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**Notes**

# Mutation in $clp_{xoo4158}$ Reduces Virulence and Resistance to Oxidative Stress in *Xanthomonas oryzae* pv. *oryzae* KACC10859

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**Cyclic AMP receptor-like protein (Clp), is known to be a global transcriptional regulator for the expression of virulence factors in *Xanthomonas campestris* pv. *campestris* (*Xcc*). Sequence analysis showed that *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) contains a gene that is strongly homologous to the *Xcc clp*. In order to determine the role of the Clp homolog in *Xoo*, a marker exchange mutant of $clp_{xoo4158}$ was generated. Virulence and virulence factors, such as the production of cellulase, xylanase, and extracellular polysaccharides (EPS) and swarming motility were significantly decreased in the $clp_{xoo4158}$ mutant. Moreover, the mutation caused the strain to be more sensitive to hydrogen peroxide and to over-produce siderophores. Complementation of the mutant restored the mutation-related phenotypes. Expression of $clp_{xoo4158}$, assessed by reverse-transcription real-time PCR and *clp* promoter activity, was significantly reduced in the *rpfB*, *rpfF*, *rpfC*, and *rpfG* mutants. These results suggest that the *clp* homolog, $clp_{xoo4158}$, is involved in the control of virulence and resistance against oxidative stress, and that expression of the gene is controlled by RpfC and RpfG through a diffusible signal factor (DSF) signal in *Xanthomonas oryzae* pv. *oryzae* KACC10859.**

***Keywords*** **:** bacterial blight, Clp, oxidative stress, *rpf* gene, *Xanthomonas oryze* pv. *oryzae*

Cell to cell communication plays an important role in the regulation of pathogenicity in many plant pathogenic bacteria (Chatterjee et al., 2002, 2008; He et al., 2008). Rpfs are well known to regulate pathogenicity by cell to cell communication in *Xanthomonas campestris* pv. *campestris* (*Xcc*), a causal agent of black rot in crucifers (Dow et al., 2000). RpfB and RpfF produce a diffusible signal factor (DSF), and RpfC and RpfG are able to sense the DSF signal and transfer it to downstream genes as a two-component system (He et al., 2008; Slater et al., 2000). In previously study, we confirmed that function of *X. oryzae* pv. *oryzae* Rpfs are similar in the *Xcc* Rpfs and lack of *X. oryzae* pv. *oryzae* Rpfs lead to deficiency of virulence (Jeong et al., 2008). Production of a number of pathogenicity factors, including extracellular polysaccharides (EPS) and enzymes, is controlled by this signal (Thowthampitak et al., 2008).

Clp (cyclic AMP receptor-like protein), a conserved global regulator which shows strong homology to the cAMP nucleotide receptor protein, Crp, in *Escherichia coli*, mediates DSF regulation of virulence factor production in *Xcc* (He et al., 2007; Ryan et al., 2007). RpfG containing HD-GYP domain, proposed function in hydrolysis of cyclic di-GMP (Ryan et al., 2007), which induces the expression of Clp (He et al., 2007). Deletion of *clp* followed by a microarray assay indicates that Clp regulates a large set of genes, including virulence genes for EPS and extracellular enzymes in *Xcc* (He et al., 2007).

The *rpf* gene homologs are well conserved in many xanthomonads and closely related bacteria (Lee et al., 2006). Our previous studies showed that functions of Rpfs were also conserved in *Xanthomonas oryzae* pv. *oryzae* (Jeong et al., 2008). Sequence analysis indicated that the *clp* homolog is well conserved in the *Xoo* genome (Lee et al., 2005). There is only 1 copy of the *clp* homolog, $clp_{xoo4158}$, which is 99% homologous to $clp_{xcc}$ in amino acid sequence and 91% in nucleotide sequence. The function and expression of $clp_{xoo4158}$ in *Xoo* KACC10859 were analyzed in this study. Results suggest that $Clp_{xoo4158}$ controls not only the production of virulence factors including cellulase, xylanase, and EPS, but also oxidative stress and iron uptake in *Xoo*.

## Reduction of virulence and virulence factors by mutation of the $clp_{xoo4158}$ gene

The $clp_{xoo4158}$ gene was cloned by PCR amplification (primers:

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**Table 1.** Virulence and virulence-related phenotypes of the $clp_{xoo4158}$ mutant and its complement strain

| Strain | Lesion lengths[a] | | Phenotype assay[a] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Cellulase activity | | Xylanase activity | | EPS dry weight | |
| | cm (Aver.) | fold (% WT) | cm (Aver.) | fold (% WT) | O.D590 (Aver.) | fold (% WT) | mg (Aver.) | fold (% WT) |
| *Xoo* KACC10859 | 25.5a | 100 | 4.5a | 100 | 0.085a | 100 | 85.5a | 100 |
| CBNUXO12 (*clp*::Tn*5*) | 7.2b | 28 ± 4.5 | 2.5b | 55 ± 0.5 | 0.053b | 62 ± 0.5 | 50.4b | 59 ± 5.5 |
| CBNUXO13 (*clp*::Tn*5*/*clp*) | 24.5a | 96 ± 2.5 | 4.3a | 95 ± 3.0 | 0.081a | 96 ± 3.0 | 82.1a | 96 ± 1.5 |

[a]Means followed by a common letter are not significantly different at the 5% level by Duncan's multiple range test.

5'-ATGAGCTCAGCAAACACGAC-3' and 5'-CAGCCTGCAGCTTCTTGAG-3') and inactivated by marker exchange in *Xoo* KACC10859 using the EZ-Tn*5* <KAN-2> Transposome™ (Jeong et al., 2008). The lesion length of CBNUXO12 ($clp_{xoo4158}$::Tn*5*) on the susceptible rice cultivar Milyang 23ho was reduced to less than 30% of the wild type (Table 1). EPS and enzymes including xylanase, cellulase, and swarming motility determined as previously described (Jeong et al., 2008; Shen et al., 2001), were also significantly reduced in CBNUXO12 ($clp_{xoo4158}$::Tn*5*) (Table 1). Complementation with $clp_{xoo4158}$ caused restoration in virulence and production in cellulase, xylanase, and EPS (Table 1). The swarming motility of CBNUXO12 ($clp_{xoo4158}$::Tn*5*) was greatly reduced, compared with *Xoo* KACC10859. The complemented strain, CBNUXO13 ($clp_{xoo4158}$::Tn*5*/$clp_{xoo4158}$), had swarming motility restored (Fig. 1). These results indicate that the mutation in $clp_{xoo4158}$ reduces xylanse, cellulase, EPS, and motility, which are known to be important virulence factors in rice pathogen (Dharmapuri et al., 2001; He et al., 2007; Rajeshwari et al., 2005).

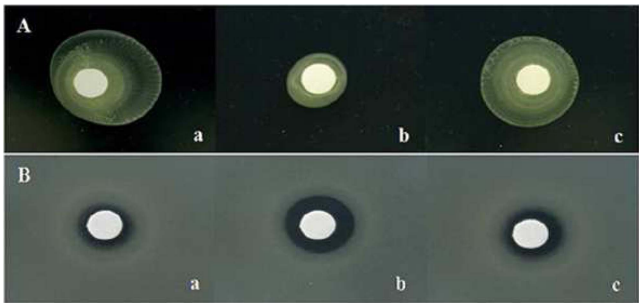


**Fig. 1.** Swarming motility (A), and sensitivity to hydrogen peroxide (B) of the $clp_{xoo4158}$ mutant and its complement strain. a: wild type strain, *Xoo* KACC1085, b: CBNUXO12 ($clp_{xoo4158}$::Tn*5*), c: CBNUXO13 ($clp_{xoo4158}$::Tn*5*/$clp_{xoo4158}$).

## Hypersensitivity to hydrogen peroxide and over-production of siderophores in the $clp_{xoo4158}$ mutant

Sensitivity to hydrogen peroxide was assayed by the disk diffusion method of growth inhibition. The growth inhibition zone was significantly increased in CBNUXO12 ($clp_{xoo4158}$::Tn*5*) than in the wild type strain, *Xoo* KACC10859 and the complementation strain, CBNUXO13 ($clp_{xoo4158}$::Tn*5*/$clp_{xoo4158}$) (Fig. 1). This result suggests that the $clp_{xoo4158}$ mutant is less tolerant to oxidative stress. The $clp_{xoo4158}$ mutant strain produced many more siderophores than the wild type strain, *Xoo* KACC10859 and the complementation strain, CBNUXO13 ($clp_{xoo4158}$::Tn*5*/$clp_{xoo4158}$), as revealed by the chrome azurole S (CAS) assay (Chatterjee et al., 2002; Schwyn et al., 1987) (Fig. 2). In many plant pathogenic bacteria, iron is the critical factor for survival and infection of the host (Subramoni et al., 2005).

These results suggest that the $clp_{xoo4158}$ gene is involved in resistance to oxidative stress and iron uptake in *Xoo* KACC10859. He et al. (2007) conducted a microarray analysis and suggested that $clp_{xcc}$ gene product is involved in iron uptake. However, our result was the first to suggest Clp involvement in resistance to oxidative stress.

## Expression of $clp_{xoo4158}$ controlled by RpfC and RpfG

Expression of $clp_{xoo4158}$ was analyzed by reverse-transcription real-time PCR and an assay of $clp_{xoo4158}$ promoter

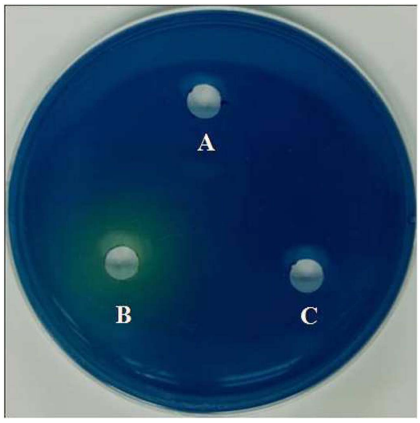


**Fig. 2.** Siderophores production of the $clp_{xoo4158}$ mutant and its complement strain on peptone sucrose agar-chorome azurole S (PSA-CAS). A: wild type strain, *Xoo* KACC1085, B: CBNUXO12 ($clp_{xoo4158}$::Tn*5*), C: CBNUXO13 ($clp_{xoo4158}$::Tn*5*/$clp_{xoo4158}$).

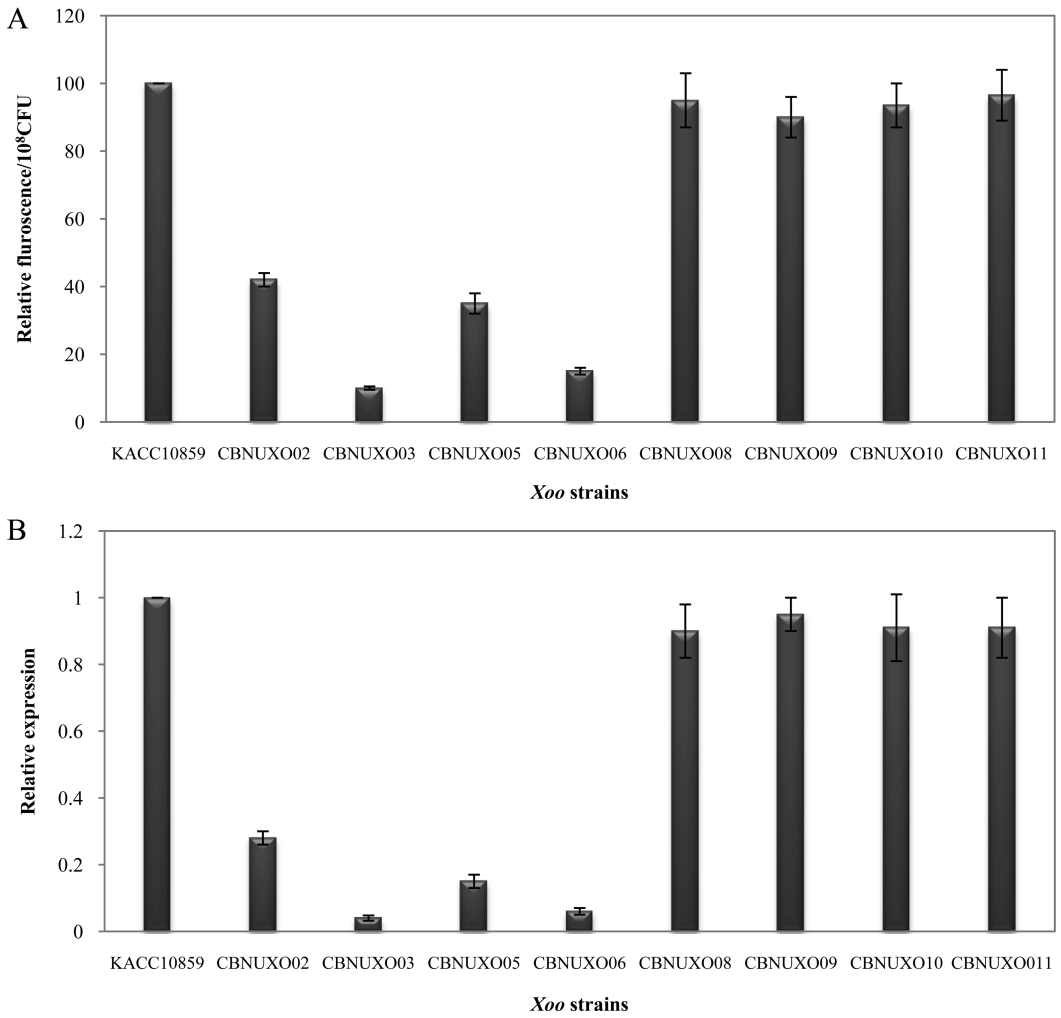


**Fig. 3.** Expression of the $clp_{xoo4158}$ gene in the different *rpfs* mutant, which were determined by promoter activity (A), and reverse-transcription real-time PCR (B). The KACC10859 (wild type), CBNUXO02: *rpfB* mutant strain (*rpfB*::Tn*5*), CBNUXO03: *rpfC* mutant strain (*rpfC*::Tn*5*), CBNUXO05: *rpfF* mutant strain (*rpfF*::Tn*5*), CBNUXO06: *rpfG* mutant strain (*rpfG*::Tn*5*), CBNUXO8: *rpfB* complementation strain, (*rpfB*::Tn*5*/*rpfB*) CBNUXO9: *rpfC* complementation strain, (*rpfC*::Tn*5*/*rpfC*), CBNUXO10 *rpfF* complementation strain, (*rpfF*::Tn*5*/*rpfF*), CBNUXO11: *rpfG* complementation strain, (*rpfG*::Tn*5*/*rpfG*). Mean and standard deviation were calculated from three independent experiments.

activity, in which the green fluorescent protein (GFP) was used as a reporter. The methods of DeAngelis et al. (2005) and Csaki et al. (2003) were used for reporter gene fusion and GFP fluorescence measurement. The expression of $clp_{xoo4158}$ was significantly reduced in CBNUXO02 (*rpfB*::Tn*5*), CBNUXO03 (*rpfC*::Tn*5*), CBNUXO05 (*rpfF*::Tn*5*) and CBNUXO06 (*rpfG*::Tn*5*) (Fig. 3). The expression of $clp_{xoo4158}$, analyzed by reverse-transcription real-time PCR, was similar in the different *rpf* mutants, as showed by the GFP reporter and all gene expression were restore in their complementation strains (Fig. 3). These results suggest that expression of $clp_{xoo4158}$ is controlled by RpfB, RpfF, RpfC, and RpfG. RpfG has been shown to control *clp* in *Xcc* (He et al., 2007; Ryan et al., 2007), and our previous study (Jeong et al., 2008) suggests that the functions of RpfB, RpfF, RpfC, and RpfG were well conserved in *Xoo*. Taken together with the results of previous studies, our results demonstrate that expression of $clp_{xoo4158}$ is controlled by RpfC and RpfG, a two component system, which perceives the signal from DSF and transfers it to the downstream genes.

The results of this study suggest that the function of the *clp* homolog, $clp_{xoo4158}$ is well conserved in *Xoo* KACC10859. Clp is highly homologous to nucleotide receptor proteins such as Crp and Vrf. *Escherichia coli* Crp and *Psedomonas aeruginosa* Vfr are known to control a set of various genes as global regulators (Suh et al., 2002; Zheng et al., 2004). Several previous studies suggest that Clp controls a wide range of genes involved in the production of virulence factors, iron uptake, and transcription

factors in *Xcc*. The DSF signaling system is involved in the regulation of virulence factor production has been previously indicated in *Xoo* (He et al., 2010). The *rpfC* and *rpfG*, as two-component system, play a role in DSF signal perception and signal transduction to transcriptional regulator, such as Clp. Taken together, we suggest that signal received Clp, regulated virulence factor by controlling transcription factor lead to affect virulence of *Xoo*. In turn, cell to cell signaling via DSF was crucial role in controlling virulence of *Xoo*. Mutation of $clp_{xoo4158}$ reduces numerous different virulence factors including EPS, extracellular enzymes, swarming motility and iron uptake in *Xoo* KACC10859. In addition, the mutation clearly demonstrated a reduction in resistance to oxidative stress, which is a new function of Clp identified in this study.

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