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Transcriptomic analysis reveals new regulatory roles of Clp signaling in secondary-metabolite biosynthesis and surface motility in *Lysobacter enzymogenes* OH11

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Abstract

Lysobacter enzymogenes is a bacterial biological-control agent emerging as a new source of antibiotic metabolites, such as HSAF (Heat-Stable Antifungal Factor) and the antibacterial factor WAP-8294A2. The regulatory mechanism(s) for antibiotic-metabolite biosynthesis remains largely unknown in *L. enzymogenes*. Clp, a cAMP-receptor-like protein, is shown to function as a global regulator in modulating biocontrol-associated traits in *L. enzymogenes*. However, the genetic basis of Clp signaling remains unclear. Here, we utilized transcriptome/microarray analysis to determine the Clp regulon in *L. enzymogenes*. We showed that Clp is a global regulator in gene expression, as the transcription of 775 genes belonging to 19 functional groups was differentially controlled by Clp signaling. Analysis of the Clp regulon detected previously characterized Clp-modulated functions as well as novel loci. These include novel loci involved in antibiotic-metabolite biosynthesis and surface motility in *L. enzymogenes*. We further showed experimentally that Clp signaling played a positive role in regulating the biosynthesis of HSAF and WAP-8294A2, as well as surface motility which is a Type-IV-pilus-dependent trait. The regulation by Clp signaling of antibiotic (HSAF and WAP-8294A2) biosynthesis and surface motility was found to be independent. Importantly, we identified a factor Lat (*Lysobacter* acetyltransferase), a homologue of histone acetyltransferase Hpa2, which was regulated by Clp and involved in HSAF biosynthesis, but not associated with WAP-8294A2 production and surface

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motility. Overall, our study provided new insights into the regulatory role and molecular mechanism of Clp signaling in *L. enzymogenes*.

Keywords

Lysobacter enzymogenes; HSAF; WAP-8294A2; Surface motility; Clp

Introduction

The genus of *Lysobacter* in the *Xanthomodaceae* family is one of the most ubiquitous environmental bacterial genera. A characteristic feature of many *Lysobacter* species is the yellow-orange appearance (Christensen and Cook 1978). This yellow-pigment was recently characterized as a non-carotenoid polyene secondary metabolite (Wang et al. 2013). In addition, this genus displays other characteristic features, such as high genomic G+C content (approximately 70%), flagella-independent surface motility and production of abundant lytic enzymes (Christensen and Cook 1978; Zhang and Yuen 1999). In this genus, *L. enzymogenes*, first proposed by Christensen and Cook in 1978, is most extensively studied and is emerging as a potentially novel biocontrol agent against pathogens of crop plants (Giesler and Yuen 1998; Qian et al. 2009). The antagonistic nature of this bacterium is thought to be due at least in part to the production of a large number of lytic enzymes capable for degrading the cell walls of pathogens (Christensen and Cook 1978; Kobayashi et al. 2005) and to the antimicrobial metabolites HSAF (dihydromaltophilin; heat-stable and broad-spectrum antifungal compound with a novel mode of action) (Li et al. 2006; Yu et al. 2007) and the cyclic lipodepsipeptide WAP-8294A2, which also exhibits anti-MRSA (methicillin-resistant *Staphylococcus aureus*) activity (Zhang et al. 2011). In addition to its antagonism, *L. enzymogenes* directly attaches itself to the hypha of fungal pathogens and subsequently causes a pathogenic infection to the fungal host (Mathioni et al. 2013; Patel et al. 2011). The ability of *L. enzymogenes* to attach to fungal hypha was shown to be dependent on type IV pilus (T4P), which is a hair-like appendage found on the surface of a wide range of bacteria that is responsible for various roles such as surface motility, host attachment and pathogenesis (Burdman et al. 2011; Mattick 2002; Patel et al. 2011). T4P are formed from thousands of pilin (PilA) subunits, and a mutation of *pilA* impairs T4P biosynthesis in a wide range of bacteria (Burdman et al. 2011).

We recently obtained the draft genome sequence of strain OH11, a Chinese *L. enzymogenes* isolate (Qian et al. 2009, 2013; Lou et al., 2011). In the genome, the biosynthetic gene clusters for the two above-mentioned antibiotic metabolites (HSAF and WAP-8294A2) have been identified and characterized (Lou et al. 2011; Zhang et al. 2011). The unique mechanism for HSAF biosynthesis was experimentally characterized (Lou et al. 2011), whereas that of WAP-8294A2 was only proposed (Zhang et al. 2011). In addition to secondary metabolites, the genes encoding for T4P biogenesis were also identified from the genome sequence of *L. enzymogenes* strain C3 (Patel et al. 2011). Nevertheless, the factors/systems controlling the biosynthesis of these metabolites and T4P remained to be explored in *L. enzymogenes*.

The cyclic AMP receptor protein (Crp) is a well-characterized global regulatory protein in bacteria (Zheng et al. 2004). This protein contains a conserved cNMP binding domain and DNA-binding domain (Pesavento and Hengge 2009). The cNMP binding domain of Crp not only binds cAMP, but also can interact with other nucleotides such as cGMP, although Crp can only be activated by specifically binding cAMP (Gorshkova et al. 1995; Takahashi et al. 1989). The binding of Crp to cAMP causes a conformational change in Crp and is mandatory for the regulatory activity of Crp (Qu et al. 2013). The cAMP-Crp complex binds to a symmetrical consensus box sequence TGTGA-N₆-TCACA located within the upstream of DNA regions of the target genes (Gunasekera et al. 1992). It has been observed that the cAMP-Crp complex primarily acts as a positive regulator, but has been shown in some cases to have a negative effect on transcription (Aiba 1985; Musso et al. 1977; Pastan and Adhya 1976). The Crp regulator is best studied in *Escherichia coli*, in which it has been shown to regulate genes primarily involved in carbon metabolism (Gosset et al. 2004). In plant-associated bacteria, Crp family regulators are best characterized for their global control of traits related to pathogenesis as is the case for *Erwinia charyanthemi* (Reverchon et al. 1997). In the opportunistic human pathogen *Pseudomonas aeruginosa*, the Crp-family regulator Vfr is also a global regulator of virulence factor expression (Beatson et al. 2002).

In contrast to Crp, the Clp (cAMP receptor-like protein) regulator has been shown to specifically interact with c-di-GMP (3',5'-cyclic diguanylic acid), but not cAMP in *Xanthomonas campestris* pv. *campestris*, a model strain in plant pathology (Mansfield et al. 2012; Tao et al. 2010). The ligand specificity between Crp and Clp is probably due to their sequence variation in their cNMP binding domains (Tao et al. 2010). The roles and intracellular signaling pathway of Clp has recently been characterized in *X. campestris* pv. *campestris*. In this bacterium, Clp is shown to function in two ways: (i) as a global regulator in DSF (Diffusible Signal Factor) signaling pathway to regulate DSF-controlled genes/functions responsible for virulence, such as genes encoding extracellular enzymes, exopolysaccharides (EPS) biosynthesis and flagellum synthesis and (ii) in DSF-independent modulation of biofilm formation (He et al. 2007). In *L. enzymogenes* C3, a Clp homologue was shown to be involved in lytic-enzyme production, antimicrobial activity and attachment to fungal hyphae (Kobayashi et al. 2005; Kobayashi and Yuen 2005). However, the molecular mechanism of Clp signaling in *L. enzymogenes* remained unknown.

Searching the genomic sequence of strain OH11, we identified a Clp orthologue (Lys1661), which is identical in sequence at the amino-acid level to that of strain C3 (Fig. S1 in the Supplementary Material). Mutation of *clp* dramatically impaired the antimicrobial activities and lytic-enzyme production in strain OH11 (Fig. S2 in the Supplementary Material). These *clp*-modulated phenotypes in strain OH11 were previously investigated in strain C3 (Kobayashi et al. 2005), which indicated that Clp signaling played a conserved role in the modulation of biocontrol-associated traits between strains OH11 and C3. We then compared the genome-wide gene expression profiles between wild-type OH11 and a *clp*-deletion mutant in order to investigate the Clp regulon. In the present study, we showed that Clp is a global regulator in gene expression. The regulon consisted of loci previously known to be regulated by Clp and of several new loci encoding antibiotic metabolite biosynthesis and surface motility. Experimental evidences supported these novel findings of Clp signaling on the regulation of antibiotic metabolite (HSAF and WAP-8294A2) biosynthesis and of

surface motility. Importantly, we identified a factor, Lat (*Lysobacter* acetyltransferase), a homologue of histone acetyltransferase Hpa2, was modulated by Clp signaling at transcriptional level and involved in the regulation of HSAF biosynthesis, but not of WAP-8294A2 production and surface motility in *L. enzymogenes*.

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* strain DH5 α , used for plasmid construction, was grown in LB broth at 37 °C. Unless otherwise stated, *L. enzymogenes* strains were grown in 10% TSB (Tryptic Soy Broth, Sigma, St. Louis, MO, USA) medium at 28 °C. When required, antibiotics were added to the medium as the following final concentrations: kanamycin (Km), 50 g/mL; gentamicin (Gm), 20 and 150 g/mL for *E. coli* and *L. enzymogenes*, respectively.

Generation of deletion mutants and complemented strains of target genes in *Lysobacter enzymogenes*

The wild-type strain OH11 (CGMCC No. 1978) of *L. enzymogenes* was used as an original strain to generate the in-frame deletion mutants. Construction of gene-deletion mutants and complemented strains was performed as described previously (Qian et al. 2013). Primers and the constructed vectors are provided in Table S1 in the Supplementary Material and Table 1, respectively.

HSAF extraction and detection

Extraction and HPLC (High-Performance Liquid Chromatography) detection of HSAF from *L. enzymogenes* were performed as described previously (Qian et al. 2013, 2014; Yu et al. 2007). In the HPLC analysis, standard HSAF were used as a control. Three replicates were used for each treatment, and the experiment was performed three times.

Extraction and Analysis of WAP-8294A2

Extraction and HPLC analysis of WAP-8294A2 from *L. enzymogenes* were performed as described previously (Zhang et al. 2011, 2014). Three replicates were used for each treatment, and the experiment was performed three times. Finally, the ESI-MS (LTQ Orbitrap XL, Thermo, Waltman, MA, USA) was used to verify the mass of the main components of the WAP-8294A2.

Surface motility assay

The medium used for surface motility assay was 1/20 TSA with high-percentage agar (1.8%), as this medium is effective for observation of surface gliding motility in *L. enzymogenes* (Gary Y. Yuen, unpublished data). In brief, surface motility assays were performed as follows: The sterilized microslides were placed inside the plate which covered a water-soaked filter paper. Then, 1-mL 1/20 TSA medium (1-mL, 80°C) containing, agar (1.8%) was carefully distributed on the surface of the microslides. The sterilized coverslips, dipped with a small amount of overnight culture of various *L. enzymogenes* strains, were

used to gently cover the surface of the microslides containing agar. After incubation for 24 h, these microslides were directly used for the surface-motility assay. A classical phenomenon of surface motility is finding of many “bacterial cells” on the edge of a bacterial colony (Agarwal et al. 1997). Photos were taken under a microscope with a 640-fold magnification. Three replicates for each treatment were used, and the experiment was performed three times.

Microarray analysis

The microarray data of the present study was deposited into the NCBI database with an accession number of GSE58846 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=mncrkycsrrgfdof&acc=GSE58846>). In brief, the experiment of microarray was performed as follows: The wild-type OH11 and *clp*-deletion mutant (*clp*) were grown in 10% TSB broth until to an exponential phase ($OD_{600}=1.0$). The cells of each strain were collected by centrifugation ($10,000\times g$ at 4 °C for 3 min), and were used for RNA extraction according to standard manipulation. The RNA was purified with NucleoSpin® RNA clean-up (MACHEREY-NAGEL, Düren, NW, Germany), and its purity was further detected by formaldehyde agarose gel electrophoresis. Additionally, the RNA concentration was quantitatively determined by using a spectrophotometer (Agilent Nanodrop, Santa Clara, CA, USA).

Before reverse transcription, poly A were added to RNA with poly A polymerase. Then, cDNA was acquired by reverse transcription with T7-oligo(dT) primer. The microarray chips were designed based on the genome sequence of *L. enzymogenes* strain OH11 (unpublished, but can be found by clicking the GPL18866 linker in the website above). The information of the microarray chips can be found with NimbleGen design with the ID 35739 by clicking the GPL18866 linker in the website above. After these operation, hybridization, washing, scanning and data analysis were followed. All the manipulation were carried out according to NimbleGen's Expression user's guid (Guo et al. 2005; He et al. 2012).

Microarray software (SAM, version 3.02) was used to identify significantly differentially expressed genes between wild-type OH11 and *clp*-deletion mutant (*clp*). Genes were determined to be significantly differentially expressed with a selection threshold of false discovery rate, $FDR<5\%$ and fold change ≥ 2.0 (the genes are positively or negatively regulated at the transcriptional level by the Clp regulator by two-fold or more). For the microarray assay, two replicates for each strain (wild type or mutant) were used.

Real-time PCR

The quantitative real time PCR assay, including RNA extraction, cDNA synthesis and real-time PCR was performed as described previously (Qian et al., 2013). Primer sequences used in this assay are listed in Table S2 in the Supplemental Material.

Data analysis

All analyses were conducted using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). The hypothesis test of percentages (*t*-test, $P=0.05$) was used to determine significant differences

in the production of antibiotic metabolites and gene expressions between mutants and the wild-type OH11.

Results

Clp regulated the transcriptional expression of a wide range of genes belonging to diverse functional groups in *L. enzymogenes*

To determine the Clp regulon in *L. enzymogenes*, we performed a transcriptomic analysis by making use of microarrays. We first determined the growth curves in 10% TSB broth of wild-type OH11 and the *clp* mutant. We found that mutation of *clp* slightly reduced the growth ability of wild-type OH11 under test conditions (Fig. S3 in the Supplementary Material). To prove the initial harvested cell density used for RNA extraction is the same between wild-type OH11 and the *clp* mutant, we selected the time point 10 h for wild-type OH11 and 11.5 h for the *clp* mutant after the initial incubation to collect cells for RNA extraction, because, based on the growth curve (Fig. S3 in the Supplementary Material), both the wild-type OH11 and the *clp* mutant reached to the logarithmic phase and their cell density was the same ($OD_{600nm}=1.0$) at the selected time points (10 h for wild type; 11.5 h for the *clp* mutant). Whether *L. enzymogenes* can produce antibiotic metabolites (one of the concerned phenotypes of the present study), such as HSAF after 10 h of growth was tested. As shown in Fig. S4 in the Supplementary Material, HSAF production was detected at that time point (10 h) in *L. enzymogenes*. This result suggests that the biosynthetic genes responsible for HSAF production have been expressed at that time. This point was verified by the transcriptomic data presented in this study (Table 2). Based on these results, we therefore selected this logarithmic phase (10 h of growth for wild type) to collect *Lyso bacter* cells for analysis of gene expressions. Next, RNA was isolated from the wild-type strain OH11 and its *clp* mutant derivative, and used for the DNA microarray. As shown in Fig. S5 and Table S3 in the Supplementary Material, 775 genes, belonging to 19 functional categories, were differentially expressed (showing 2-fold change) between the *clp* mutant and the wild-type strain. Of the Clp regulon, 303 and 472 genes were up- and down-regulated in the *clp* mutant, respectively, when compared to the wild-type strain. Subsequently, a set of 20 genes distributed among the Clp regulon were chosen for validation with qRT-PCR amplification. Most of these 20 selected genes were highly associated with our concerned phenotypes, including antibiotic and lytic-enzyme production, motility and signal transduction (Table 2). As shown in Table 2, the transcriptional levels of these selected genes determined by qRT-PCR were consistent with that of the microarray.

Systematic analysis of the Clp regulon identified a number of genes responsible for lytic-enzyme production (Table S4 in the Supplementary Material), which were previously shown to be Clp regulated (Kobayashi et al. 2005). Bioinformatic analysis showed that the regulator Clp regulated the expression of 20 gene clusters, of which most of them are functionally unknown (Fig. 1; Table S5 in the Supplementary Material). Further analysis suggested that 2 gene clusters have been previously characterized (namely Cluster A and B) and are responsible for antibiotic metabolite biosynthesis (Fig. 2; Table S6 and S7; Fig. S6 in the Supplementary Material), for HSAF (Cluster A) and for WAP-8294A2 biosynthesis (Cluster B), respectively. Furthermore, we also identified another gene cluster (Cluster D)

linked to the biosynthesis of an uncharacterized secondary-metabolite, as this cluster contained a *pks* (encoding a polyketide synthase) and a *nrps* (encoding a nonribosomal peptide synthetase) gene. Both of these latter type of genes are known for their involvement in the biosynthesis of natural products in microorganisms, such as antibiotics, pigments and siderophores (Donadio et al. 2007; Xie et al. 2012).

In addition to secondary-metabolite biosynthesis, we detected 4 clusters (E to H in Fig. 1 and Table S5 in the Supplementary Material) associated with cell motility. Although *Lysobacter* lacks flagella (Christensen and Cook 1978), we are surprised to find by a Blastp analysis that most genes of Cluster E belonging to the Clp regulon shared high similarity at amino acid level to the reported proteins of flagella biosynthesis in diverse bacteria (Table S5 in the Supplementary Material). We currently did not know the precise function of this cluster in *L. enzymogenes*, which is deserved for a further investigation. Clusters F, G and H contained the genes that encode for proteins that act as the major and accessory proteins for T4P biosynthesis in bacteria. The other 12 clusters (Cluster I to T) with unknown functions were differentially regulated at the transcriptional level by Clp signaling, and are worthy for further investigation.

Clp signaling played an important role in antibiotic-metabolite biosynthesis

As noted above, two gene clusters related to the biosynthesis of HSAF and WAP-8294A2 were regulated at the transcriptional level by Clp signaling. We further studied the phenotypical regulation by *clp* on these two metabolites. As shown in Fig. 2, the mutation of *clp* dramatically reduced HSAF and WAP-8294A2 production, and the complementation restored the production of these two antibiotic metabolites in the *clp* mutant (Table S6 and S7; Fig. S6 in the Supplementary Material). To further confirm these results, the expression levels of the genes responsible for HSAF and WAP-8294A2 biosynthesis were tested using qRT-PCR analysis. Here, the *pks/nrps* gene encoding a hybrid polyketide synthase/nonribosomal peptide synthetase is critical for HSAF biosynthesis, whereas the *wapsI* gene encoding a nonribosomal peptide synthetase is essential for WAP-8294A2 biosynthesis in *L. enzymogenes* (Lou et al. 2011; Zhang et al. 2011). As expected, the transcriptional levels of *pks/nrps* and *wapsI* were both significantly reduced in the *clp* mutant compared to wild-type OH11 (Table 2); this was consistent with the finding of HSAF and WAP-8294A2 production in the *clp* mutant being reduced. Taken together, these results revealed that Clp signaling played a critical role in the positive modulation of antibiotic-metabolite biosynthesis in *L. enzymogenes*.

Clp signaling was involved in surface motility through regulation of T4P in *L. enzymogenes*

Three gene clusters associated with T4P were detected in the Clp regulon of *L. enzymogenes*. As T4P is important for surface motility in diverse bacteria as noted in the Introduction section, we tested whether T4P is required for surface motility in *L. enzymogenes*. Thus, we in-frame deleted *pilA* (encoding the major pilin subunit of T4P) and tested its role in surface motility. This gene (*pilA*) was numbered as *lys1803* and designated as *orf4* in cluster F (Fig. 1 and Table S5 in the Supplementary Material). As expected, mutation of *pilA* impaired surface motility, as no bacterial cells were observed at the colony-

edge of this mutant (Fig. 3). Under the same testing conditions, the *pilA* complemented strain exhibited wild-type surface motility (Fig. 3).

The regulation of Clp signaling on *pilA* transcription (Table 2), suggests that Clp signaling plays a regulatory role on the surface motility in *L. enzymogenes*. To further address this possibility, we compared the ability of wild-type OH11 and the *clp*-deletion mutant in this function. As shown in Fig. 3, the wild-type strain OH11 exhibited clear surface motility. On the edge of a single colony of strain OH11, bacterial cells could be observed. Mutation of *clp* however impaired surface motility, as no bacterial cells could be detected at the edge of the *clp* mutant colonies. Under the same conditions, the *clp* mutant containing the empty vector was also deficient in surface motility, whereas the *clp* complemented strain exhibited wild-type surface motility. We also introduced the *pilA* complemented construct into the *clp* mutant to test whether expression of *pilA* can fully or partially restore the deficiency of the *clp* mutant in surface motility. However, expression of *pilA* in the background of the *clp* mutation did not restore the deficiency of the *clp* mutant in surface motility (Fig. 3). This result is also reasonable, because as noted in Fig. 1, three clusters (F, G and H) relating to T4P were all reduced at the transcriptional level in the *clp* mutant. Therefore, it is possible that the regulation of *clp* on surface motility is due to the modulation of gene clusters but not a single component (*pilA*) associated with T4P. Nevertheless, our results indicated that the Clp regulator plays a positive regulatory role in the transcription of T4P genes responsible for surface motility in *L. enzymogenes*.

Involvement of *clp* in the regulation of antibiotic-metabolite biosynthesis and surface motility is independent in *L. enzymogenes*

The above findings suggest that *clp* regulates both antibiotic-metabolite biosynthesis and the surface motility in *L. enzymogenes*. However, it is unknown whether Clp signaling utilizes a similar or different regulatory cascade in order to regulate these two functions. To address this point, we generated the HSAF- and WAP-8294A2-deficient mutant or double-deficient mutant to determine their effects on surface motility in *L. enzymogenes* (Fig. S7 in the Supplementary Material). As shown in Fig. S8 in the Supplementary Material, we clearly observed that individual disruption of HSAF and WAP-8294A2 or double mutations did not show any visible effect on surface motility. Thus, it is likely that the regulation of Clp signaling on surface motility is independent of the production of antibiotic metabolites. Moreover, we investigated the role of T4P on antibiotic biosynthesis. We observed that the *pilA* mutant (mutant deficient of T4P) exhibited wild-type production of HSAF and WAP-8294A2 (Fig. S9; Table S6 and S7 in the Supplementary Material). Taken together, these results suggest that Clp signaling acted independently in the regulation of antibiotic biosynthesis and surface motility in *L. enzymogenes*.

A *clp*-controlled factor Lat was involved in the modulation of HSAF biosynthesis, but not of surface motility

Regulators, such as transcriptional regulators with the ability to modulate gene transcriptional activity, were found to play important roles in signal pathway in bacteria (He et al. 2007). Therefore, to address the potential regulatory pathway of Clp signaling in antibiotic biosynthesis, we focused on searching for these types of regulators from Clp

regulons by using bioinformatics. This led to the identification of 8 candidate regulators from the Clp regulon. We then deleted 8 regulators to investigate their roles in *clp*-controlled antibiotic biosynthesis (Table S3; Fig. S10A in the Supplementary Material). Due to the rather laborious techniques, we initially focused on HSAF regulation. If any of these 8 regulators was found to be involved in the regulation of HSAF biosynthesis, we then also tested its role in WAP-8294A2 production. In this way, we identified a protein/regulator (Lys893) that was involved in HSAF biosynthesis, but not associated with WAP-8294A2 production (see below). The other 7 regulators tested were not involved in HSAF biosynthesis (Fig. S10B; Table S6 in the Supplementary Material). Sequence analysis showed that the protein Lys893 is a homologue of a histone acetyltransferase HPA2-like acetyltransferase from *Hahella chejuensis* (E-value: 3e-71 and Identity: 67%) (Fig. S11 in the Supplementary Material). It also shared high similarity at the amino-acid sequence level to other counterparts (*N*-acetyltransferase), such as those from *Rhodanobacter fulvus* (E-value: 3e-70 and Identity: 67%) and *Bacillus subtilis* (E-value: 5e-64 and Identity: 60%) (Fig. S11 in the Supplementary Material). Based on the sequence similarity, we defined this protein as Lat (Lysobacter acetyltransferase) in *L. enzymogenes*.

The role of *lat* in antibiotic-metabolite biosynthesis was then investigated. As shown in Fig. 4A, HSAF production in the *lat* mutant was almost abolished as determined by HPLC. Furthermore, qPCR results showed that the expression of *pks/nrps*, the critical gene for HSAF biosynthesis was dramatically reduced in the *lat* mutant (Fig. 4B). All of these phenotypes could be partially restored in the corresponding complemented strain. These results suggested that Clp signaling probably utilized a *lat*-dependent pathway to regulate HSAF biosynthesis. However, mutation of *lat* did not show any visible effect on WAP-8294A2 production (Fig. S12A; Table S7 in the Supplementary Material), indicating that the regulation of Clp signaling on these two antibiotic biosynthetic pathways was probably different. As shown in Fig. S12B in the Supplementary Material, mutation of *lat* did not impair wild-type surface motility, indicating that *lat* was not involved in surface motility in *L. enzymogenes*.

Discussion

The roles of the Clp signaling pathway in lytic-enzyme production, antifungal activity, and biocontrol efficiency have been reported in *L. enzymogenes* C3 (Kobayashi et al. 2005). Here, we demonstrated new roles of this signaling in antibiotic-metabolite biosynthesis and surface motility in *L. enzymogenes* OH11. We studied the Clp regulon at the genome-wide level and determined that Clp is a global regulator in gene expression. A new *clp*-controlled regulator (Lat) was identified to be involved in HSAF biosynthesis.

Phenotypic and gene-expression analysis showed that *clp* controls the biosynthesis of HSAF and WAP-8294A2, two characterized antibiotic metabolites in *L. enzymogenes*. However, we did not find the putative binding motif of the well-characterized Crp (TGTGANNNNNTCACA) of *E. coli* (Zheng et al. 2004) in the promoter of *pks/nrps* and *wapsI*, two important genes responsible for HSAF and WAP-8294A2 biosynthesis, respectively. These results indicate that the Clp (Crp-like protein) regulator of *L.*

enzymogenes might utilize an indirect way to regulate these loci or the binding motif of Clp regulator in *L. enzymogenes* might be different with that of Crp in *E. coli*.

The nature of the factor(s) in the Clp signaling pathway that is responsible for *clp*-controlled antibiotic-metabolite biosynthesis is currently unknown. In *X. campestris* pv. *campestris*, two transcriptional factors, Zur and FhrR (a TetR-type transcription factor) are located downstream of Clp signaling, and both are involved in the regulation of a wide range of *clp*-modulated functions/genes (He et al. 2007). In general, Zur is involved in iron uptake, TCA cycle, aerobic respiration, multidrug resistance and detoxification, whereas FhrR plays an important role in the Hrp system, flagella genes and ribosomal proteins (He et al. 2007). We did not detect an FhrR homologue in the genomic sequence of *L. enzymogenes* OH11. Although we identified a homologue of Zur (Lys4487) in the genome of strain OH11, it was not detected in the Clp regulon (Table S3 in the Supplementary Material). Moreover, mutation of *zur* did not show any effect on HSAF and WAP-8294A2 production (Fig. S13; Table S6 and S7 in the Supplementary Material), suggesting that the regulator Clp also did not use a *zur* or *fhrR*-dependent mechanism to regulate the biosynthesis of these two antibiotic metabolites. In the present study, a novel regulator (Lat), probably acting as a downstream component of the Clp signaling pathway, was identified as a regulator of HSAF biosynthesis, but not WAP-8294A2. This suggested that the regulator Clp probably used a Lat dependent as well as a Lat independent mechanism to regulate HSAF and WAP-8294A2 biosynthesis in *L. enzymogenes*, respectively. Interestingly, we also did not find the putative *E. coli* Crp binding site in the upstream region of the *lat* gene. Therefore, these results indicate that the Clp (Crp-like protein) regulator of *L. enzymogenes* might utilize an indirect way to regulate the transcriptional expression of *lat* or the binding motif of Clp in *L. enzymogenes* might be different with that of Crp in *E. coli*.

Lat belongs to the family of *N*-acetyltransferases (NATs), also called the Gcn5-related *N*-acetyltransferase (GNAT), which encompasses enzymes that catalyze the transfer of an acetyl group from acetyl-CoA to a primary amine of acceptor molecules (Dyda et al. 2000). The members of this superfamily are characterized by four conserved sequence motifs (A-D) arranged in the order C-D-A-B in the primary sequence. All known crystal structures of GNAT proteins suggest that these homologous regions are involved in acetyl-CoA binding (Dyda et al. 2000). This superfamily spans all life kingdoms and includes enzymes with a wide range of substrates and cellular functions. These enzymes are reported to acetylate various proteins, including the N-terminal [α]-amino group of proteins, the [ϵ]-amino group of lysine residues, aminoglycoside antibiotics, spermine/spermidine and arylalkylamines such as serotonin (Neuwald and Landsman 1997). The GNAT superfamily has been shown to play important roles in gene regulation, antibiotic resistance and hormonal regulation of circadian rhythms (Neuwald and Landsman 1997).

Histone acetylation has been recognized as an important factor modulating gene expression via its effects on chromatin structure and assembly (Kuo and Allis 1998). In the present study, sequence alignment showed that Lat shares the highest similarity to histone acetyltransferase Hpa2-like acetyltransferase (HAT), which belongs to the GNAT superfamily. Hpa2, a member of this family, is from *Saccharomyces cerevisiae* and has been shown to acetylate specific lysine residues of histone H3 and H4 (Angus-Hill et al. 1999).

Hpa2 is also able to autoacetylate itself in an intramolecular reaction (Angus-Hill et al. 1999). The present work showed that Lat, a homologue of Hpa2 was involved in antibiotic-metabolite (HSAF) biosynthesis, suggesting an important role of Lat in biocontrol-related trait in *L. enzymogenes*. The counterpart of histone in bacteria is H-NS (Histone-like nucleoid structuring protein) (Atlung and Ingmer 1997). H-NS binds to A-T-rich bent regions of DNA and modulates the expression of a wide range of environmentally controlled genes (Atlung and Ingmer 1997; Ussery et al. 1994; Williams and Rimsky 1997). Whether H-NS or other DNA binding regulator plays a role in HSAF biosynthesis in *L. enzymogenes* is unexplored. If yes, whether Lat can acetylate H-NS or other DNA binding regulator, modulating its activity, resulting in regulating HSAF biosynthesis is also unknown. We currently did not find available reports about the later point. Addressing these issues will be important for deep understanding the regulatory mechanism of Lat in HSAF biosynthesis in *L. enzymogenes*.

T4P are hair-like appendages found on the surface of a wide range of bacteria belonging to the β -, γ -, and δ -*Proteobacteria*, *Cyanobacteria* and *Firmicutes* (Burdman et al. 2011). Tens of genes are involved in T4P synthesis and regulation, with the majority of them being generally named *pil/fim* genes, including *pilA*, the gene encoding the major subunit (pilin) of the T4P filament (Burdman et al. 2011). The multiple functionalities of T4P are best studied in animal pathogenic bacteria and also recently investigated in plant bacterial pathogens (Burdman et al. 2011). However, relatively little attention has been given to the role of T4P in biocontrol bacteria. Interestingly, it is known that T4P is involved in the attachment and infection of *L. enzymogenes* C3 to the hyphae of fungal hosts (Patel et al. 2011). Mutation of *clp* affected this function similarly to that of the T4P mutant (Kobayashi and Yuen 2005). This finding provided a link between Clp signaling and T4P. The present study provided evidence to support that Clp signaling controlled T4P synthesis in *L. enzymogenes*, since most genes responsible for T4P synthesis at the transcriptional level were modulated by Clp signaling (Fig. 1; Table 2 and S5 in the Supplementary Material). This regulation was subsequently confirmed by the overlap and similar function of Clp signaling and T4P in surface motility, which is a representative characteristic of T4P in diverse bacteria. This and previous findings indicate that Clp signaling used a T4P dependent mechanism(s) to attach and infect hyphae of fungal hosts. However, we did not find the putative binding-motif of an *E. coli* Crp on the promoter of *pilA*, suggesting that the regulatory role of Clp (Crp-like protein) on Type IV pili (T4P) could be possibly be indirect or the binding-motif of Clp in *L. enzymogenes* might be different than that of Crp in *E. coli*. More importantly, mutation of *lat* did not show any effect on surface motility (Fig. S12B in the Supplementary Material), providing evidence that suggested the regulation of Clp on T4P was probably independent of the Lat regulator in *L. enzymogenes*.

In summary, the present study showed that Clp signaling positively and independently modulated the biosynthesis of the antibiotic metabolites (HSAF and WAP-8294A2) and surface motility in *L. enzymogenes*. A new *clp*-controlled regulator Lat was identified to be involved in the regulation of HSAF biosynthesis. The regulation of WAP-8294A2 production and surface motility by Clp signaling is *lat* independent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

The gene clusters regulated by Clp in *Lysobacter enzymogenes*. The red and blue arrow indicate that these genes are negatively and positively modulated in Clp regulons, respectively. The white arrow indicates that these genes were not detected in the Clp regulon. Detailed information is provided in Table S5 in the Supplementary Material.

Fig. 2.

Mutation of *clp* impaired HSAF (A) and WAP8294A2 (B) production levels in *Lysobacter enzymogenes*. Peak area indicates the areas of HSAF or WAP-8294A2 determined by HPLC method (Table S6 and S7 in the Supplementary Material). OD_{600nm} represents the growth status of tested strains at the time points used for the extraction of HSAF or WAP-8294A2 in *L. enzymogenes*. OH11, the wild-type strain *L. enzymogenes*; *clp*, the *clp* deletion mutant of strain OH11, *clp*(*clp*), the complemented strain of *clp*; *clp* (pBBR), *clp* mutant harboring the empty vector. WAP is an abbreviation of WAP-8294A2. Vertical bars represent standard errors. Different letters above the bars indicate a significant difference between the wild-type strain OH11 and the tested strains ($P < 0.05$; *t*-test).

Fig. 3.

Mutation of *pilA* and *clp* impaired surface motility in *Lysobacter enzymogenes*. A classical phenomenon of surface motility is always linked with the finding of many “bacterial cells” on the edge of a bacterial colony. Mutation of *pilA* and *clp* resulted in no “bacterial cells” on the edge of corresponding mutant colonies. OH11, the wild-type strain *L. enzymogenes*; *pilA*, the *pilA* deletion mutant of strain OH11, *pilA(pilA)*, the complemented strain of *pilA*; *pilA* (pBBR), *pilA* mutant harboring the empty vector; *clp*, the *clp* deletion mutant of strain OH11, *clp(clp)*, the complemented strain of *clp*; *clp* (pBBR), *clp* mutant harboring the empty vector; *clp(pilA)*, expression of *pilA* in the *clp* mutant.

Fig. 4.

Mutation of *lat* impaired HSAF production in *Lysobacter enzymogenes* OH11. A, quantitative determination of HSAF production in tested *Lysobacter* strains; B, qRT-PCR analysis of the transcriptional level of *pks/nrps* in tested *Lysobacter* strains. Peak area indicates the areas of HSAF determined by HPLC method (Table S6 in the Supplementary Material). OD_{600nm} represents the growth status of tested strains at the time points used for the extraction of HSAF in *L. enzymogenes*. *pks/nrps*, a key gene for HSAF biosynthesis (Lou et al. 2011). OH11, the wild-type strain *L. enzymogenes*; Δlat , the *lat* deletion mutant of strain OH11, $\Delta lat(lat)$, the complemented strain of *lat*; $\Delta lat(pBBR)$, the *lat* mutant harboring the empty vector. Vertical bars represent standard errors. Different letters above the bars indicate a significant difference between the wild-type strain OH11 and the tested strains ($P < 0.05$; *t*-test).

Table 1

Bacterial strains and plasmids used in this study.

Strains and plasmids	Characteristics ^a	Source
<i>Lysobacter enzymogenes</i>		
OH11	Wild type (CGMCC No. 1978) , Km ^R	Qian et al. (2009)
<i>clp</i>	<i>clp</i> in-frame deletion mutant, Km ^R	This study
<i>clp(clp)</i>	<i>clp</i> harboring plasmid pBBR- <i>clp</i> , Gm ^R , Km ^R	This study
<i>c/p(pBBR)</i>	<i>clp</i> harboring plasmid pBBR1-MCS5, Gm ^R , Km ^R	This study
<i>clp(pilA)</i>	<i>clp</i> harboring plasmid pBBR- <i>pilA</i> , Gm ^R , Km ^R	This study
<i>pilA</i>	<i>pilA</i> in-frame deletion mutant, Km ^R	This study
<i>pilA(pilA)</i>	<i>pilA</i> harboring pBBR- <i>pilA</i> , Gm ^R , Km ^R	This study
<i>pilA</i> (pBBR)	<i>pilA</i> harboring plasmid pBBR1-MCS5, Gm ^R , Km ^R	This study
<i>pks</i>	<i>pks</i> in-frame deletion mutant, Km ^R	This study
<i>wapsI</i>	<i>wapsI</i> in-frame deletion mutant, Km ^R	This study
<i>pks wapsI</i>	Double deletion mutant of <i>pks</i> and <i>wapsI</i>	This study
<i>zur</i>	<i>zur</i> in-frame deletion mutant, Km ^R	This study
<i>lat</i>	<i>lat</i> in-frame deletion mutant, Km ^R	This study
<i>lat(lat)</i>	<i>lat</i> harboring plasmid pBBR- <i>lat</i> , Gm ^R , Km ^R	This study
<i>lat</i> (pBBR)	<i>lat</i> harboring plasmid pBBR1-MCS5, Gm ^R , Km ^R	This study
0093	0093 in-frame deletion mutant, Km ^R	This study
0394	0394 in-frame deletion mutant, Km ^R	This study
1044	1044 in-frame deletion mutant, Km ^R	This study
1456	1456 in-frame deletion mutant, Km ^R	This study
3322	3322 in-frame deletion mutant, Km ^R	This study
4987	4987 in-frame deletion mutant, Km ^R	This study
5155	5155 in-frame deletion mutant, Km ^R	This study
<i>Escherichia coli</i>		
DH5α	<i>supE44lacU169(lacZAM15) hsdR17 recA lenda lgyrA96 thi-1 relA11</i>	Qian et al. 2013
Plasmids		
pEX18GM	Suicide vector with a <i>sacB</i> gene, Gm ^R	Hoang et al. (1998)
pBBR1-MCS5	Broad-host-range vector with a P _{lac} promoter, Gm ^R	Kovach et al. (1995)
pEX18- <i>clp</i>	pEX18GM with two flanking fragments of <i>clp</i> , Gm ^R	This study
pEX18- <i>pilA</i>	pEX18GM with two flanking fragments of <i>pilA</i> , Gm ^R	This study
pEX18- <i>pks</i>	pEX18GM with two flanking fragments of <i>pks</i> , Gm ^R	This study
pEX18- <i>wapsI</i>	pEX18GM with two flanking fragments of <i>wapsI</i> , Gm ^R	This study
pEX18- <i>lat</i>	pEX18GM with two flanking fragments of <i>lat</i> , Gm ^R	This study
pEX18-0093	pEX18GM with two flanking fragments of <i>clp</i> , Gm ^R	This study
pEX18-0394	pEX18GM with two flanking fragments of 0394, Gm ^R	This study
pEX18-1044	pEX18GM with two flanking fragments of 1044, Gm ^R	This study
pEX18-1456	pEX18GM with two flanking fragments of 1456, Gm ^R	This study

Strains and plasmids	Characteristics ^a	Source
pEX18-3322	pEX18GM with two flanking fragments of 3322, Gm ^R	This study
pEX18-4987	pEX18GM with two flanking fragments of 4987, Gm ^R	This study
pEX18-5155	pEX18GM with two flanking fragments of 5155, Gm ^R	This study
pEX18- <i>zur</i>	pEX18GM with inner flanking fragments of <i>zur</i> , Gm ^R	This study
pBBR- <i>clp</i>	pBBR1-MCS5 cloned with a 1250-bp fragment containing intact <i>clp</i> and its predicted promoter, Gm ^R	This study
pBBR- <i>pilA</i>	pBBR1-MCS5 cloned with a 1105-bp fragment containing intact <i>pilA</i> and its predicted promoter, Gm ^R	This study
pBBR- <i>lat</i>	pBBR1-MCS5 cloned with a 978-bp fragment containing intact <i>lat</i> and its predicted promoter, Gm ^R	This study

^aKm^R, Gm^R = Kanamycin-, Gentamicin-resistant, respectively.

Table 2

Comparison of 20 selected gene expressions between qRT-PCR and Microarray

Selected Gene ID ^a	Blastp homolog	Gene name	Microarray (<i>clp</i> /OH11)	qRT-PCR (<i>clp</i> /OH11)
Genes positively modulated by Clp				
LysEGL000394	GntR family transcriptional regulator	0394	0.438	0.1883±0.0294
LysEGL000893	Histone acetyltransferase HPA2-like acetyltransferase	<i>lat</i>	0.4618	0.2964±0.1263
LysEGL001319	Hypothetical protein STIAU_8011	1319	0.3248	0.0687±0.0027
LysEGL001351	GGDEF family protein	1351	0.1815	0.1231±0.0118
LysEGL001456	Transcriptional regulator	1456	0.4172	0.4278±0.0113
LysEGL001801	Tfp pilus assembly pathway ATPase PilB	<i>pilB</i>	0.4447	0.6671±0.0696
LysEGL001803	Fimbrial protein EcpC	<i>pilA</i>	0.4073	0.2133±0.0225
LysEGL002168	WAPS2	<i>waps2</i>	0.1004	0.0037±0.0009
LysEGL002170	WAPS1	<i>waps1</i>	0.0367	0.0018±0.0014
LysEGL002648	Ox2	Ox2	0.0712	0.0006±0.0003
LysEGL002651	Hybrid polyketide synthase and nonribosomal peptide synthetase	<i>pks-nrps</i>	0.0529	0.0006±0.0002
LysEGL004691	Chitinase A	<i>chiA</i>	0.2465	0.0177±0.0016
LysEGL004987	MerR family transcriptional regulator	4987	0.2459	0.3579±0.0750
LysEGL005055	Threonine dehydratase	5055	0.4159	0.4244±0.3241
LysEGL005155	Putative regulatory protein, TfoX family	5155	0.4666	0.4535±0.0108
Genes negatively modulated by Clp				
LysEGL001352	Rhs family protein	1352	7.1914	57.3349±17.0155
LysEGL001365	Flagellar hook capping protein	1365	5.8559	15.2282±2.9075
LysEGL001371	Flagellar M-ring protein FlhF	<i>flhF</i>	3.7637	5.7277±1.0353
LysEGL001381	Flagellar biosynthetic protein FlhB	<i>flhB</i>	7.9342	9.5038±0.9710
LysEGL001382	Flagellar biosynthesis pathway protein FlhR	1382	5.743	5.8081±1.2667

^aGene ID was based on the genome sequence of *L. enzymogenes* strain OH11, which could be found with the accession number of GSE58846 in NCBI.