*clpX* mutant) were approximately 1.04 cm (Fig. 3). These results indicated that the mutation in the *clpX* gene attenuated the virulence of *X. campestris* pv. *campestris*. Inactivation of the *clpX* gene is shown to decrease the virulence of various

Fig. 2 Effects of mutation of *clpX* on extracellular enzyme activities in *X. campestris* pv. *campestris*. The activity of extracellular cellulase, mannanase, pectinase, and protease was evaluated using the substrate-supplemented plate assay as described in the "Material and methods" section. The tested strains included: 1 (Xcc17v, wild-type strain Xcc17 carrying empty vector pRK415); 2 (J67v, *clpX* mutant J67 carrying pRK415); and 3 (J67c, complemented *clpX* mutant). The diameter of the colony and hydrolysis zone of each strain were measured and the ratio of hydrolysis zone size-to-colony diameter is calculated. Values presented are the mean ± standard deviation (*n* = 3)

	Mean diameter of hydrolysis zone (cm) ± SD[a]			
Strain	Cellulase	Mannanase	Pectinase	Protease
1 (Xcc17v, wild type)	2.09 ± 0.13 [4.74]	1.97 ± 0.08 [3.11]	1.23 ± 0.05 [1.74]	1.90 ± 0.24 [2.30]
2 (J67v, *clpX* mutant)	1.53 ± 0.12 [3.77]	0.88 ± 0.16 [1.63]	0.78 ± 0.04 [1.26]	1.34 ± 0.23 [1.73]
3 (J67c, complemented)	2.09 ± 0.09 [4.86]	1.97 ± 0.05 [3.04]	1.28 ± 0.04 [1.73]	1.95 ± 0.27 [2.37]

[a] Values in brackets indicate ratio of hydrolysis zone size to colony diameter.

bacteria, such as *Bacillus anthracis* (McGillivray et al. 2009), *B. cenocepacia* (Veselova et al. 2016), *D. dadantii* (Li et al. 2010), and *S. aureus* (Frees et al. 2003).

The *clpX* gene is required for growth during heat stress and is important for puromycin and SDS tolerance

As the formation of a biofilm presumably functions as a protective role that allows bacteria to survive under harsh conditions (Dow et al. 2003; Buttner and Bonas 2010), the reduced biofilm formation of the *clpX* mutant (Fig. 1) suggested that *clpX* gene might implicated in stress tolerance. To examine whether ClpX contributes to stress adaptation of *X. campestris* pv. *campestris*, the sensitivity of the *clpX* mutant together with the wild-type and complemented strains was tested under a range of stresses, including heat (37 °C), H_2O_2 (3%), polymyxin B (5 mg/mL), puromycin (5 mg/ml), and SDS (5%). At physiological temperature (28 °C), the growth of all tested strains was similar (Fig. 4a, upper). When bacterial strains were grown at elevated temperature (37 °C), the growth of *clpX* mutant J67v was inhibited and genetic complementation restored this deficiency (Fig. 4a, down). The *clpX* mutant did not differ from the wild-type strain in susceptibility to H_2O_2 and polymyxin B (data not

shown). Compared to wild-type Xcc17v, the *clpX* mutant J67v was more sensitive to puromycin (Fig. 4b) and SDS (Fig. 4c). Taken together, these results demonstrated that ClpX is required for tolerance to heat, puromycin and SDS stresses. Several reports have indicated that *clpX* mutants of bacteria are more susceptible to various stresses. In the presence of oxidative stress or at low temperature, ClpX is important for growth in *S. aureus*, whereas inactivation of *clpX* in *S. aureus* leads cells to growth at higher temperature or in the presence of a higher puromycin concentration than the wild-type cells (Frees et al. 2003). In *B. anthracis*, ClpX function protects bacteria against antimicrobial peptide killing (McGillivray et al. 2009).

Inactivation of *clpX* has broad effects on the proteome of *X. campestris* pv. *campestris*

To acquire further insights into additional physiological roles of ClpX in *X. campestris* pv. *campestris*, we used quantitative proteome analysis to examine differences in protein expression profile between the wild-type Xcc17 and the *clpX* mutant J67. In total, 615 and 551 proteins were identified in Xcc17 and J67, respectively (Table S2). Among these proteins, the expression patterns of 211 proteins (155 proteins were downregulated while 56 proteins were upregulated

Table 1 Effect of *clpX* mutation on the expression of attachment-related genes, extracellular enzyme encoding genes, putative ATP-dependent Clp protein encoding genes, and virulence-associated genes

| Gene ID[a]	Gene name	Protein name	Fold change (J67/Xcc17)[b]	
			Proteomic	RT-qPCR
Attachment-related genes				
XC_3767 [M]	*galE*	UDP-glucose 4-epimerase	ND	0.09
XC_1930 [M]	*galU*	UTP-glucose-1-phosphate uridylyltransferase	0.72	0.68
XC_4290 [N]	*ha*	Hemagglutinin	†	0.14
XC_2160 [S]	*yapH*	YapH protein	0.54	0.07
Extracellular enzyme genes				
XC_0639 [G]	*engA*	Cellulase	0.58	0.62
XC_2458 [G]	*manA*	Mannan endo-1,4-beta-mannosidase	ND	0.18
XC_3591 [G]	*pelA*	Pectate lyase	ND	0.40
XC_3379 [O]	*prt1*	Extracellular protease	0.71	0.28
Clp protein encoding genes				
XC_2218 [O]	*clpA*	ATP-dependent Clp protease ATP-binding subunit	1.08	1.01
XC_3264 [O]	*clpX*	ATP-dependent Clp protease ATP-binding subunit	0.10	0.06
XC_0668 [O]	*clpY* (*hslU*)	ATP-dependent HslUV protease ATP-binding subunit	1.64	4.59
XC_3265 [O]	*clpP*	ATP-dependent Clp protease proteolytic subunit	ND	1.03
XC_0667 [O]	*clpQ* (*hslV*)	ATP-dependent HslUV protease peptidase subunit	‡	3.61
Virulence related genes				
XC_0626 [G]	*celA*	1,4-Beta-cellobiosidase	†	0.72
XC_1965 [T]	*baeS*	Two-component system sensor protein	0.41	0.45
XC_2227 [T]	*ravS*	Two-component system sensor protein	0.34	0.49
XC_2228 [T]	*ravR*	C-di-GMP phosphodiesterase A	†	0.34
XC_2699 [O]	*mip*	Peptidyl-prolyl cis–trans isomerase	†	0.72

[a]The gene ID is based on *X. campestris* pv. *campestris* strain 8004. The letter in bracket indicates the COG functional category as shown in Fig. 5

[b]The fold change of proteomic analysis is the spectral count ratio. ND means the protein is not detected both in wild-type Xcc17 and *clpX* mutant J67; † and ‡ indicate the protein is unique to the wild-type strain Xcc17 and the *clpX* mutant J67, respectively. The fold change of RT-qPCR was calculated using $2^{-\Delta\Delta C_T}$

in the *clpX* mutant) were associated with the presence of ClpX (Fig. 5a). Of these differentially expressed (fold change ≥ 2.0) proteins (1) 99 proteins were only present in the wild type (2) 35 proteins were only present in the *clpX* mutant, and (3) 56 and 21 proteins were highly expressed in the wild type and *clpX* mutant, respectively (Fig. 5a). These data suggested that approximating 35% of the identified proteins were affected by *clpX* in *X. campestris* pv. *campestris*. The completely list of differentially expressed proteins identified between the wild-type Xcc17 and the *clpX* mutant J67 can be found in Table S3. Further, the function of the 211 differentially expressed proteins was predicted and classified using COG analysis. Specifically (1) there were 17 proteins (12 downregulated and 5 upregulated) in the unclassified category; (2) the remaining 194 *clpX*-influenced proteins were classified into different functional categories; and (3) the majority of altered proteins including proteins related to metabolism (cluster E, G, and P), cell wall biogenesis (cluster M), chaperone (cluster O) and signal transduction

(cluster T) (Fig. 5b). These differentially expressed proteins are distributed over all the functional categories of the *X. campestris* pv. *campestris* genome, indicating ClpX has a broad physiological role in *X. campestris* pv. *campestris*. Identification of such differentially expressed proteins provides new candidates for future studies that will allow assessment of their physiological roles and significance in ClpX-regulated processes.

To our knowledge, only two other reports have examined the proteome of the *clpX* mutant, which were performed in *B. cenocepacia* (Veselova et al. 2016) and *S. mutans* (Jana et al. 2016). Comparison of the proteomes from the wild-type strain and the *clpX* mutant strain revealed that at least 19 and 53 proteins had altered levels of expression in *B. cenocepacia* (Veselova et al. 2016) and *S. mutans* (Jana et al. 2016), respectively. As was the case in *B. cenocepacia*, the majority of altered proteins in *X. campestris* pv. *campestris* *clpX* mutant were involved in metabolic function and regulation. Contrary to what were seen in *B. cenocepacia* and *S.
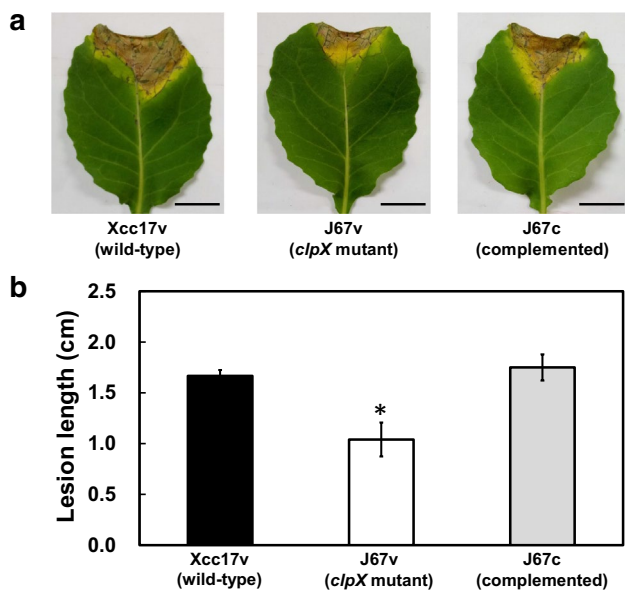
Fig. 3 Effects of mutation of *clpX* on virulence of *X. campestris* pv. *campestris* in cabbage. **a** Black rot symptoms caused by *X. campestris* pv. *campestris* strains on inoculated leaves of host cabbage plant. Images were taken at 14 day post-inoculation. **b** Average lesion lengths caused by *X. campestris* pv. *campestris*. Values presented are the means ± standard deviations from three repeats, each with six leaves. Significance was determined using the Student *t* test (* indicates significance at $p < 0.05$)

mutans, it was found that inactivation of *clpX* results in a higher prevalence of downregulated proteins in *X. campestris* pv. *campestris*. While the exact reason for the presence of downregulated proteins in the *clpX* mutant is not known,

it is speculated that some of the upregulated proteins are the substrates for ClpX. Future analysis of these candidates is necessary for identification targets recognized by ClpX.

Consistent with the observation that the *clpX* mutant showed reduced bacterial attachment and extracellular enzyme production, the protein level expressed by the attachment-related genes (*galU*, *ha*, and *yapH*) and the extracellular enzyme genes (*engA* and *prt1*) was lower in J67 by quantitative proteome analysis (Table 1). Similarly, consistent with the observation that the *clpX* mutant showed a reduction of virulence, the expression of several virulence-related proteins was significantly reduced in the *clpX* mutant, such as XC_0626, XC_1965, XC_2227, XC_2228, and XC_2699 (Table 1). It was reported that: (1) the homologues of XC_0626 and XC_1965 (XOO4035 and XOO2323) were known to have roles in the pathogenicity of *X. oryzae* pv. *oryzae* strain KACC10331 (Wang et al. 2008) (2) the homologues of XC_2227 and XC_2228 (RavS and RavR) were essential for regulation of virulence factor production and pathogenesis of *X. campestris* pv. *campestris* strain XC1 (He et al. 2009) (3) the homologue of XC_2228 of *X. campestris* pv. *campestris* strain ATCC33913 (XCC1958) and *X. oryzae* pv. *oryzae* PXO99[A] (PXO_01019) was important for virulence (Qian et al. 2008; Yang et al. 2012), and (4) XC_2228 and XC_2699 were required for virulence of *X. campestris* pv. *campestris* strain 8004 (Zang et al. 2007; Tao et al. 2014). The transcription of genes encoding these aforementioned proteins showed decreased expression level in the *clpX* mutant compared to wild type by RT-qPCR analysis (Table 1), suggesting ClpX positively regulates the expression of these virulence-related proteins transcriptionally.
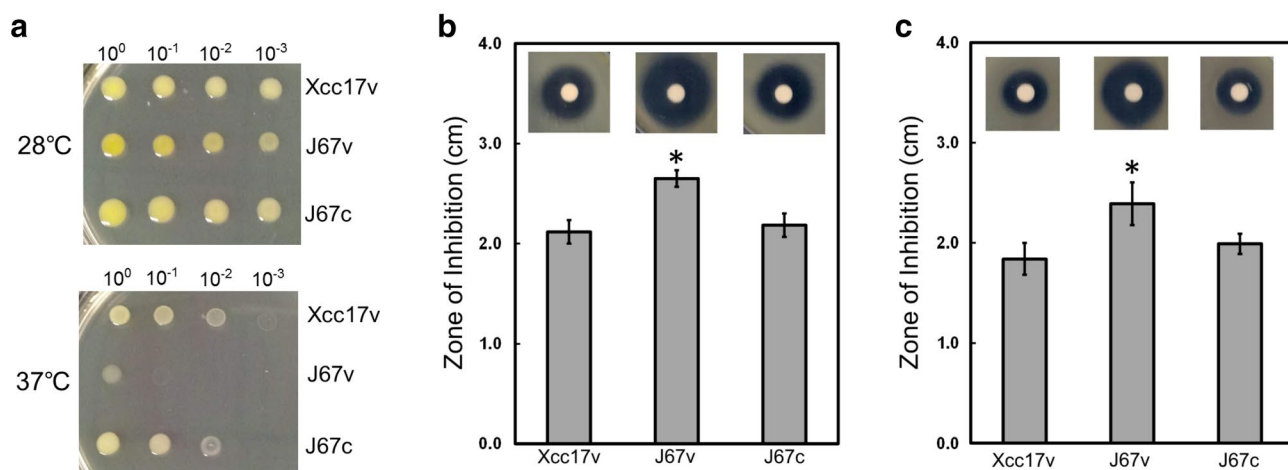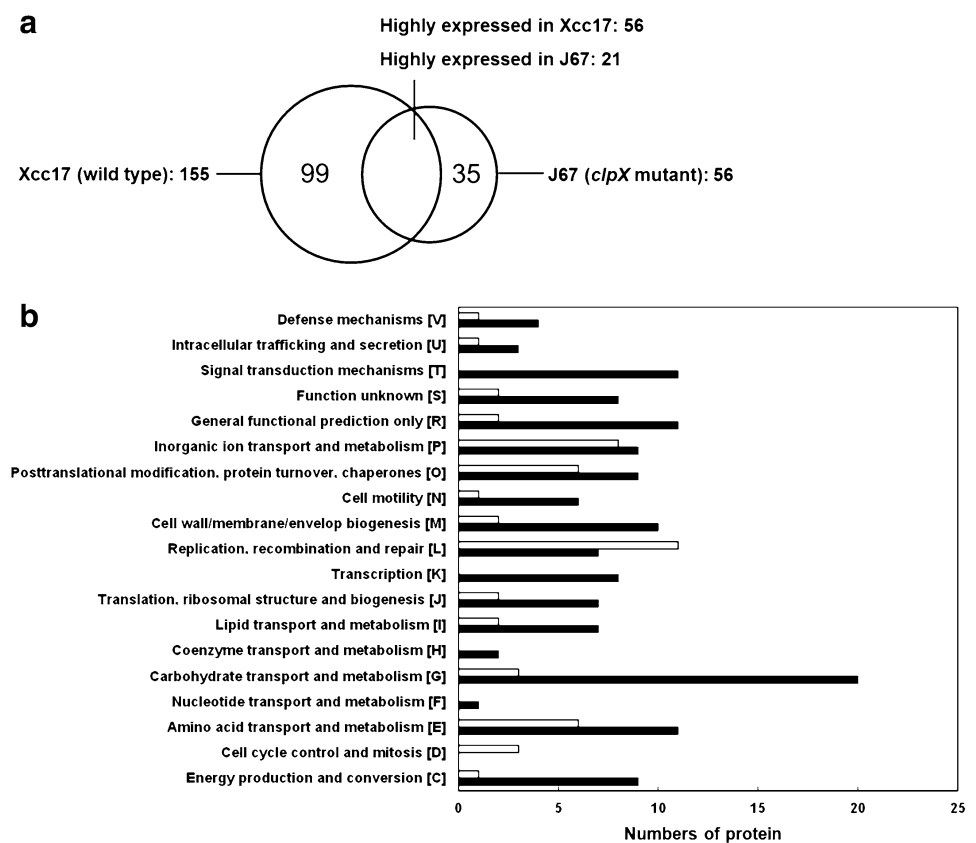

Fig. 4 Effects of mutation of *clpX* on stress tolerance. Heat tolerance (**a**) was performed with tenfold dilution of cells spotted on LB plate and incubated at 28 °C or 37 °C for 3 days. Tolerance to puromycin (**b**) and SDS (**c**) was conducted by disc diffusion assays in which exponentially growing cells were spread on LB plates and discs containing puromycin (5 mg/mL) or SDS (5%) were placed on top. After

3 days of incubation, the inhibitory-zone diameters for cultures were measured and compared. Values presented are the means ± standard deviations from three repeats. The asterisk (*) indicates $p < 0.05$. The cultures tested were: Xcc17v (wild-type Xcc17 with empty vector pRK415); J67v (*clpX* mutant J67 with pRK415); and J67c (complemented *clpX* mutant)

Fig. 5 Differentially expressed proteins identified through proteomic analysis. **a** Venn diagram presents the numbers of differentially expressed proteins found in Xcc17 (wild type) and J67 (*clpX* mutant). **b** COG function analysis of differentially expressed proteins. The black bar and white bar denote proteins in which the expression levels were upregulated and downregulated in Xcc17, respectively. Numbers of proteins in each category are presented on the X-axis. Detailed information concerning the differentially expressed proteins that were identified can be found in Table S2

a
Highly expressed in Xcc17: 56
Highly expressed in J67: 21

Xcc17 (wild type): 155 — 99 35 — J67 (*clpX* mutant): 56

b

Defense mechanisms [V]
Intracellular trafficking and secretion [U]
Signal transduction mechanisms [T]
Function unknown [S]
General functional prediction only [R]
Inorganic ion transport and metabolism [P]
Posttranslational modification, protein turnover, chaperones [O]
Cell motility [N]
Cell wall/membrane/envelop biogenesis [M]
Replication, recombination and repair [L]
Transcription [K]
Translation, ribosomal structure and biogenesis [J]
Lipid transport and metabolism [I]
Coenzyme transport and metabolism [H]
Carbohydrate transport and metabolism [G]
Nucleotide transport and metabolism [F]
Amino acid transport and metabolism [E]
Cell cycle control and mitosis [D]
Energy production and conversion [C]

Numbers of protein

In the *X. campestris* pv. *campestris* genome (da Silva et al. 2002; Qian et al. 2005; Liu et al. 2015), three Clp ATPase-encoding genes (*clpA*, *clpX*, and *clpY*) and two Clp protease-encoding genes (*clpP* and *clpQ*) were found, suggesting the bacteria's genome harbors three potential ATP-dependent Clp protease systems: ClpAP, ClpXP, and ClpYQ. The proteomic analysis showed that, in the wild-type strain and the *clpX* mutant, the expression of ClpA was at the same level, whereas the expression of ClpY and ClpQ was increased in the *clpX* mutant and the expression of ClpX was significantly reduced in the *clpX* mutant (Table 1). The altered expression of these proteins was further validated by RT-qPCR. Further, the transcription of *clpP* was unaffected through RT-qPCR analysis, even the ClpP protein was not identified by proteomic analysis. These results contrast with the results obtained for *B. anthracis*, in which loss of *clpX* results in increased expression of *clpP2* (Claunch et al. 2018). While the expression of ClpA and ClpP was not altered, it is worthy to notice that ClpYQ, another putative ATP-dependent Clp protease, was found to be upregulated in the *clpX* mutant strain, suggesting an attempt by the cells to compensate for the loss of ClpX (or ClpXP) function. At present, it is not clear why ClpX appears to influence the expression of ClpYQ, and this warrants further analysis.

Conclusion

We previously screened approximately 1000 EZ-Tn*5* mutants for their ability to form biofilm and identified several biofilm-related genes that have been further demonstrated to have a role in virulence (Liao et al. 2016, 2019; Liu et al. 2018). Here, we pursued this work and identified one transposon mutant showing reduced bacterial attachment and exhibiting an insertion in *clpX* gene. In addition to several sequenced *X. campestris* pv. *campestris* strains (da Silva et al. 2002; Qian et al. 2005; Liu et al. 2015), the *clpX* gene has been annotated in other sequenced *Xanthomonas* species, such as *X. axonopodis* pv. *citri* (da Silva et al. 2002), *X. campestris* pv. *vesicatoria* (Thieme et al. 2005), *X. gardneri* (Richard et al. 2017), *X. oryzae* pv. *oryzae* (Lee et al. 2005), and *X. oryzae* pv. *oryzicola* (Bogdanove et al. 2011). Although the *clpX* gene exists in several *Xanthomonas* species, nothing is known regarding its biological functions. In this study, we provided conclusive genetic evidence demonstrating that *clpX* gene possesses multifarious pathogenicity-related functions (such as bacterial attachment, virulence factor synthesis, and pathogenesis) and is involved in a range of stress tolerances (heat, puromycin, and SDS)

of *X. campestris* pv. *campestris*. Further, we confirmed that ClpX positively regulates the transcription of genes related to attachment and extracellular enzyme synthesis, as well as genes associated with virulence. Additionally, we employed a quantitative proteomic approach, coupling nLC to MS/MS, to compare the protein profiles between the wild type and *clpX* mutant strains to obtain a general picture of ClpX function. Through the preliminary comparison of the protein profiles of the two strains, it was found that *clpX* mutation had a considerable effect on the bacterial proteome and 211 altered proteins (a greater than twofold difference) belonging to different COG category were discerned. To the best of our knowledge, this is the first report on the functional aspects of ClpX in *Xanthomonas*.

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