Direct Regulation of Extracellular Chitinase Production by the Transcription Factor *LeClp* in *Lysobacter enzymogenes* OH11

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ABSTRACT

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Lysobacter enzymogenes is a gram-negative bacterial biological control agent that produces abundant extracellular enzymes capable of degrading the cell walls of fungal pathogens. In strain OH11, an isolate from China, the global regulator *LeClp* controls the production of extracellular chitinase by regulating the transcription of the chitinase-encoding gene *chiA*. Using a

combination of bioinformatic, genetic, and biochemical methods, we show that *LeClp* regulates *chiA* transcription by directly binding to the *chiA* promoter region. Although *LeClp* appears to be important in this role, it is not the sole regulator of *chiA* transcription. Furthermore, the sequence analysis of putative *LeClp* binding sites indicated that the *LeClp* homolog could be involved in the regulation of extracellular chitinase production in diverse *Lysobacter* spp. by a mechanism similar to that in *L. enzymogenes*. Our findings present new insights into the molecular mechanism of *LeClp* in controlling extracellular chitinase activity, providing a fundamental road to elucidate how *LeClp* regulates the production of other extracellular lytic enzymes in *L. enzymogenes*.

Lysobacter is a gram-negative bacterial genus belonging to the family Xanthomonadaceae (Christensen and Cook 1978). This genus consists of a wide range of species from diverse ecological niches, including plant surface, rhizosphere soil, and water (Christensen and Cook 1978; Hayward et al. 2010). Considerable research attention has been paid to this genus due to its capacity to produce a multitude of new antibiotics and abundant lytic enzymes (Christensen and Cook 1978; Hayward et al. 2010; Xie et al. 2012). These characteristics led some species, such as *Lysobacter enzymogenes*, *L. antibioticus*, and *L. capsici*, to be important biological control agents against a variety of crop diseases (Ji et al. 2008; Ko et al. 2009; Puopolo et al. 2014; Qian et al. 2009; Zhang and Yuen 1999). Among these species, the best-studied is *L. enzymogenes* (Christensen and Cook 1978; Kobayashi et al. 2005; Qian et al. 2012, 2013, 2014; Zhang and Yuen 1999). As denoted by its name, this species is shown to produce extracellular lytic enzymes such as chitinases, proteases, and β -1,3-glucanases (Kobayashi et al. 2005). These enzymes have important roles in the antifungal activity of *L. enzymogenes* because they can efficiently degrade the cell walls of fungi (Palumbo et al. 2005; Zhang and Yuen 2000).

The role of chitinases in control of fungal crop diseases has been well demonstrated in *L. enzymogenes*. In an early study, treatment of conidia of the fungus *Bipolaris sorokiniana* with chitinolytic fractions from *L. enzymogenes* strain C3 caused conidial deformation and abnormal germ tube formation; these effects were lost when the chitinolytic fractions were heated (Zhang and Yuen 2000). In a later report,

L. enzymogenes C3 was found to produce at least two chitinases that are antifungal, including chitinase A and an unknown chitinase with a molecular weight of 48 kDa (Zhang et al. 2001). These early reports demonstrated the importance of chitinase production to *L. enzymogenes* biocontrol activity. In this regard, understanding of the molecular mechanisms involved in the regulation of chitinase biosynthesis in *L. enzymogenes* will be helpful in developing methods to enhance chitinase production and, thus, improve biocontrol effectiveness.

In the first report on the genetic regulation of chitinase biosynthesis in *L. enzymogenes* (Kobayashi et al. 2005), chitinase production by *L. enzymogenes* C3 was shown to be modulated by the cAMP receptor-like protein (Clp) transcription factor, with inactivation of *clp* resulting in near-complete deficiency in chitinase production. Furthermore, a recent screen of a transposon-based mutant library of strain C3 led to identification of four new regulators controlling the production of extracellular chitinase: glucose-galactose transporter, disulfide bond formation protein B, Clp protease, and polyamine synthase (Choi et al. 2012). However, it is not clearly understood as to how these regulators function in controlling extracellular chitinase production in *L. enzymogenes* C3.

In agreement with the findings using strain C3, we recently showed that extracellular chitinase activity in *L. enzymogenes* OH11, a strain isolate in China, is also regulated by Clp (Qian et al. 2009; Xu et al. 2015); mutation of *clp* also completely abolished extracellular chitinase production in strain OH11. A further study showed that, although strain OH11 possesses three predicted chitinase-encoding genes, only *chiA*, whose product is chitinase A, is required for extracellular chitinase activity (Xu et al. 2015). These results suggest that Clp most likely regulates extracellular chitinase activity in *L. enzymogenes* OH11 through control of chitinase A biosynthesis or secretion. The results from investigations on strains C3 and OH11 collectively suggest that Clp plays a conserved role in controlling extracellular chitinase activity; however, the precise mechanism by which Clp functions in each strain remains unclear.

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

The objective of the present work was to determine how Clp regulates extracellular chitinase production in *L. enzymogenes*, using strain OH11 as the subject. In the present study, the Clp of *L. enzymogenes* is designated “LeClp”. We provide abundant evidence to show that LeClp directly binds the promoter region of *chiA* under both in vitro and in vivo conditions, thus resulting in a positive regulation of *chiA* transcription. Our studies establish the first detailed report on the mechanism by which LeClp exerts its regulation on extracellular chitinase production in *L. enzymogenes*. Our results also show that the direct regulatory mode of LeClp on *chiA* appears to be a conserved attribute in diverse chitinase-producing *Lysobacter* spp.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains used for plasmid construction were routinely grown in Luria broth (LB) or on LB agar plates at 37°C, supplemented with gentamicin (Gm) (25 µg/ml) or 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (100 µg/ml) as needed. *L. enzymogenes* strains were grown in LB medium or 1/10 tryptic soy broth (TSB) at 28°C. When required, antibiotics were added into the medium according to the following final concentrations: kanamycin (Km) at 100 µg/ml, Gm at 150 µg/ml, and ampicillin (Amp) at 100 µg/ml.

Bacterial one-hybrid assay. The bacterial one-hybrid reporter system was shown to be efficient for the testing of the physical interaction of the transcription factor with the promoter of target genes (Guo et al. 2009). This assay was applied to examine the potential interaction between the transcriptional regulator (LeClp) and the target DNA (the *chiA* promoter) in the present study. As described previously, the bacterial one-hybrid reporter system involved three components: plasmids pBXcmT and pTRG, which were used for cloning the bait DNA and expressing a target protein, respectively, and *E. coli* XL1-Blue MRF⁺ kan strain (Table 1), which is the host strain for propagating pBXcmT and pTRG recombinants (Guo et al. 2009). In the present study, the *chiA* promoter region (400 bp) of *L. enzymogenes* OH11 was cloned into pBXcmT, generating the recombinant vector pBXcmT-*chiA* (Table 1). Similarly, the coding region of *Leclp* (690 bp) was cloned into pTRG,

creating the final construct pTRG-*clp* (Table 1). The vector pBXcmT-*chiA* and pTRG-*clp* were transformed into XL1-Blue MRF⁺ kan strain. If the direct physical binding occurs between LeClp and the *chiA* promoter, the positive-transformed *E. coli* strain containing both pBXcmT-*chiA* and pTRG-*clp* will be expected to grow well on the selective medium, which is the minimal medium containing 5 mM 3-amino-1,2,4-triazole, streptomycin at 8 µg/ml, tetracycline at 12.5 µg/ml, chloramphenicol at 34 µg/ml, and Km at 30 µg/ml, as described previously (Guo et al. 2009). Furthermore, the cotransformant containing the vector pBX-R2031/pTRG-R3133 served as a positive control (Guo et al. 2009), while the cotransformant containing the empty pTRG and pBXcmT-*chiA* was used as a negative control in the present study. All cotransformants were spotted onto the selective medium and grown at 28°C for 3 to 4 days, then photographed.

Protein expression and purification. The assay of protein expression and purification was performed as previously described (Deng et al. 2012). In brief, the gene *Leclp* was amplified by polymerase chain reaction (PCR) with the designated primer pairs listed in Supplementary Table S1. The PCR product of *Leclp* was purified and digested by *Bam*HI and *Xho*I, and further cloned into the *Bam*HI/*Xho*I-digested expression vector pGEX-6p-1, creating the final construct pGEX-6p-1-*clp* (Table 1). This vector was transformed into *E. coli* strain BL21 (DE3) for protein expression. In brief, the resultant transformed strain was cultivated in LB medium containing Amp at 100 µg/ml overnight at 37°C. Then, a 5-ml overnight culture was transferred into a fresh 500-ml LB medium in the presence of Amp at 100 µg/ml and grown at 37°C with shaking at 200 rpm until the optical density at 600 nm (OD₆₀₀) value reached 0.4. Subsequently, isopropyl β-D-1-thiogalactopyranoside at a final concentration of 0.4 mM was added to the culture, followed by further growth at 28°C for 4 h. Then, the cells were collected by centrifugation (6,000 rpm) at 4°C and resuspended in 15 ml of phosphate-buffered saline (PBS) lysis buffer supplemented with phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 1 mM. The cells were lysed by a brief sonication and the crude cell extracts were centrifuged at 13,000 rpm and 4°C. Soluble protein fractions were collected and mixed with preequilibrated Glutathione Sepharose 4B (GE) for 6 h at 4°C, which was placed into a column and extensively washed with PBS buffer. The proteins were subsequently eluted using elution buffer that contained the

TABLE 1. Bacterial strains and plasmids used in this study

Strains, plasmids	Characteristics ^a	Source
<i>Lysobacter enzymogenes</i>		
OH11	Wild-type strain, Km ^R	Qian et al. 2009
Δ <i>Leclp</i>	<i>Leclp</i> in-frame deletion mutant of strain OH11, Km ^R	Wang et al. 2014
Δ <i>Leclp</i> (<i>Leclp</i>)	Mutant Δ <i>Leclp</i> harboring plasmid pBBR- <i>clp</i> , Km ^R , Gm ^R	Wang et al. 2014
Δ <i>Leclp</i> (pBBR)	Mutant Δ <i>Leclp</i> harboring plasmid pBBR1-MCS5, Km ^R , Gm ^R	Wang et al. 2014
Δ <i>chiA</i>	<i>chiA</i> in-frame deletion mutant of strain OH11, Km ^R	Qian et al. 2012
Δ <i>chiA</i> (<i>chiA</i>)	Mutant Δ <i>chiA</i> harboring plasmid pBBR- <i>chiA</i> , Km ^R , Gm ^R	Xu et al. 2015
Δ <i>chiA</i> (pBBR)	Mutant Δ <i>chiA</i> harboring plasmid pBBR1-MCS5, Km ^R , Gm ^R	Xu et al. 2015
Δ <i>clp</i> (<i>chiA</i>)	Mutant Δ <i>Leclp</i> harboring plasmid pBBR- <i>chiA</i> , Km ^R , Gm ^R	This study
<i>Escherichia coli</i>		
DH5a	F [−] , φ80 <i>dlacZ</i> Δ <i>M15</i> , Δ(<i>lacZYA-argF</i>) <i>U169</i> , <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (<i>r_K[−]</i> , <i>m_K⁺</i>), <i>phoA</i> , <i>supE44</i> , λ [−] , <i>thi-1</i> , <i>gyrA96</i>	Lab collection
BL21 (DE3)	F [−] , <i>ompT</i> , <i>gal</i> , <i>dcm</i> , <i>lon</i> , <i>hsdSB</i> (<i>r_B[−]</i> , <i>m_B[−]</i>), λ(<i>DE3</i>)	Lab collection
XL1-Blue MRF ⁺ Kan	Δ(<i>mcrA</i>)183, Δ(<i>mcrCB-hsdSMR-mrr</i>)173, <i>endA1</i> , <i>supE44</i> , <i>thi-1</i> , <i>recA1</i> <i>gyrA96</i> , <i>relA1</i> , <i>lac</i> , [<i>F</i> ⁺ <i>proAB lacIq</i> Δ <i>M15</i> Tn5 (Km ^R)]	Guo et al. 2009
Plasmids		
pMD19-T	ColE1 origin, T Simple vector, Amp ^R	TaKaRa
pBBR1-MCS5	Broad host range cloning vector, <i>lacZ</i> , Gm ^R	Kovach et al. 1995
pBBR- <i>chiA</i>	pBBR1-MCS5 with 2.5kb fragment including <i>chiA</i> , Gm ^R	Xu et al. 2015
pGEX-6p-1	vector for protein expression, Amp ^R	Deng et al. 2012
pGEX-6p-1- <i>clp</i>	pGEX-6p-1 with the coding region of <i>Leclp</i> , Amp ^R	This study
pTRG	Plasmid used for protein expression in bacterial one-hybridization assay, Tet ^R	Guo et al. 2009
pTRG- <i>clp</i>	pTRG with the coding region of <i>Leclp</i> , Tet ^R	This study
pBXcmT	Plasmid used for DNA cloning in bacterial one-hybridization assay, Chlo ^R	Guo et al. 2009
pBXcmT- <i>chiA</i>	pBXcmT with the predicted <i>chiA</i> promoter region, Chlo ^R	This study

^a Km^R, Gm^R Amp^R, Tet^R, and Chlo^R = resistant to kanamycin, gentamicin, ampicillin, tetracycline, and chloramphenicol, respectively.

reduced glutathione. Protein purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration was determined using a Bradford protein assay kit (Bio-Rad).

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay (EMSA) was performed as described previously (Wang et al. 2013). In brief, DNA probes (the 400-bp *chiA* promoter region) were amplified by PCR with the primers *pchiA*-F and *pchiA*-R. The DNA probes used in this study were labeled with [α - 32 P]-dCTP by DNA polymerase Klenow fragments, and free [α - 32 P]-dCTP was removed by ProbeQuant G-50 column (GE). The GST-tagged protein *LeClp* was incubated with 6 fmol of radiolabeled oligonucleotide duplex in a 20- μ l reaction containing 10 mM Tris (pH 7.0), 50 mM KCl, 1 mM dithiothreitol (pH 7.5), 2.5% glycerol, 5.0 mM MgCl₂, 1.0 μ g poly(dI:dC), 0.05% NP-40, and 10 mM EDTA for 20 min at room temperature. In competition experiments, unlabeled probes were added during the preincubation period. Free DNA and protein-bound DNA were separated on 5% nondenaturing polyacrylamide gel in 0.5 $\times$ Tris-boric acid-EDTA. After electrophoresis, gels were blotted onto filter paper, dried, and autoradiographed.

Chromatin immunoprecipitation PCR. The chromatin immunoprecipitation (ChIP) assay was conducted as previously described (Wang et al. 2013). In brief, *L. enzymogenes* strain OH11 was incubated in LB medium at 28°C with shaking at 200 rpm, until the value of OD₆₀₀ reached 1.0. Then, the cells were harvested by centrifugation at 6,000 rpm and suspended in an equivalent volume of 10% TSB broth at 28°C in a rotary shaker at 200 rpm for 1 h. All bacterial cells were incubated with 1% formaldehyde for 10 min at room temperature for cross-linking and subsequently quenched with 0.5 M glycine for 5 min. For DNA preparation, the cells were further washed with 10 ml of cold PBS buffer twice, then scraped into 1.2 ml of ChIP lysis buffer (10 mM Tris-HCl, pH 8.0; 20% sucrose; 50 mM NaCl; and 10 mM EDTA) and 4.8 ml of ChIP buffer (50 mM HEPES-KOH, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% Triton X 100; 0.1% sodium deoxycholate; 0.1% SDS; and 1 mM PMSF). These lysates were sonicated using a Diagenode Bioruptor UCD-300 (Diagenode) to generate DNA fragments that ranged in the size from 100 to 500 bp. After centrifugation, a 100- μ l aliquot was kept as the positive control DNA (Input). The 800- μ l aliquot was incubated with 2 μ l of the respective antibody (anti-Clp; gift from W. Qian, Chinese Academy of Science) and 30 μ l of protein A/G agarose beads overnight at 4°C (Sample). As a negative control, only 30 μ l of protein A/G agarose beads were added in another 800- μ l aliquot (Mock). The sepharose beads were collected by centrifugation and washed with immunoprecipitation (IP) buffer, IP buffer with 500 mM NaCl, wash buffer, and Tris-EDTA buffer. Then, the beads were suspended in 100 μ l of elution buffer (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; and 1% SDS), and incubated for 1.5 h at 42°C with RNase A and an additional 6 h at 65°C with proteinase K to reverse protein/DNA cross-links. Finally, these samples were processed for DNA purification by phenol-chloroform extraction and ethanol precipitation. The quantity of DNA was detected by the NanoDrop ND-1000 (NanoDrop). Finally, the PCR assay was performed in 25 μ l with the input DNA, sample DNA, and mock DNA, respectively, with the relevant primer pairs. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. For further validation, these PCR products were also sequenced in the present study.

Western blot analysis. The Western blot analysis was performed according to the laboratory protocol, with a minor modification (Xu et al. 2015). In brief, GST-tagged protein *LeClp* was separated by SDS-PAGE and immobilized onto a polyvinylidene difluoride membrane using the semidry blot machine (Bio-Rad). Membranes were probed with the specific primary anti-GST (M20007; Abmart), followed by an HRP-conjugated antimouse secondary antibody (M21001; Abmart) for signal detection.

Determination of extracellular chitinase activity. The production of extracellular chitinase from *L. enzymogenes* strains

was determined as described previously (Qian et al. 2009; Xu et al. 2015). In brief, the test *L. enzymogenes* strains were incubated in LB medium at 28°C with shaking at 200 rpm, until the value of OD₆₀₀ reached 1.0. Then, 2 μ l of bacterial culture was spotted on LB plates contained colloidal chitin. After 96 h of incubation at 28°C, chitinase activity was assessed according to the cleaning zones around the bacterial colonies. Each treatment involved three replications, and the same experiment was performed three times.

RESULTS

***LeClp* bound directly to the promoter region of *chiA* in vitro.** To explore how *LeClp* regulates the transcription of *chiA*, we tested whether *LeClp* can directly bind the promoter of *chiA* via the C-terminal helix-turn-helix (HTH)-DNA binding domain of *LeClp*. To test this point, the characterized binding consensus sequence (5'-NTGTGA-N6-TCACANTTTN-3') of *E. coli* CRP (<http://regulondb.ccg.unam.mx/>), the homolog of *LeClp*, was chosen for predicting whether the promoter of *chiA* possessed any potential DNA binding site of *LeClp*. This led to identification of a potential binding site (5'-TTGTACGCGGCCAGTTTC-3') of *LeClp* at the *chiA* promoter (Fig. 1A). This result supported our initial idea and further suggested that *LeClp* could directly bind to the *chiA* promoter. To validate this point, we employed the *E. coli*-based one-hybrid reporter system (described in detail in Materials and Methods). We clearly observed that the test *E. coli* strain containing both *LeClp* and the *chiA* promoter grew very well on the selective medium, similar to the positive control, whereas the negative control failed to grow (Fig. 1B). This result indicated that a direct binding of *LeClp* on the promoter of *chiA* occurred under the test conditions.

To further confirm the above finding, an EMSA/gel shift assay was performed. We found that the GST-tagged *LeClp* formed a clear protein/DNA complex with the test DNA probe, whereas no shift band was observed between the empty GST and DNA probe under the similar test conditions (Fig. 2). Furthermore, addition of an unlabeled probe (cold probe) at 10- and 1,000-fold excesses to the reaction mixtures competitively inhibited the binding of *LeClp* to the labeled DNA probe. This EMSA result, together with the data from Figure 1, strongly showed that *LeClp* directly bound to the promoter region of *chiA* under the in vitro condition.

A direct in vivo physical interaction occurred between *LeClp* and the *chiA* promoter. Next, we attempted to test whether an in vivo binding of *LeClp* on the promoter of *chiA* occurs in *L. enzymogenes* cells. To achieve this point, the ChIP-PCR assay was carried out, whose scheme is presented in Figure 3A (described in detail in Material and Methods). Clearly, a 212-bp promoter region of *chiA* was amplified by PCR from the designated Sample (the chromatin DNA of strain OH11 immunoprecipitated with the respective antibody), which was similar to the case from the positive control Input (Fig. 3B). However, under the same condition, the negative control Mock (the chromatin DNA of strain OH11 immunoprecipitated without the respective antibody) failed to amplify the corresponding PCR products (Fig. 3B). These results suggested that *L. enzymogenes* *LeClp* bound to the promoter region of *chiA* in vivo. Collectively, all of the above results as well as our previous report (Wang et al. 2014) clearly reveal that *LeClp* exerted its positive regulation on *chiA* transcription through a direct interaction with the *chiA* promoter region in *L. enzymogenes*.

***LeClp* is an important but not the sole regulator of *chiA* transcription.** To address whether *LeClp* is the sole regulator of *chiA* transcription, the plasmid-borne *chiA* under the control of its native promoter was introduced into the *Leclp* mutant. This introduction caused a minor but visible effect in restoring the deficiency of the *Leclp* mutant in producing extracellular chitinase (Fig. 4). However, the *Leclp* mutant possessing an empty vector was still fully deficient in this ability. Furthermore, as controls, the *Leclp*

or *chiA* mutant completely lost the ability to produce extracellular chitinase, where the trait was restored in the respective complemented strain to the wild-type level (Fig. 4). Taken together, these results suggested that, although *LeClp* was important, it was not the sole regulator of *chiA* transcription in *L. enzymogenes*.

The direct interaction between *LeClp* and the *chiA* promoter appears to be a conserved mechanism in diverse chitinase-producing *Lysobacter* spp. Recently, the genomes of four representative chitinase-producing *Lysobacter* spp., including *L. enzymogenes* C3, *L. antibioticus* ATCC29479, *L. capsici* 55, and

Fig. 1. *LeClp* directly bound to the promoter of *chiA* in the *Escherichia coli*-based one-hybrid assay. **A**, Alignment of the binding consensus sequence of *E. coli* CRP with the promoter region of *chiA* in *Lysobacter enzymogenes*. Conserved bases are highlighted in black. **B**, Direct physical interaction between *LeClp* and the *chiA* promoter region was detected in *E. coli*. Experiments were performed according to the procedures described in the Materials and Methods section. BOH-CK(+) = cotransformant containing pBX-R2031 and pTRG-R3133, used as a positive control; pTRG/pchiA = cotransformant containing pBXcmT-*chiA* and the empty pTRG, served as a negative control; and pTClp/pchiA = cotransformant possessing pTRG-*clp* and pBXcmT-*chiA* (Table 1). Rows: -3AT-Str^r = no selective medium plate (3AT = 3-amino-1,2,4-triazole and Str = streptomycin) and +3AT+Str^r = selective medium plate.

Fig. 2. Electrophoretic mobility shift assay showing the direct binding of *LeClp* to the promoter of *chiA* in vitro. **A**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the purified GST-tagged *LeClp*, which was validated by **B**, Western blot using α -GST, the anti-GST antibody. **C**, The unlabeled probe (cold probe) at 10- and 1,000-fold excesses to the reaction mixtures competitively inhibited the binding of *LeClp* to the labeled DNA probe. GST was used as a negative control. Free DNA and DNA-protein complex are indicated by arrows, respectively.

Fig. 3. *LeClp* directly bound the promoter of *chiA* in *Lysobacter enzymogenes* cells. **A**, Scheme of chromatin immunoprecipitation used in this study, including 1, formaldehyde cross-linking; 2, sonication; 3, immunoprecipitation; and 4, reverse-crosslink. **B**, Results of chromatin immunoprecipitation polymerase chain reaction assay. A specific 212-bp band, belonging to the *chiA* promoter region, was amplified from the chromatin immunoprecipitated sample and validated by sequencing. Input = the DNA fragments that ranged in the size from 100 to 500 bp after sonication (positive control), Sample = the chromatin DNA immunoprecipitated with the anti-cAMP receptor-like protein (Clp) antibody, Mock = the chromatin DNA immunoprecipitated without the anti-Clp antibody (negative control), - = double-distilled H₂O (negative control), and + = the intact DNA of the wild-type strain OH11 (positive control).

Fig. 4. *LeClp* was an important but not the sole regulator of *chiA* transcription. Production of extracellular chitinase was reflected by the hydrolyzing zone of various *Lysobacter enzymogenes* strains on the selective medium. OH11, the wild-type strain of *L. enzymogenes*; $\Delta Leclp$, the *clp*-deletion mutant of *L. enzymogenes*; $\Delta Leclp(Leclp)$, the complemented strain of $\Delta Leclp$; $\Delta Leclp(pBBR)$, $\Delta Leclp$ with an empty vector; $\Delta Leclp(chiA)$, $\Delta Leclp$ containing an overexpressed *chiA*; $\Delta chiA$, the *chiA*-deletion mutant of *L. enzymogenes*; $\Delta chiA(chiA)$, the complemented strain of $\Delta chiA$; and $\Delta chiA(pBBR)$, $\Delta chiA$ possessing an empty vector. Diameter of the hydrolyzing zone was indicated by a bar corresponding to 1 cm.

Fig. 5. Both the homolog of *LeClp* and its potential binding site on the promoter region of *chiA* were conserved in diverse chitinase-producing *Lysobacter* spp. **A**, Alignment of *LeClp* and its homologs from other *Lysobacter* spp., whose genomes were recently reported (de Bruijn et al. 2015). The N-terminal cyclic nucleotide monophosphate (cNMP)-binding and C-terminal helix-turn-helix (HTH)-CRP domain of *LeClp* are indicated with black bars. **B**, Multiple alignment of the *Escherichia coli* CRP binding sequence with the predicted promoter region of *chiA* and its homologs from diverse *Lysobacter* spp. Conserved bases are highlighted in black and gray.

L. gummosus 3.2.11, were reported (de Bruijn et al. 2015). To explore whether the direct interaction between *LeClp* and the *chiA* promoter in strain OH11 is also conserved in those abovementioned *Lysobacter* spp., a comparative sequence alignment was performed. Clearly, all of the sequenced *Lysobacter* spp. possessed a highly conserved *LeClp* homolog (Fig. 5), and their corresponding HTH-binding domain at the amino-acid sequence level was almost same as that in strain OH11 (Fig. 5A). Furthermore, the promoter regions of *chiA* from all *Lysobacter* spp. were predicted to have a DNA binding consensus sequence of *E. coli* CRP (Fig. 5B), which was similar to the case from strain OH11. Collectively, the sequence analysis of the putative *LeClp* binding site indicated that the *LeClp* homolog could be involved in the regulation of extracellular chitinase production in those closely related *Lysobacter* spp. described above, by a mechanism similar to that in *L. enzymogenes* OH11.

DISCUSSION

As a global regulator, *LeClp* makes an important contribution to the production of diverse extracellular lytic enzymes in *L. enzymogenes* (Kobayashi et al. 2005; Wang et al. 2014); however, the corresponding molecular mechanism is unknown. In the present study, we provided the first report to show that the regulation of *LeClp* on the production of extracellular chitinase was, at least in part, due to the direct binding of *LeClp* to the promoter of *chiA*. Such a regulatory mechanism seems to be conserved in diverse chitinase-producing *Lysobacter* spp. This new knowledge will be helpful to generate high chitinase-producing *L. enzymogenes* strains for better application in the biocontrol of crop fungal disease.

Although this study provides abundant evidence of the direct interaction between *LeClp* and the *chiA* promoter, *LeClp* appears not to be the sole regulator of *chiA* transcription (Fig. 4). It is possible that, in addition to *LeClp*, there is another unidentified transcription factor that can bind with the native *chiA* promoter, causing some leaky transcription of *chiA* from the introduced *chiA* plasmids (maybe hundreds). However, in the chromosomal background of the *Leclp* mutant, there is only one copy of *chiA*; hence, the phenotype rescue phenomenon is less obvious.

The regulation of Clp on enzyme biosynthesis has been well characterized in the phytopathogenic bacterium *Xanthomonas campestris* pv. *campestris* (He et al. 2007). In this bacterium, the regulator Clp serves as a downstream regulator of the diffusible signal factor (DSF) cell-to-cell signaling to modulate diverse enzyme production (He et al. 2007). Although *L. enzymogenes* also contains a functional *LeDSF* system, this system did not regulate the biosynthesis of extracellular lytic enzymes such as chitinase (Qian et al. 2013). Therefore, in contrast to the findings in *X. campestris*, *LeClp* appears to utilize an *LeDSF*-independent manner to regulate the production of extracellular chitinase. These results suggested that the Clp family proteins have evolved to acquire species-adaptive mechanisms to control respective enzyme biosynthesis in *X. campestris* and *L. enzymogenes*, two related but different bacterial species at taxonomy.

In *X. campestris*, the regulation of Clp on extracellular enzyme biosynthesis is dependent on c-di-GMP, a newly identified bacterial second messenger (Chin et al. 2010; Tao et al. 2010). A representative example is the endoglucanase enzyme encoding by *engXCA* that contributes to *X. campestris* virulence through its cellulolytic activity (Gough et al. 1990). In *X. campestris* cells, Clp is a c-di-GMP receptor with high affinity (Chin et al. 2010; Tao et al. 2010). Clp directly binds the promoter of *engXCA*, resulting in a positive control of its transcription. High-level c-di-GMP inhibits such binding, thus suggesting that c-di-GMP makes a negative contribution to formation of Clp-DNA complex (Chin et al. 2010; Tao et al. 2010). Although *LeClp* shares a high amino-acid similarity with the Clp of *X. campestris* (Kobayashi et al. 2005), it remains unclear whether the direct regulation of *LeClp* in transcription of *chiA* is c-di-GMP dependent, which needs further investigations.

In summary, our findings present new insights into how the global regulator *LeClp* controls the production of extracellular chitinase, which provides useful guidance to understand the regulation of *LeClp* in control of other extracellular lytic enzymes, such as β -1,3-glucanase in *L. enzymogenes*. Also, our studies may trigger the study of other chitinase-producing and underexplored *Lysobacter* spp. possessing the homologs of both *LeClp* and *ChiA*.

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