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LotS/LotR/Clp, a novel signal pathway responding to temperature, modulating protease expression via c-di-GMP mediated manner in *Stenotrophomonas maltophilia* FF11

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ABSTRACT

Stenotrophomonas maltophilia as one of increasing food spoilage bacteria and fish pathogens has become a threat to aquiculture industry. A major factor contributing to the success of bacterium is its outstanding ability to secrete protease at low temperatures. Here, a cAMP receptor like protein (Clp) shows a positive regulation on this protease, named *S. maltophilia* temperature-response protease (SmtP). Interestingly, a two-component system, comprising of LotS sensor and LotR regulator, for low-temperature response is also confirmed to modulate SmtP expression with similar effect to Clp. Evidence is presented that LotS/LotR modulates *smtP* (coding SmtP) expression via Clp: *clp* promoter activity was reduced significantly at low temperatures and protease activity was partially restored by Clp overexpressed in *lotS* or *lotR* deletion strain. Furthermore, as a Clp negative effector, the binding ability of c-di-GMP with Clp is not impacted by temperature. c-di-GMP level was increased in *S. maltophilia* growing at high temperature, but not exhibited significantly in *lotR* deleted strain, these indicate that LotR is required for temperature modulating c-di-GMP level, although the synthesis or degradation activity of c-di-GMP by LotR was not detected. These findings suggest that LotS/LotR/Clp play an important role in responding to temperature stimuli via c-di-GMP mediated manner.

1. Introduction

Bacteria are constantly subjected to different environmental stimuli, mainly related to the cellular metabolism process or lifestyle. A key factor influencing bacterial processes is temperature (Guijarro et al., 2015). Especially for the seafood processing and aquaculture industry, the regulation of gene expression of microorganisms in response to low temperatures seems to be particularly important (Guijarro et al., 2015; Kranzler et al., 2016; Meng et al., 2017). In many cases, the changes of many cellular processes are caused even by small temperature shifts, and therefore bacteria must possess sophisticated thermosensing devices in order to respond and adapt to changing temperature (Cybulski et al., 2010). Two-component systems (TCS) that link environmental signals to cellular responses are viewed as one of the primary modes of signal transduction in prokaryotes (Ulrich et al., 2005). A prototypical TCS usually consists of a membrane-bound sensor histidine kinase and a cognate cytoplasmic response regulator. The histidine kinase perceives environmental stimuli and causes an autophosphorylation of the kinase. The phosphoryl group is transferred to the cognate response regulator, resulting in the activation of the C-terminal output domain of the response regulator (Casino et al., 2010; Willett et al., 2013). The majority of experimentally characterized temperature-responsive TCS, such as DesKR from *Bacillus subtilis*, CorRS from the plant pathogenic bacterium *Pseudomonas syringae pv. Glycinea* PG4180 and LtrK/LtrR from *Methanococcoides burtonii* regulate gene expression at the level of transcription using the DNA-binding helix-turn- helix (HTH) output domains of the response regulators (Aloza-Vazquez and Bender, 1998; Hunger et al., 2004; Smirnova and Ullrich 2004; Najnin et al., 2016). Other regulation forms of TCSs such as binding to RNA, protein or performing enzyme activities (Fouhy et al., 2006; Casino et al., 2010) was reported previously.

The cyclic AMP (cAMP) receptor protein (Crp), a well-studied transcriptional regulator present in *Escherichia coli* (*E.coli*) that responds to environmental stimuli (Zheng et al., 2004). This protein contains a conserved N-terminal cAMP-binding domain and a C-terminal helix-turn-helix (HTH) DNA-binding domain (Pesavento and Hengge 2009).

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The binding of cAMP to Crp causes a conformational change in Crp and is indispensable for the regulatory activity of Crp (Sureka et al., 2014). A recent study also indicated that transcription factor Clp, a homologue of the cyclic AMP receptor protein, is involved in regulation of secondary metabolite biosynthesis and several virulence factors in *Xanthomonas campestris pv. campestris* (Andrade et al., 2006; Hsiao et al., 2009; Wang et al., 2014). In contrast to Crp, the Clp regulator has been shown to specifically interact with c-di-GMP (3′, 5-cyclic diguanylic acid), but not cAMP (Tao et al., 2010; Zhang 2010). The interaction of c-di-GMP with Clp abolishes the ability of Clp to bind to its target gene promoter, indicating that c-di-GMP acts as a negative effector for downstream gene expression via changing Clp conformation in *Xanthomonas campestris pv. campestris* (Chin et al., 2010). In *S. maltophilia*, it has been reported that a cyclic AMP receptor protein relating to cell-cell communication system that mediates expression of a FecA (ferric citrate receptor) homologue in *S. maltophilia* (Huang and Wong 2007). Nonetheless, there has been little information concerning transcriptional regulator for regulating temperature-dependent genes expression in *S. maltophilia*.

S. maltophilia, belonging to the subclass of γ-β-*proteobacteria* with a high G + C content, is gram-negative bacillus widely distributed in a variety of environmental habitats such as soil, foods, plant rhizospheres and hospital disinfectant solutions (Brooke 2012). Also, *S. maltophilia* as fish emerging pathogens has become a great threat for cultured channel catfish (Geng et al., 2010), African catfish (Abraham et al., 2016), Yellowtail (Furushita et al., 2005), Gilthead (Kapetanovic et al., 2006) and Zebrafish (Ferrer-Navarro et al., 2013). The extracellular protease of *S. maltophilia* that isolated from channel catfish (*Ictalunes punctatus*) has been verified for the virulence factor (Huang and Geng 2006; Du et al., 2011). And as food spoilage bacteria *S. maltophilia* isolated from mahi-mahi (Frank et al., 1985), raw milk (Tzanetakis et al., 1995), albacore tuna (Ben-Gigirey et al., 2000) and beef (Fujita et al., 2007) have also been reported. The protease production of *S. maltophilia* was also investigated for relevant interest for their potential role in the spoilage of fish (Ben-Gigirey et al., 2000). However, the regulatory mechanism(s) for the temperature-dependent protease secretion remains unclear.

In our previous study, *S. maltophilia* FF11, isolated from frozen Antarctic krill, has been reported to secrete more proteases at low temperatures (Wang et al., 2016). In this study, Results of quantitative RT-PCR (qRT-PCR) and promoter activity assay showed that *smtP* expression was coordinated by temperature at the transcription level. Transcription factor Clp and two-component regulatory system of LotS/LotR involved in SmtP production were confirmed to respond to low temperature stimuli, based on assay of genes deletion in *S. maltophilia* FF11. Furthermore, the change of *clp* promoter activity and protease production after Clp overexpression in *lotS* or *lotR* deleted strain were detected compared to *S. maltophilia* FF11. Additionally, the function of LotR containing putative GGDEF and EAL related to c-di-GMP synthesis or degradation was investigated in vitro by biochemical analysis. c-di-GMP level in different strains and the binding ability of Clp with c-di-GMP in vitro were also assayed. Our results reveal that a novel signal transduction pathway of LotS/LotR/Clp for regulation of gene expression in response to temperature stimuli via a c-di-GMP mediated manner in *S. maltophilia* FF11.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All bacterial strains and plasmids used in this work are listed in Tables 1 and 2. *S. maltophilia* FF11 (GenBank accession number KP399636) was used as wild-type strain. *E. coli* JM109 and S17-1λpir were used for cloning and conjugation, respectively. *E. coli* BL21 (λDE3) cells were employed in expression of recombinant protein Clp. Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7.5) was used to grow all strains. Temperature experiments were performed by inoculating overnight-grown *S. maltophilia* FF11 in a 1:100 dilution in LB medium followed by cultivation with shaking 180 rpm/min for different times unless otherwise noted at 15 °C, 25 °C and 37 °C, respectively. *E. coli* strains were grown at 37 °C (Clp expression induced at 18 °C). If required, antibiotics were used at the following final concentrations 50 μg/mL kanamycin, 100 μg/mL ampicillin, 15 μg/mL tetracycline (Tet) for *E. coli* and 125 μg/mL for *S. maltophilia*, chloramphenicol (Cm), 25 μg/mL for *E. coli* and 150 μg/mL for *S. maltophilia*.

2.2. Construction of the clp, lotS and lotR gene deletion and complement strains

DNA primers used in this study are listed in Table 3. The deletion mutants of Δ*clp* (*clp* gene deletion), Δ*lotR* and Δ*lotS* derived from *S. maltophilia* FF11 were constructed by homologous recombination as described previously by using the suicide vector pEX18Tc (Lin et al., 2009). The upstream and downstream fragments flanking both sides of the *clp* gene were amplified using primers *clp* up F/up R and *clp* down F/down R, respectively. Meanwhile, cm^r gene with its promoter region was amplified by PCR with the primers *clp* cm^r F/R. These three fragments were used as templates in an overlapping PCR, resulting in a fragment with a deletion in *clp* and insertion of cm^r. Then, the PCR product was digested with *Bam*H I and *Hind* III restriction enzymes and ligated to the suicide vector pEX18Tc, resulting in plasmid pEX18Tc-*clp*. With the similar procedure, the upstream and downstream fragments flanking both sides of the *lotR* and *lotS* gene were amplified using primers *lotR* up F/up R, *lotR* down F/down R and *lotS* up F/up R, *lotS* down F/down R, respectively. After a second fusion PCR, the overlapping fragments was digested with *Kpn* I and *Bam*H I (*lotS*) or *Bam*H I and *Hind* III (*lotR*) restriction enzymes and cloned into the suicide vector pEX18Tc, generating plasmids pEX18Tc-*lotS* and pEX18Tc-*lotR*. pEX18Tc-*clp*, pEX18Tc-*lotR* and pEX18Tc-*lotS* were transformed into *S17-1λpir* and introduced into *S. maltophilia* FF11 via conjugation (Okazaki and Avison 2008; Lin et al., 2009). The single-crossing transconjugants were selected by Cm and Tet resistance and verified by PCR. *S. maltophilia* FF11 with pEX18Tc-*clp*, pEX18Tc-*lotR* and pEX18Tc-*lotS* inserted into the chromosome were plated onto LB agar medium supplemented with Cm (150 μg/mL) and 8% sucrose. The successful Δ*clp*, Δ*lotS* and Δ*lotR* were screened and verified by PCR. For complementation analysis, the coding regions of *clp*, *lotS* and *lotR* were amplified by PCR using the primers listed in Table 2. Then, the PCR products were digested with *Eco*R I and *Hind* III (*clp*) or *Bam*H I and *Hind* III (*lotS* and *lotR*) and cloned into expression vector pLAFR3 under the control of lac promoter, respectively. The recombinant plasmids pLAFR3-*clp*, pLAFR3-*lotR* and pLAFR3-*lotS* were transferred into respective deletion strains via conjugation, generating complementation strains Δ*clp* (C), Δ*lotS* (C) and Δ*lotR* (C), respectively. Gene sequences of *clp*, *lotR* and *lotS* have been deposited to GenBank database, and the accession number was MG776304 (*clp*), MG776305 (*lotR*) and MG776306 (*lotS*), respectively. To assay of promoter activity, the reporter vector pL6gusA with fusing a promoterless *gusA* (β-glucuronidase) gene was used as described previously (Jiang et al., 2008). A 452-bp upstream fragment of *smtP* gene was amplified by PCR, cloned into pL6gusA and pL6*smtP*-gusA obtained. Similarly, a 534-bp upstream fragment containing *clp* gene promoter was amplified and ligated into the reporter vector pL6gusA, named pL6*clp*-gusA.

2.3. Extracellular protease activity and zymogram assay

Bacteria growth was determined when strains were cultivated (180 rpm/min) at 15 °C, 25 °C and 37 °C, respectively. The samples were collected every 12 h and detected at OD_{600}. Protease activity was determined using a modified method described by Ribitsch et al. (2012). The detailed procedures were referred to our previous study (Wang

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