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Research Article

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Cloning and expression of *cpxA* gene from *Pectobacterium carotovorum subsp. carotovorum*

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ABSTRACT

Histidine kinase CpxA, a kind of membrane protein, is a pivotal member in Cpx two-component regulatory systems, which consist of CpxA, CpxR, and CpxP. Of those, CpxA is the most important part with the function of acting as sensor kinase. In order to clarify the specific mechanism of CpxA in Cpx system, some experiments must be carried out in vivo and in vitro, so the urgent problem is to express the CpxA protein in vitro. In this study, therefore, cpxA gene from Pectobacterium carotovorum subsp. carotovorum was cloned and inserted into pET-15b plasmid, and the recombinant plasmid (pET-15b/cpxA) transformed into BL21 (DE3). Additionally, the cpxA gene was expressed through the induction of IPTG, under the control of lac promoter.

Key words: *Pectobacterium carotovorum* subsp. *carotovorum* (PC1), Cpx, CpxA , cloning, expression

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INTRODUCTION

To survive, all bacteria have to adapt to the changing environment through signal transduction systems, among which two-component regulatory systems (TCRS) are the most important and well known (Xu, 2014). Each of these systems are composed of a histidine kinase (HK) in the inner membrane and a cytosolic response regulator (RR), resulting in a cellular response. HK and RR exchange information via conserved phosphorylation and dephosphorylation reactions(Jung, 2012). When the HK detects a specific signal, it first autophosphorylates and then transfers the phosphate group to the RR, allowing the RR to act as a transcription factor to alter gene expression in most cases(Vogt， 2012). As an important member in TCRS, Cpx two-component regulatory systems are prevalent in gram-negative bacteria.

The Cpx system was first discovered when mutations in the chromosomal *cpxA* locus were found to reduce expression of the F-plasmid conjugative pilus in *Escherichia coli* (McEwen，1980). Cpx systems consist of membrane-anchored sensor kinase CpxA, cytosolic response regulator CpxR, and the accessory protein CpxP in the periplasm (Ruiz, 2005). The Cpx system is involved in the process of bacterial adherence, invasion, motility, and other activities (Raivio, 2001). Some data suggested that the Cpx response has an important effect on the bacterial virulence and pathogenicity (Humphreys, 2004; Mitobe-Arakawa, 2005).

The most important factor of the Cpx system is the inner membrane protein CpxA with its central function as a sensor kinase (Sabine, 2012). CpxA consists of the input domain and the transmitter domain. The cytosolic transmitter domain is composed of kinase core (Dhp domain and C-terminal CA domain) (MacRitchie, 2008) and HAMP linker, which communicates the input domain with transmitter domain (Fig. 1). CpxA responds to envelope stresses through autophosphorylation, likely in a conserved histidine residue, and subsequently transfers the phosphate groups to CpxR (Raivio, 1997). The isolated kinase core of CpxA exhibits both kinase and phosphatase activities (Yamamoto, 2005). The complexity of natural membranes complicates the analysis of these interactions *in situ*, so these studies are commonly carried out in artificial amphiphile systems (Levi, 2003). Therefore, the urgent

problem is to obtain the CpxA protein through the constructed engineering bacteria *in vitro*.

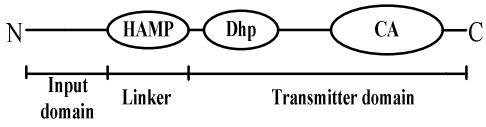

Fig. 1. The structure of CpxA. CpxA integrate signals through the periplasmic input domain and the cytosolic transmitter domain. The HAMP linker transmits signals from the input domain to the transmitter domain (Sabine, 2012).

Pectobacterium carotovorum subsp. carotovorum, classified previously as *Erwinia carotovora subsp. carotovora* (Hong, 2006), belongs to the family of *Pectobacterium carotovorum* of plant pathogenic bacteria (Yang, 2012), which can be found worldwide in surface waters, carrier invertebrates, as well as plant hosts (Mole, 2010). It degrades macromolecules into small molecules within the plant cell wall, leading to maceration of the plant tissue (Kim, 2012). The bacierium can also infect a host plant by multiple routes and can elicit diseases on leaves and stems, as well as in tubers (Masrhi, 2010; Mole, 2010; Park, 2012). The disease outbreaks are generally triggered by environmental factors such as rain or hot weather and can strike during tuber storage, leading to total crop loss (Charkowski, 2009). Soft rot and blackleg of vegetables and other typical symptoms can be caused by the diseases (Fig. 2)(Toth, 2003). Further insight into membrane protein CpxA in *Pectobacterium carotovorum subsp. carotovorum* contributes towards distinctly understanding the role of CpxA in the Cpx system and the pathogenic mechanism; thus, crop loss caused by the diseases can be avoided to a certain extent. Data suggested that some genes such as *caroDK*, *caroDI* (Roh, 2010), *casF*, and *casB* (Kim, 2012) are associated with diseases, and in this article the *cpxA* gene in *Pectobacterium carotovorum subsp. carotovorum* was reported for the first time.

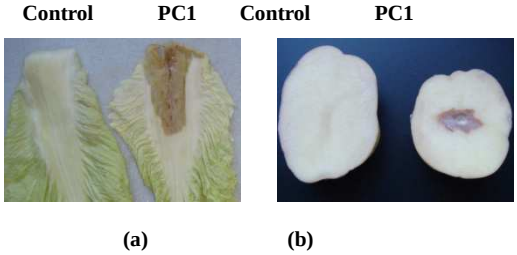

Fig.2. (a) Phenotypes of brassica pekinensis infected with water (Control) and Pectobacterium carotovorum subsp. carotovorum (PC1) for 5 d. (b) Phenotypes of solanum tuberosum infected with water (Control) and Pectobacterium carotovorum subsp. carotovorum (PC1) for 5 d.

In this research, the *cpxA* gene in *Pectobacterium carotovorum subsp. carotovorum* was inserted into the plasmid vector pET-15b, the recombinant plasmid (pET-15b/*cpxA*) was transformed into the strain BL21 (DE3), and through isopropyl β-D-1-thiogalactopyranoside (IPTG) induction a large quantity of target protein CpxA is produced.

EXPERIMENTAL SECTION

Strains and plasmid

Pectobacterium carotovorum subsp. carotovorum (PC1) DSM 30169 was purchased from Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Plasmid pET-15b was kindly provided by professor Hunke. Competent Top10 was purchased from TIANGEN BIOTECH Co.,Ltd. (Beijing, China).

Main reagents

Pfu DNA polymerase (GenStar, China); *Xho* I and *BamH* I restriction enzymes and T4 DNA polymerase (Thermo Fisher, USA); Bacterial genome extraction kit (Tiangen, China); Plasmid Mini Kit (Omega, USA); Agarose gel extraction kit (Transgen, China).

PCR amplification

The *cpxA* gene was amplified by PCR using the genomic DNA of PC1 as a template. The primers (P1 and P2) were designed with prime primer 5.0.

P1:5'-CCGCTCGAGCAGCTTACCGCCCTG C-3'
P2:5'-GCGGATCCTTACCGTTGATGCAATGG-3'

The *Xho* I and *BamH* I restriction sites were underlined. The primers were synthesized by the Beijing Invitrogen

Biotechnology Company.

According to the bacterial genome extraction kit manual, the total genome of PC1 was extracted for *cpxA* gene amplification. PCR reaction (50.0 μL) conditions were as follows: 95□ 2 mins; 95□ 30 sec, 58□ 30 sec, 72□ 2 mims and 30 sec, 35 cycles; and 72□ 5 mins. The PCR was carried out by using the Gene Amp PCR system 9700 (Applied Biosystms). PCR products were confirmed on 1.0% agarose gels and recycled by the agarose gel extraction kit (Macherey-Nagel, Germany).

Cloning of *cpxA* gene

PCR products were digested by *Xho* I and *BamH* I and recycled by the agarose gel extraction kit. The purified PCR products were directly subcloned into the multiple cloning sites of pET-15b, which was also digested with *Xho* I and *BamH* I. 5 μl digested-plasmid DNA and 3.5 μl digested PCR products were mixed with 1.0 μl T4 DNA ligase buffer and 0.5 μl T4 DNA ligase, overnight at 16 □. The 10.0 μl ligation products were transformed into competent Top10 and incubated at 37 □ overnight on agarose plate (containing 100 $\mu g \cdot ml^{-1}$ Ampicillin).

Identification of recombinant expression vector

To confirm whether *cpxA* gene was successfully inserted into plasmid pET-15b, the recombinant plasmids (pET-15b/*cpxA*) were digested with *Xho* I and *BamH* I. According to the electrophoresis, it could be determined that the *cpxA* gene was inserted. And the recombinant plasmids were sent to the Beijing Invitrogen Biotechnology Company for sequencing.

Expression of *cpxA* gene

BL21(DE3) strains, each containing recombinant plasmid, were grown over night in 50 mL Luria-Bertani (LB) medium (100 $\mu g \cdot ml^{-1}$ Ampicillin) at 37□ and 160 rpm. The overnight culture was used to inoculate (20 ml/l) LB medium，supplemented with 100 $\mu g \cdot mL^{-1}$ Ampicillin. The cells were grown at 37℃ to OD value (600 nm) 0.4-0.6 and subsequently induced with different concentrations (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0 mM) of isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 30℃ and 160 rpm.

When the OD value (600 nm) reached to 2, the cells were harvested by centrifugation at 4 ℃ and 10,000 g for 10 min. The pellets from 100 ml culture were resuspended in 10 mL buffer (50 mM Tris-HCl, 0.15 M NaCl, 20% Glycerol, pH 7.5). Then, the suspension was disrupted by sonication on ice and analyzed by SDS-PAGE.

RESULTS

Extraction of the total genome and PCR

The extracted genome of PC1 was detected by 1% agarose gel. The *cpxA* gene was cloned by PCR using the genomic DNA of PC1 as a template, P1 and P2 as primers. The results showed that PCR product contained 1272 bp (Fig. 3).

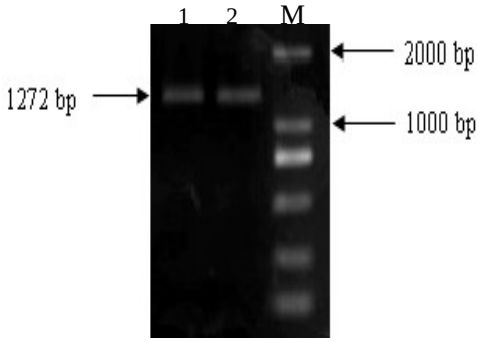

Fig. 3. Cloning and identification of the cpxA in PC1. 1: PCR products of cpxA; 2: PCR products of cpxA; M: DNA marker. The arrows show the targed fragment

Identification of the recombinant plasmid (pET-15b/*cpxA*)

The recombinant plasmid (pET-15b/*cpxA*) was confirmed by double-enzyme digestion (Fig. 4) and gene sequencing.

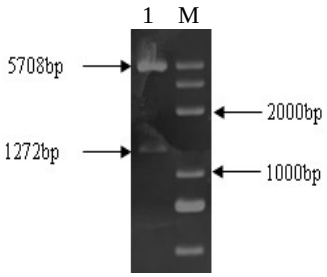

Fig. 4. Identification of recombinant plasmid (pET-15b/*cpxA*). 1: recombinant plasmid digested by *BamH* I and *Xho* I; M: DNA marker. The arrows show the targed fragment

The results showed that after digestion with *Xho* I and *BamH* I, the recombinant plasmid (pET-15b/*cpxA*) had two fragments. The size of lower one (lane 1) was around 1272 bp and was consistent with the PCR product. This indicated that the *cpxA* gene was subcloned into pET-15b plasmid, and the expression vector was constructed successfully.

Expression of recombinant plasmid (pET-15b) and SDS-PAGE

The *cpxA* gene expression was induced by IPTG (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0 mM). The amount of CpxA protein was identified by SDS-PAGE (Fig. 5).

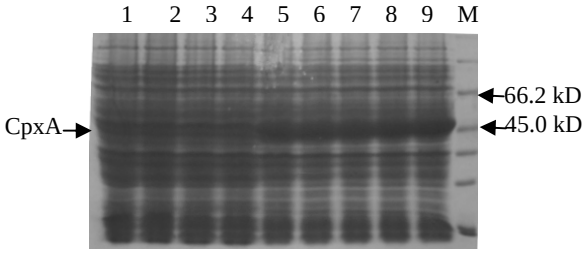

Fig. 5. Identification of the CpxA protein by SDS-PAGE. 1-9: the amount of CpxA protein at different concentrations of IPTG (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0 mM); M: protein marker. The arrows show the targed fragment.

The recombinant plasmid (pET-15b/*cpxA*) was transformed into BL21 (DE3) and was induced by different kinds of concentrations (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0 mM) of IPTG. The protein expression was investigated by the 10% SDS-PAGE. As is shown in Fig. 5, the results showed that the CpxA protein is 50.1 kD, indicating that the *cpxA* gene was successfully expressed in BL21 (DE3). The concentration of IPTG is a critical factor, considering the cost and the production of the target protein (Olaofe, 2010). So, the study was conducted with different concentrations of IPTG with the aim of determining the appropriate concentration. When the concentration ranges from 0 to 0.01 mM, the target gene was lowly expressed, and when the concentration was equal to or greater than 0.05 mM (0.05, 0.1, 0.2, 0.5, 1.0 mM), the *cpxA* gene was highly expressed. Therefore, the optimal concentration of IPTG was 0.05 mM, which resulted in the highest expression of the target protein at a low cost.

DISCUSSION

Bacteria are equipped with two-component systems to cope with environmental changes, and auxiliary proteins provide response to additional stimuli. The Cpx two-component system is the global modulator of cell envelope stress in Gram-negative bacteria that integrates very different signals and consists of the kinase CpxA, the regulator CpxR, and the dual function auxiliary protein CpxP (Zhou, 2011). CpxA is a sensor histidine kinase of a two-component system, Cpx, which is induced by a variety of envelope stresses and has been implicated in expression of key virulence regulators.

The results from the present study showed that the *cpxA* gene from PC1 was successfully inserted into the pET-15b plasmid, and the CpxA protein was expressed in BL21 (DE3).

At present, and for the first time, CpxA protein has been obtained from PC1. Although the CpxA protein was not crystallized, it is clear that these results lay the foundation for analyzing the structure of the CpxA protein and further research of the role of CpxA in Cpx system and bacterial infection. Some data showed that direct protein-protein interaction of CpxA and CpxP existed in *Escherichia coli* (Fleischer, 2007), so in PC1, how does

CpxP interact with CpxA and is the mechanism a protein-protein interaction? Based on the structure of the CpxA as a histidine kinase, how does the CpxA function as an autokinase, a CpxR kinase, and a CpxR-P phosphatase (Raivio, 1999)? Further, site-substitution should be applied to the acquisition of different kinds of CpxA mutant protein *in vivo*. Thus, by investigating the gain and loss of function of CpxA, we can correlate the interaction of the CpxA with CpxP and CpxR.

In conclusion, the present study revealed that the *cpxA* gene could be highly expressed in BL21 (DE3) through the induction of IPTG, and additional studies are required for further structural and functional studies in order to clarify the specific mechanism of CpxA in the Cpx system and subsequently the role of Cpx response in the pathogenicity of *Pectobacterium carotovorum subsp. carotovorum*.

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