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Functional Characterization and Transcriptional Analysis of *clpP* of *Xanthomonas campestris* pv. *campestris*

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Abstract

The caseinolytic protease (Clp) system is essential for survival under stress conditions and for virulence in several pathogenic bacteria. *Xanthomonas campestris* pv. *campestris* (Xcc) is a plant pathogen which causes black rot disease in crucifers. In this study, the Xcc *clpP* gene which is annotated to encode the proteolytic core of Clp was characterized. Mutation of *clpP* resulted in susceptibility to high temperature and puromycin stresses. Site-directed mutagenesis revealed that S105, H130, and D179 are critical amino acid residues for ClpP function in puromycin tolerance. Inactivation of *clpP* also revealed an attenuation of virulence on the host plant and a reduction in the production of extracellular cellulase, mannanase, pectinase, and protease. The affected phenotypes of the *clpP* mutant could be complemented to wild-type levels by the intact *clpP* gene. Transcriptional analysis revealed that expression of *clpP* is induced under heat shock condition.

Introduction

Pathogenic bacteria often encounter different stresses exerted by the external environment. These stresses include antimicrobial chemicals, changes in osmolarity, pH, and temperature [1]. To conquer harmful situations, bacterial cells are equipped with various mechanisms to enable them to adapt to heterogeneous environments. Caseinolytic protease (Clp) system plays an essential role in protein quality control and stress management [2]. In addition to its involvement in protein homeostasis and stress tolerance, the Clp protein has a wide range of functions, such as expression of pathogenicity factor and regulation of developmental process in bacteria [2, 3].

The members of the Clp family composed of two functional units: proteolytic core (ClpP and ClpQ) and ATPase-active chaperone rings (ClpA, ClpC, ClpE, ClpX, and ClpY) [4]. The ClpP protease can interact with different chaperons, namely ClpA, ClpC, ClpE, and ClpX; while ClpQ associates with ClpY to form active proteolytic machinery [4]. The ClpP is a conserved protein that is present in nearly all sequence eubacterial genomes [2]. ClpP is a serine protease characterized by a unique arrangement of the active site triad, Ser-His-Asp and is found to be involved in the proteolysis of damaged and misfolded proteins, regulatory proteins, and ribosome-stalled proteins in several species [5]. In addition, ClpP is important for the degradation of proteins involved in biofilm formation, cell motility, heat stress response, metabolism, nutrient starvation, and stationary phase adaptation [5]. In a number of pathogenic bacteria, ClpP function plays a vital role in infectivity and virulence [5].

Xanthomonas campestris pv. *campestris* (Xcc), a plant pathogen, is the causal agent of black rot on leaves of economically important crops such as cabbage, cauliflower, and radish [6]. Xcc is known to produce a variety of virulence factors such as exopolysaccharide and extracellular enzymes (cellulase, mannanase, pectinase, and protease) [7, 8]. In the sequenced Xcc genome, several putative *clp* genes (such as *clpA*, *clpP*, *clpQ*, *clpX*, and *clpY*) were annotated [9–11]. Despite Clp proteins being studied extensively in other bacteria, the specific functions of Clp proteins in Xcc are nearly unknown. Till now, only one report regarding Xcc Clp proteins was found in the available literature. It was indicated that the Xcc *clpX* gene is important for bacterial attachment, stress tolerance, and virulence [12]. Therefore, in the present study, we explored the function and transcription of *clpP* to gain more insight into the role and regulation of Clp proteins in Xcc.

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Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in the present study are listed in Table 1. *E. coli* strains were cultured in Luria–Bertani (LB) medium [13] at 37 °C. Xcc strains were grown in LB medium or XOLN medium [14] at 28 °C. For liquid cultures, bacteria were shaken at 180 rpm. Agar (1.5%) was added for solid media. The concentrations of the antibiotics used were as follows: ampicillin (50 μg/mL), gentamycin (15 μg/mL), kanamycin (50 μg/mL), and tetracycline (15 μg/mL).

DNA Techniques

Molecular biology protocols were as described by Sambrook et al. [13]. Polymerase chain reaction (PCR) was done as previously described [15] using the primers listed in Table 2. DNA sequencing was performed by Mission Biotech Co., Ltd. (Taipei, Taiwan). Transformation of *E. coli* and Xcc was performed by the standard method [13] and electroporation [16], respectively.

Mutant Construction and Complementation

The *clpP* mutant was generated by insertional inactivation of Xc17 and designated as CE17. First, the 717-bp *Pst*I-*Xba*I fragment containing the Xc17 *clpP* gene was amplified by

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
E. coli		
ECOS™ 101	*endA1 recA1 relA1 gyrA96 hsdR17*(r_K^-, m_K^+) *phoA supE44 thi-1* Δ(*lacZYA-argF*) *U169* ϕ80Δ(*lacZ*)M15 F^-	Yeastern
X. campestris pv. campestris		
Xc17	Virulent wild-type strain isolated in Taiwan, Ap^R	[42]
CE17	Xc17-derived mutant with a Gm^R cartridge inserted in *clpP* gene, Ap^R, Gm^R	This study
Plasmid		
yT&A	PCR cloning vector, Ap^R	Yeastern
pTclpP	A 717 bp RCR amplified fragment from *clpP* (nucleotides –77 to + 640 relative to the translation start site) and cloned into yT&A	This study
pTclpPS105A	pTclpP derivative carrying an S to A mutation at positon 105 of *clpP* gene product	This study
pTclpPH130A	pTclpP derivative carrying an H to A mutation at positon 130 of *clpP* gene product	This study
pTclpPD179A	pTclpP derivative carrying a D to A mutation at positon 179 of *clpP* gene product	This study
pOK12	*E. coli* general cloning vector, P15A *ori*, *lacZα* fragment, Km^R	[17]
pUCGM	Small broad-host-range Gm^R cartridge contained in pUC1918, a pUC19 derivative, Ap^R, Gm^R	[18]
pOKclpP	The 717 bp *Pst*I-*Xba*I fragment of the pTclpP cloned into the *Pst*I and *Xba*I sites of pOK12	This study
pOKclpPG	The 855 bp *SmaI* fragment of the pUCGM cloned into the *EcoR*V site of pOKclpP	This study
pRK415	Broad-host-range vector, RK2 *ori*, Tc^R	[19]
pRKclpP	The 717 bp *Pst*I-*Xba*I fragment of the pTclpP cloned into the *Pst*I and *Xba*I sites of pRK415	This study
pRKclpPS105A	The 717 bp *Pst*I-*Xba*I fragment of the pTclpPS105A cloned into the *Pst*I and *Xba*I sites of pRK415	This study
pRKclpPH130A	The 717 bp *Pst*I-*Xba*I fragment of the pTclpPH130A cloned into the *Pst*I and *Xba*I sites of pRK415	This study
pRKclpPD179A	The 717 bp *Pst*I-*Xba*I fragment of the pTclpPD179A cloned into the *Pst*I and *Xba*I sites of pRK415	This study
pFY13–9	Promoter-probing vector derived from pRK415, using *lacZ* as the reporter, Tc^R	[21]
pFYclpP1	The 375-bp fragment, − 384/− 10 relative to *clpP* translation start site, cloned into the *Xho*I/*Xba*I sites of pFY13-9	This study
pFYclpP2	The 245-bp fragment, − 254/− 10 relative to *clpP* translation start site, cloned into the *Pst*I/*Xba*I sites of pFY13-9	This study
pFYclpP3	The 187-bp fragment, − 196/− 10 relative to *clpP* translation start site, cloned into the *Xho*I/*Xba*I sites of pFY13-9	This study

Ap^R ampicillin-resistant, Gm^R gentamycin-resistant, Km^R kanamycin-resistant, Tc^R tetracycline-resistant

Table 2 Primers used in this study

Primer	Sequence[a]	Direction and use
1315XhoI	5′-CTCGAGGGGTTCATGGACGCCGCT-3′	F, promoter analysis
1445XhoI	5′-CTCGAGGAGCCGGAACAGGTCATTGA-3′	F, promoter analysis
1622PstI	5′-CTGCAGCCCGCACCCGTTTAGCCA-3′	F, mutant construction, confirmation and complementation
1689XbaI	5′-TCTAGATGTTGTGGCAGCGGCCTGTG-3′	R, promoter analysis
2338XbaI	5′-TCTAGAGGCGTCTGCGACTCAAGACG-3′	R, mutant construction, confirmation and complementation
S105AF	5′-GCCAGGCGGCC**GCG**ATGGGCGCG-3′	F, site-directed mutagenesis
S105AR	5′-CGCGCCCAT**CGC**GGCCGCCTGGC-3′	R, site-directed mutagenesis
H130AF	5′-CGCGCGTGATGATC**GCT**CAGCCGCTGGGC-3′	F, site-directed mutagenesis
H130AR	5′-GCCCAGCGGCTG**AGC**GATCATCACGCGCG-3′	R, site-directed mutagenesis
D179AF	5′-CGACACCGAACGC**GCC**AACTTCAAGAGCG-3′	F, site-directed mutagenesis
D179AR	5′-CGCTCTTGAAGTT**GGC**GCGTTCGGTGTCG-3′	R, site-directed mutagenesis

F forward direction, *R* reverse direction

[a]Added restriction enzyme sites are underlined; the mutated bases are in boldface and underlined

PCR using primer pair 1622PstI/2338XbaI and ligated into the yT&A cloning vector (Yeastern), giving pTclpP. After sequencing verification, the 717-bp *Pst*I/*Xba*I fragment from pTclpP was cloned into pOK12 [17], generating pOKclpP. Then, the gentamycin resistance gene (Gm^R cartridge) from pUCGM [18] was inserted into the *EcoR*V site within the pOKclpP insert. The resultant plasmid, pOKclpPG, was electroporated into Xc17 allowing for double crossover. Insertion of Gm^R cartridge into *clpP* gene was confirmed by PCR.

For complementation, the 717-bp *Pst*I-*Xba*I fragment from pTclpP was cloned into pRK415 [19]. The generating plasmid, pRKclpP, was then introduced into the *clpP* mutant CE17 by electroporation, resulting in the complemented strain CE17(pRKclpP). In parallel, the empty vector pRK415 was transferred into Xc17 and CE17, giving Xc17(pRK415) and CE17(pRK415) for comparison.

Site-Directed Mutagenesis

The site-directed mutagenesis of ClpP was conducted using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), according to the manufacturer's instructions. The mutation was constructed in the highly conserved active site residues of ClpP (S105, H130, or D179), using pTclpP as a template and the primers listed in Table 2, generating pTclpPS105A, pTclpPH130A, and pTclpPD179A. After DNA sequence confirmation, the mutated *clpP* was cloned into pRK415 to give pRKclpPS105A, pRKclpPH130A, and pRKclpPD179A. These constructs were separately electroporated into CE17, generating CE17(pRKclpPS105A), CE17(pRKclpPH130A), and CE17(pRKclpPD179A).

Stress Tolerance Assay

The methods for temperature tolerance assay and evaluating the sensitivity of bacteria to hydrogen peroxide (H_2O_2), sodium dodecyl sulfate (SDS), and puromycin are according to previously study [12]. The effect of temperature on bacterial growth was also determined quantitatively in liquid culture. Strains to be assayed were grown in LB medium and incubated at 28 °C or 37 °C with shaking (180 rpm). Samples were taken at designated intervals, and the growth of each tested strain was evaluated by measuring the OD_{550} values. These experiments were performed at least three times.

Virulence Assay and Extracellular Enzyme Activity Analysis

The virulence of Xcc to host plant cabbage was assayed by leaf-clipping method [20] and the lesion lengths were measured 14 days post-inoculation. Six replicates were performed for each strain and three independent experiments were carried out. The activities of extracellular enzymes (cellulase, mannanase, pectinase, and protease) were assessed by substrate-supplemented plate assay method as described previously [20]. The substrates supplemented were carboxymethyl cellulose (CMC, for cellulase), locust bean gum (LBG, for mannanase), sodium polypectate (for pectinase), and skim milk (for protease). The activity of cellulase and mannanase was examined by staining CMC- and LBG- supplemented plates with Congo red, and pectinase activity was examined by staining sodium polypectate containing plates with ruthenium red. Substrate degradation by extracellular enzyme depleted the plate of stain-binding material, forming clearing zones. Protease activity was examined directly by

the appearance of clear zones surrounding the colonies on plates containing skim milk. Each experiment was done at least three times.

Promoter Activity Determination

The upstream region of *clpP* gene was PCR-amplified and cloned into pFY13–9, which is a promoter-probing vector using *lacZ* as the reporter [21]. The resulting constructs pFYclpP1, pFYclpP2, and pFYclpP3 carried nt − 384/− 10, − 254/− 10, and − 196/− 10 regions relative to the *clpP* translational start site, respectively. These constructs were separately introduced into Xc17 by electroporation. Strains carrying these reporter constructs were cultured overnight and then inoculated into fresh media to adjust an initial OD_{550} value of 0.35. Sampling interval was designated and promoter activity was monitored by measuring the β-galactosidase activity of the reporter constructs according to previously described method [22]

Statistical Analysis

Data are presented as the mean of triplicate per measurement, and each experiment was repeated at least three times. Student's *t*-test was used to determine the statistical significance of differences between means.

Results and Discussion

clpP is Required for Heat and Puromycin Tolerance

In the genome of the Xcc strain Xc17, the locus_tag AAW18_RS04850 was annotated to encode ClpP (GenBank accession no. NZ_CP011946) [9]. The Xcc *clpP* gene encodes a 22-kDa product and displays similarity to the family of ClpP proteins found in several bacteria, such as *E. coli* (75.6% identity) and *Bacillus subtilis* (71.4%). Sequence analysis also revealed that the Xcc ClpP protein contains the conserved amino acid residues Ser-105, His-130, and Asp-179 that have been shown to constitute the catalytic triad in *E. coli* [5].

To determine the functions of ClpP in Xcc, the *clpP* mutant designated as CE17 was first constructed by insertional inactivation using wild-type Xc17 as the parental strain. Complementation of the mutant CE17 was performed by transforming plasmid pRKclpP and designated as CE17(pRKclpP). In parallel, parental strain Xc17 and the *clpP* mutant CE17 were transformed with pRK415 [19] to control for any effects of the vector itself and named as Xc17(pRK415) and CE17(pRK415). Further, the *clpP* mutant CE17 was transformed with mutated versions pRKclpPS105A, pRKclpPH130A, and pRKclpPD179A, in which the three above-mentioned amino acids (S105, H130, and D179) were substituted by alanine, to evaluate the role of these potential catalytic residues.

It has indicated that bacteria Clp protease has a multitude of functions, such as stress tolerance and virulence factor expression [3]. To validate the involvement of *clpP* in stress adaptation of Xcc, the wild-type Xc17(pRK415), *clpP* mutant CE17(pRK415), and complemented strain CE17(pRKclpP) were subjected to heat (37 °C), oxidative stress (1% H_2O_2), detergent (5% SDS), and antibiotic (5 mg/mL puromycin) treatments. Under standard culture conditions, i.e., 28 °C on LB plate, the *clpP* mutant grew as well as the wild-type and the complemented strains (Fig. 1a, upper). However, at 37 °C on LB plate, the growth of *clpP* mutant was completely arrested (Fig. 1a, lower). This growth deficiency was restored by genetic complementation (Fig. 1a, lower). Comparison of the growth curves of wild-type and mutant strains revealed no significant difference in LB medium at 28 °C (Fig. 1b, upper), whereas the growth of *clpP* mutant at 37 °C was much slower than that of both wild-type and complemented strains (Fig. 1b, lower). These results demonstrated that ClpP is not required for optimal growth at physiological temperature, whereas it has an important role in growth of Xcc at high temperature. The *clpP* mutant did not differ from the parent strain in sensitivity to H_2O_2 and SDS (Fig. 2a, b). However, the *clpP* mutant CE17(pRK415) was more sensitive to puromycin than its parent Xc17(pRK415) (Fig. 2c). The sensitivity of the *clpP* mutant to puromycin was complemented in CE17(pRKclpP), whereas the ability to restore bacteria growth was eliminated when CE17 was complemented with mutated versions (pRKclpPS105A, pRKclpPH130A, and pRKclpPD179A) (Fig. 2c). Taken together, these results imply that ClpP is required for heat and puromycin tolerance and the potential catalytic amino acid residues (S105, H130, and D179) are associated with the full function of ClpP in puromycin tolerance.

The ClpP is involved in the degradation of abnormal or damaged proteins that arise in response to stress treatment [23, 24]. Growth at elevated temperature or in the presence of puromycin, an aminoacyl-tRNA analogue which prematurely aborts protein translation, leads to the accumulation of misfolded proteins [25]. Here, we observed that the *clpP* mutant of Xcc exhibited a reduced growth rate at high temperature and an impaired resistance to puromycin treatment compared to the wild type. The failure of *clpP* mutant to grow at elevated temperature or in the presence of puromycin suggesting ClpP is required for Xcc to deal with conditions that are known to cause proteins to misfold. Several previous studies have demonstrated growth deficits resulting from ClpP deficiency under heat treatment, including *Actinobacillus pleuropneumoniae* [26], *Lactococcus lactis* [24], *Staphylococcus aureus* [25], *Streptococcus pneumonia* [23],

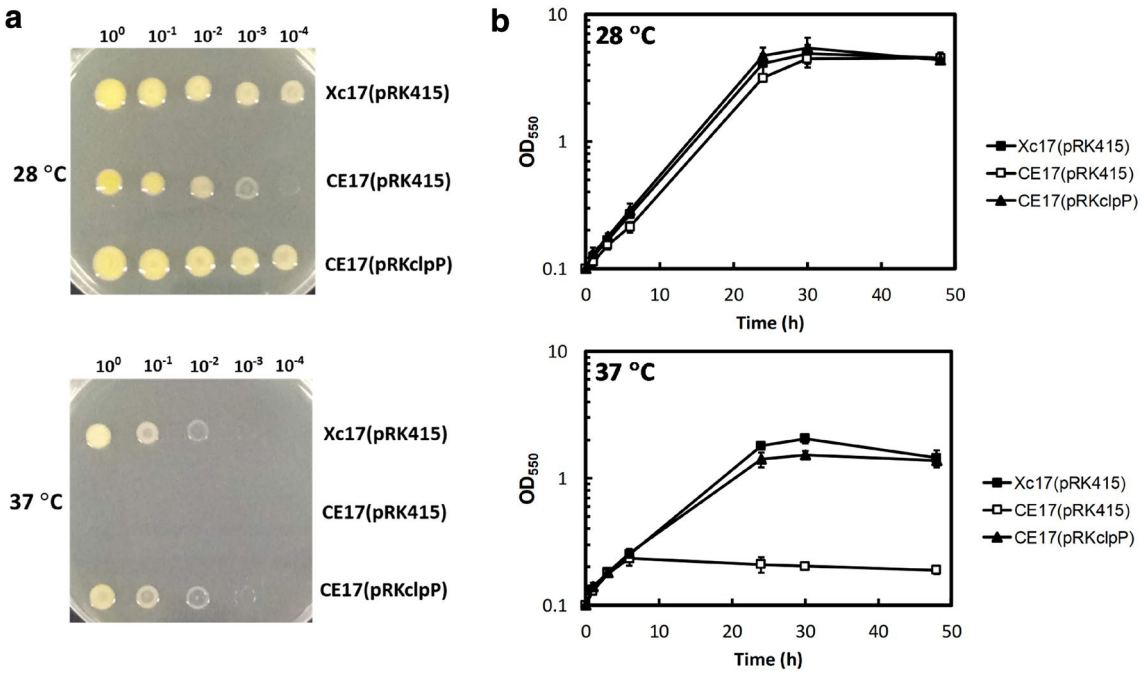

Fig. 1 Effects of mutation of *clpP* on cell growth under heat stress. **a** Bacterial cells were spotted on LB plates and incubated at 28 °C or 37 °C for 3 days. **b** Cells were grown in LB medium at 28 °C or 37 °C and the cell density was measured at OD_{550} following cell growth. Results are the means of at least three independent experiments. Error bars indicate standard deviations

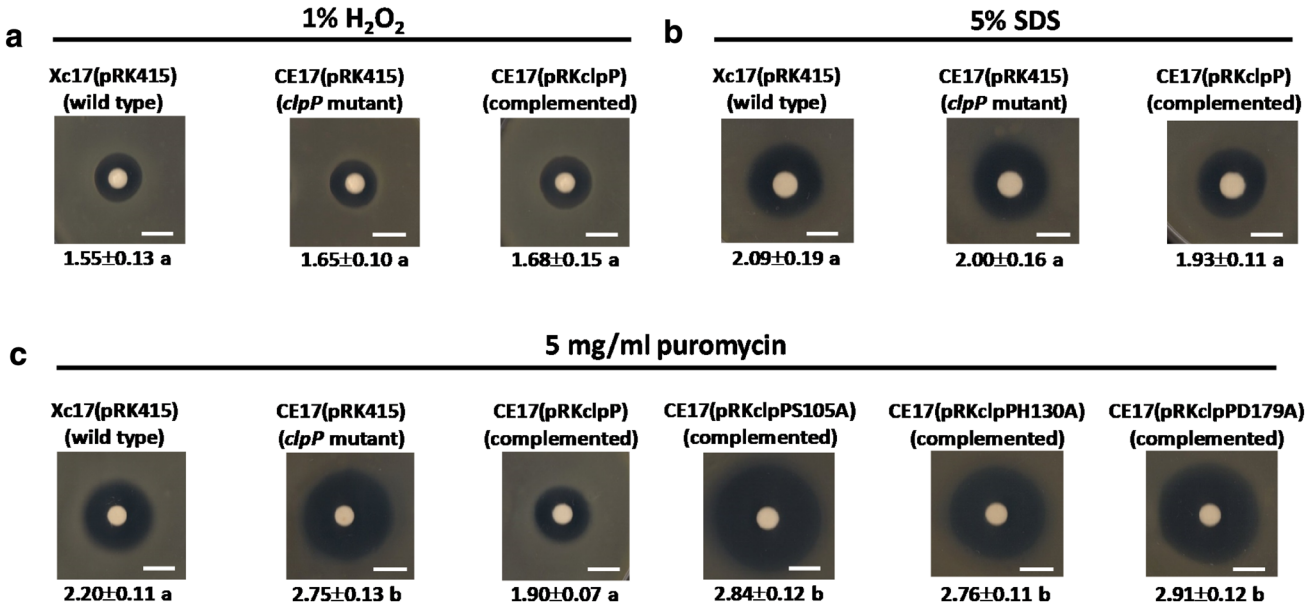

Fig. 2 Sensitivity of *clpP* mutant to H_2O_2, SDS, and puromycin stresses. Bacterial cells were spread on LB plates and disks containing 10 µl of 1% H_2O_2 (**a**), 5% SDS (**b**), and 5 mg/mL puromycin (**c**) were placed on top. After 3 days of incubation, the diameters of inhibition zones were measured. Values under each photographs represent the mean diameter of inhibition zone (in cm) (mean ± standard deviation) from three independent experiments. Different letters following the values indicate significant difference (*t*-test, $P < 0.01$)

and *Streptococcus suis* [27]. The *clpP* mutants of *L. lactis*, *S. aureus* and *S. pneumonia* were more sensitive to puromycin than wild-type cells [23–25].

clpP is Essential for Full Virulence of Xcc and is Involved in Extracellular Enzyme Production

To investigate whether *clpP* is involved in the pathogenicity of Xcc, the constructed strains were inoculated onto host plant cabbage by leaf-clipping methods. Fourteen days after inoculation, the *clpP* mutant CE17(pRK415) produced disease symptom with a mean lesion length of 0.93 cm on the infected leaves, whereas typical V-shaped disease symptoms with mean lesion lengths of 1.70 and 1.75 cm were observed on the leaves inoculated with the wild-type strain Xc17(pRK415) and the complemented strain CE17(pRK415), respectively (Fig. 3a). Xcc produces exopolysaccharide and a range of extracellular enzymes which collectively essential for pathogenesis. To evaluate whether *clpP* influences the synthesis of these virulence factors, the exopolysaccharide yields and the activities of four extracellular enzymes produced by the *clpP* mutant were determined. The results revealed that the amounts of exopolysaccharide produced by the mutant were similar to those produced by the wild type (data not shown). However, the *clpP* mutant produced a lower level of extracellular enzymes, including cellulase, mannanase, pectinase, and protease (Fig. 3b). The diameters of hydrolytic zone produced by the *clpP* mutant were significantly lower than those observed for the wild-type and complemented strains (Fig. 3b). Taken together, these results demonstrated that *clpP* is essential for full virulence of Xcc and is required for the ability to synthesize extracellular enzymes.

The ClpP plays an important role in virulence of several pathogens, such as *Dickeya dadantii* [28], *Listeria monocytogene* [29], *S. aureus* [25], *S. pneumonia* [23], and *S. suis* [27]. In *D. dadantii*, inactivation of *clpP* reduced the production of pectinolytic enzyme, which has a major role in the pathogenicity of this organism [28]. In *L. monocytogene*, mutation of *clpP* reduced the haemolytic activity of the major listeria virulence factor, listeriolysin O [29]. The amount of extracellular enzymes and toxins in *S. aureus* is reduced in the absence of ClpP [25]. The expression of *apuA* gene which has been linked to virulence in *S. suis* is reduced in *clpP* mutant [27]. Our observations showing decreased virulence of *clpP* mutant and inactivation of *clpP* affected the ability of the mutant to synthesize a range of extracellular enzymes suggested that reduced virulence factor synthesis might be the main reason for the attenuated virulence. The ClpP might be involved in the expression of these virulence genes and/or the turnover of them, which remain to be characterized. Xcc genome annotation revealed that several virulence factors are related to bacterial pathogenesis. Whether decreased virulence of Xcc *clpP* mutant is linked to the expression of other unidentified pathogenicity determinant(s) merits further investigation.

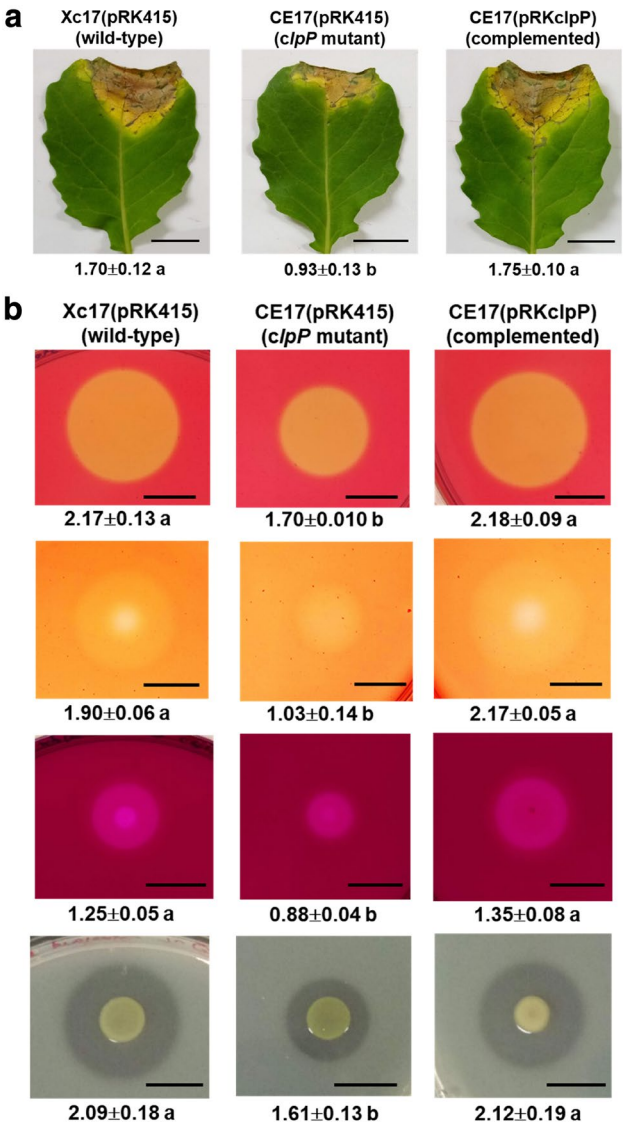

Fig. 3 Effects of mutation of *clpP* on Xcc virulence and extracellular enzyme production. **a** Black rot symptoms caused by Xcc strains on cabbage leaves inoculated by leaf-clipping method. Images were taken at day 14 post-inoculation. Scale bars = 1 cm. Values under each leaf are the mean lesion length (in cm) (mean ± standard deviation) from three repeats, each with six leaves. Different letters following the values indicate significant difference (*t*-test, $P < 0.01$). **b** The activity of extracellular cellulase, mannanase, pectinase, and protease (from top to down) was evaluated using the substrate-supplemented plate assay. Values under each photographs are the mean diameter of hydrolysis zone (in cm) (mean ± standard deviation) from three independent experiments. Different letters following the values indicate significant difference (*t*-test, $P < 0.01$)

In *E. coli*, over 60 proteins involved in numerous biological functions were identified as potential substrates of a proteolytically inactive variant of ClpP [30]. A similar repertoire of substrate candidates has been found for

B. subtilis ClpP [31]. Identification of potential targets of ClpP in these model bacteria indicates that ClpP has a wide range of impact on the bacterial proteome. In addition to *E. coli* and *B. subtilis*, the ClpP is found to be important for the degradation of various proteins, such as damaged and misfolded proteins, regulatory proteins, as well as proteins involved in heat stress response, metabolism, and virulence in several bacteria [5]. Our observations showing ClpP has multiple roles, including heat and puromycin tolerance, extracellular enzyme production, as well as virulence, suggested the involvement of ClpP in several cellular processes in Xcc. Though the potential targets of ClpP are unknown, it is reasonable to suggest that ClpP might be involved in the regulation of the Xcc proteome. Whether ClpP has an impact on Xcc proteome requires further evaluation.

clpP Expression is Induced by Heat Shock

To investigate the expression of *clpP* in Xcc, the upstream regions of *clpP* gene were cloned ahead of a promoterless *lacZ* gene in pFY13–9 [21]. Three reporter constructs (pFYclpP1, pFYclpP2, and pFYclpP3) (Fig. 4a) were introduced into Xc17, generating reporter strains named Xc17(pFYclpP1), Xc17(pFYclpP2) and Xc17(pFYclpP3). The β-galactosidase activity produced by the obtained reporter strains grown in XOLN medium containing glycerol at 28 °C or 37 °C was measured. As shown in Fig. 4b, the β-galactosidase production from the same constructs did not change over the course of different time interval at 28 °C, indicating that the *clpP* promoter is independent on the growth phase of the cell under physiological temperature. At 28 °C, both Xc17(pFYclpP1) and Xc17(pFYclpP2) produced similar β-galactosidase activities (2124 U and 2273 U at 24 h, respectively), whereas Xc17(pFYclpP3) showed the same levels of activity as those of Xc17 carrying vector pFY13–9 (42 U) (Fig. 4b). At 37 °C, Xc17(pFYclpP1) and Xc17(pFYclpP2) expressed 4762 U and 4716 U of β-galactosidase, representing increases of 2.24- and 2.08-fold over the same strains grown under heat stress treatment, respectively. Taken together, these results demonstrated that

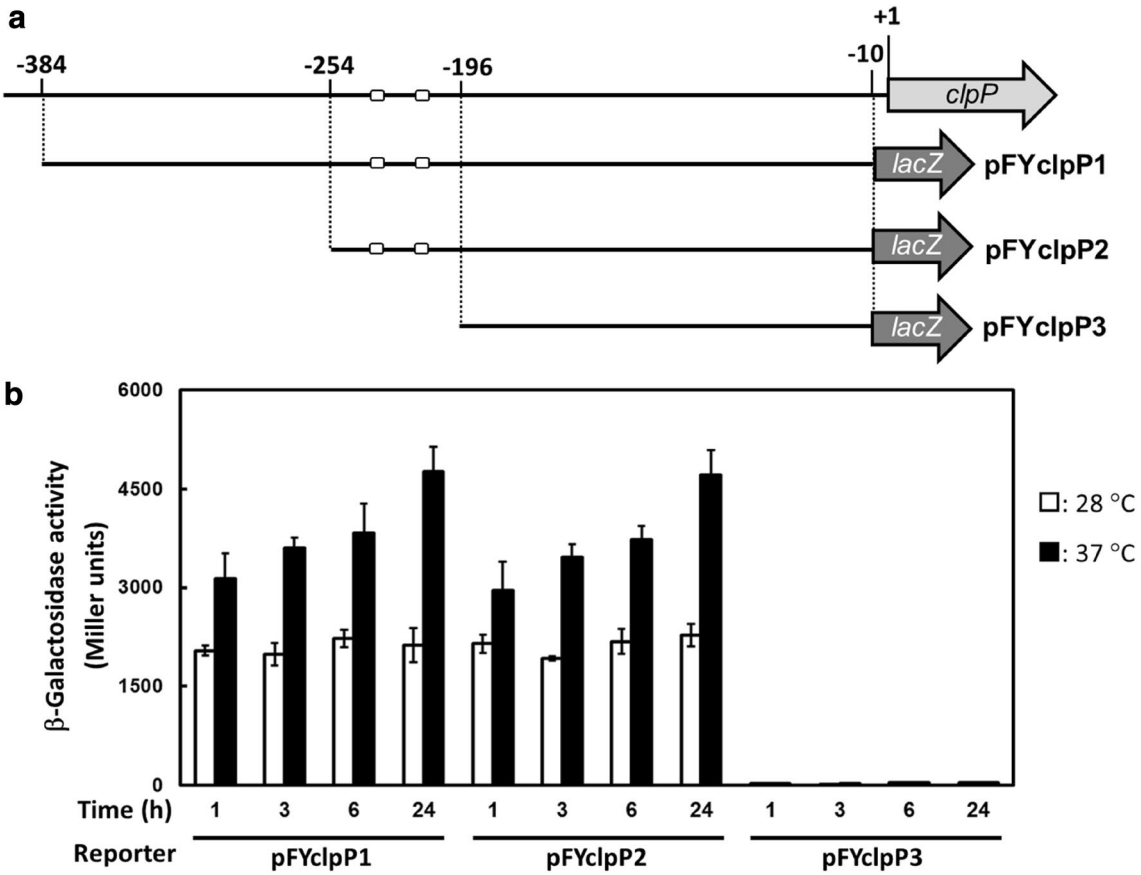

Fig. 4 Characterization of the *clpP* gene upstream region. **a** The upstream regions of the *clpP* gene cloned into the promoter-probing vector pFY13−9 to form reporter constructs pFYclpP1, pFYclpP2, and pFYclpP3. The regions containing the putative σ^{32} promoter elements are thickened. **b** Promoter activities expressed from different reporter constructs in Xc17. Promoter activity was measured as β-galactosidase activity (Miller units). Results are the means of at least three independent experiments. Error bars indicate standard deviations

the *clpP* expression is growth phase-independent and is under heat shock control. The expression of *clpP* is induced after heat treatment suggesting that ClpP is implicated in the degradation of aberrant proteins occurring after heat shock. Sequence analysis revealed that the upstream regions of the *clpP* gene presence of a putative σ^{32} promoter elements: a − 35 box (TTGAGA) and a − 10 box (CCCCAA CT) located at − 229 and − 207 (with a spacer of 14 nucleotides) relatives to the translation start site, respectively (Fig. 4a). A predicted ribosome-binding site (GGAG) was located 6 nt upstream of the translation stat codon. The predicted − 35/− 10 region (TTGAGA/CCCCAACT) was a close match (underlined bases) to the consensus sequence (TTGAAA/CCCCATNT) of *E. coli* promoters known to be recognized by the σ^{32} factor [32]. In addition, the 14 bp spacing between the − 35 and − 10 regions was as well conserved as those of σ^{32} promoters [32]. The observations that (i) expression from pFYclpP1 and pFYclpP2 (carrying nt − 384/− 10 and − 254/− 10 regions relative to the *clpP* translation start site, which included potential − 35/− 10 elements) gave similar activity and induction fold; and (ii) the level of Xc17(pFYclpP3) (carrying nt − 196/− 10 regions, which did not include − 35/− 10 boxes) was the same as Xc17(pFY13–9), suggesting the − 254/− 10 regions contain the complete promoter sequence and is required for heat stress response.

The expression of *clpP* has been shown to be induced by heat shock in several Gram-positive bacteria, including *Bacillus subtilis* [33, 34], *L. lactis* [24], *L. monocytogene* [29], *S. aureus* [25], *Streptococcus mutans* [35], and *S. pneumoniae* [23]. A putative CtsR (class three stress gene repressor) DNA binding motif was present in the upstream region of *clpP* in *B. subtilis* [36], *L. lactis* [24], *L. monocytogene* [29], and *S. pneumoniae* [23]. Limited reports are available regarding the *clpP* expression in Gram-negative bacteria. The *clpP* of *E. coli* is a σ^{32}-dependent heat shock gene and a twofold induction of transcription is observed after heat treatment [37, 38]. The transcription of *Caulobacter crescentus clpP* was found to be induced by heat shock and the strength of the *clpP* promoter is dependent on the growth phase of the cells [39]. In addition, the upstream region of *clpP* of *C. crescentus* shown to resemble the previously identified σ^{32}-dependent heat shock promoter [39]. σ^{32} controls the expression of heat shock genes in *E. coli* and is broadly distributed in proteobacteria [32]. Here, a typical σ^{32}-dependent promoter was identified upstream of the Xcc *clpP* and transcription of *clpP* was under heat control indicating the *clpP* gene is regulated by σ^{32} in this organism. Little information is available regarding the σ^{32}-dependent promoter in Xcc. Prior to the present study, it was only known that the transcription of *grpE*, *dnaK,* and *hspA* with σ^{32}-dependent promoter is induced in the presence of heat stress [40, 41]. Our results extend the insights of σ^{32} regulon in Xcc, although the regulatory mechanism remains to be elucidated.

Conclusion

In this work, we investigated the function and transcription of *clpP* gene of Xcc, the causative agent of black rot disease brassica corps worldwide. We constructed the *clpP* mutant by homologous recombination to examine the function of ClpP. The *clpP* mutant was shown to display a pleiotropic phenotype, including an impaired growth at high temperature, a reduced tolerance in response to puromycin treatment, an attenuation of virulence, and a defect in extracellular enzyme production. Genetic complementation of wild-type *clpP* could restore these altered phenotypes. Promoter activity assays indicated that *clpP* expression was induced by heat shock. The ClpP proteolytic activity is activated upon association with members of the ATPase-active chaperons [4], and such members have been annotated in Xcc genome [9–11]. The ClpX is involved in bacterial attachment, stress tolerance, and virulence in Xcc [12]. In our future work, we will determine whether ClpX (or ClpA) interacts with ClpP and what role they play in modulating the proteolytic activity in Xcc under physiological and stress conditions.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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