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ChiS histidine kinase negatively regulates the production of chitinase ChiC in *Streptomyces peucetius*

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ABSTRACT

Computational analysis of sequence homology of the *chiSRC* gene cluster, encoding a chitinase in *Streptomyces peucetius*, showed that the gene cluster could be a two-component regulon comprising a sensor kinase (*chiS*) and a response regulator (*chiR*). To prove that the *ChiSRC* is an authentic two-component system, the *chiS* gene was cloned and expressed in *E.coli* and the purified protein was used for biochemical analysis. In this report, we provide biochemical evidence to show that the sensor kinase encoded by *chiS* gene indeed is a histidine kinase capable of autophosphorylation and the histidine 144 residue of the ChiS protein is the phosphate acceptor. An insertion mutation at the *chiS* locus led to overproduction chitinase protein in *S. peucetius* implying that the *chiC* gene is negatively regulated by the two-component system.

1. Introduction

Streptomyces are sporulating, soil dwelling, gram-positive eubacteria known to produce a wide range of secondary metabolites, as well as enzymes. Unique capability of *Streptomyces* to utilize multiple carbon sources arises from their ability to produce various catabolic enzymes such as chitinases, proteases, amylases, xylanases and cellulases (Gilbert et al. 1995; Vetrivel et al. 2001) which are regulated significantly. *Streptomyces peucetius*, a producer of anti-tumour antibiotics doxorubicin and daunorubicin, can grow on chitin and produces chitinase encoded by *chiC* gene (Vetrivel and Dharmalingam 2000; Vetrivel et al. 2001; Paranthaman and Dharmalingam 2003).

S. peucetius being a soil dwelling organism is exposed to multiple environmental stimuli and thus requires efficient mechanisms to sense and respond specifically to the stimuli. Bacterial gene expression in response to environmental stimuli is often regulated by two component systems (TCS) (Mizuno 1998). TCS functions as intracellular information processing pathways that link external stimuli to specific adaptive responses towards that stimulus (West and Stock 2001). Transient phosphorylation of the sensor kinases and response regulators at specific conserved residues is required for transducing intracellular and extracellular signals and this reversible phosphorylation leads to an appropriate cellular response (Stock et al. 2000). A typical TCS consists of a sensor kinase (SK) also referred to as sensor histidine kinase (SHK) or histidine kinase (HK), a response regulator (RR) and its target genes. Genome sequencing of *S. coelicolor* has identified 84 genes which encode SKs. Among the 84 SKs, 67 are paired while 17 are unpaired (Hutchings et al. 2004). Chitinase enzymes are shown to be under the control of TCS in *S. coelicolor* (Kormanec et al. 2000) and *S. thermoviolaceus* (Tsujibo et al. 1999).

Histidine kinases are mostly intrinsic membrane proteins with two or more transmembrane α-helices at the N-terminus (Mascher et al. 2006), these domains are important for proper positioning of the protein in membrane (Yamamoto et al. 2005). The C-terminus of histidine kinases forms an independently folded catalytic domain that extends into cytoplasm, and binds to ATP. HKs also contain various domains such as sensing domain, histidine containing phosphotransfer domain, linker domain, and a kinase catalytic core. The cytoplasmic domain of HKs harbours the conserved motifs that include H-box, N-box, F-box and G-boxes. The H-box contains the histidine residue which is the phosphate acceptor in the autokinase reaction. The highly conserved N-box also plays a significant role in the kinase activity (Hsing et al. 1998).

Earlier report from our lab has shown that the nucleotide sequence upstream of *S. peucetius chiC* shares homology with *chiR* genes of *S. coelicolor* and *S. thermoviolaceus* (Vetrivel et al. 2001).

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In this study, we examined the autokinase activity of ChiS, and the site of phosphorylation in ChiS. Role of ChiS in the production of Chitinase ChiC is also explored. To the best of our knowledge, this is the first report that has demonstrated the biochemical characteristics of a TCS histidine kinase involved in regulating chitinase production from *S. peucetius*.

2. Materials and methods

2.1. Reagents and chemicals

Ni-Sepharose was purchased from GE Healthcare (Pittsburgh, USA) and [γ ^{32}P]ATP, [α ^{32}P]dCTP were supplied by Board of Radiation and Isotope Technology (Hyderabad, India). All other chemicals were purchased from Sigma (St. Louis, USA) and Merck (Mumbai, India). Oligonucleotides used in this work were synthesized at Microsynth GmbH (Balgach, Switzerland). Enzymes used in PCR and cloning were purchased from GE Healthcare (Pittsburgh, USA), Promega (Madison, USA), New England Biolabs (Ipswich, USA), Finnzymes (Mumbai, India) and MBI Fermentas (Mumbai, India). Bacterial culture media components were obtained from standard commercial manufacturer (HiMedia chemicals, Mumbai, India).

2.2. Bacterial strains and growth conditions

E. coli and *Streptomyces* strains used in this study are listed in Table S1 provided in the supplementary materials. Plasmids used in this study are listed in Table S2. Growth, maintenance and transformation of *E. coli* were performed as described by Sambrook et al. (2001). *S. peucetius* for routine use was grown and maintained of SM agar plates (Kieser et al. 2000). To measure chitinase production, *S. peucetius* was grown in nitrate defined medium containing chitin (NDMC) as described earlier (Paranthaman and Dharmalingam 2003).

2.3. DNA techniques

DNA cloning was carried out essentially as described by Sambrook et al. (2001). Total genomic DNA was isolated from *S. peucetius* as described earlier (Kieser et al. 2000). For southern hybridization, DNA was transferred to N^+ nylon membranes using a semidry vacuum blotting system. The hybridization conditions and subsequent detection were in accordance with the manufacturer's instructions. Probe for southern blot was prepared using Prime-a-Gene kit (Promega, Madison, USA) following the manufacturer's instructions.

2.4. Construction of ChiS expression plasmid

Plasmid DNA from *E. coli* cells was prepared by alkaline lysis mini prep method as outlined by Sambrook et al. (2001). *chiS* gene was PCR amplified by *Pfu* polymerase using primers ChSF and ChSR from pSPVC2.3 and cloned into pGEM-T vector according to manufacturer's instructions, the resulting recombinant plasmid was designated as pChiS. Further *chiS* gene was sub-cloned into pQE-30 as a *Bam*HI and *Hind*III fragment to obtain pQEChiS. Similarly, *chiS* coding region corresponding to 105-352 amino acids (Cytoplasmic domain) was PCR amplified using primers ChiSCDF and ChiSCDR from pSPVC2.3 and cloned into pQE-30. Sequences of oligonucleotides used are shown in Table S3. The clones obtained were confirmed by sequencing before any further experiments.

2.5. Site directed mutagenesis

Site directed mutagenesis was performed by overlap extension PCR (Ho et al. 1989). Sequences of oligonucleotides used in PCR reactions are shown in Table. S3 PCR was performed using Phusion high fidelity polymerase (Finnzymes, Mumbai, India) and the obtained amplicon was cloned into pGEM-T to generate pChiSH144A. Presence of mutation was confirmed by sequencing both strands of the insert using T7 and SP6 universal primers. To clone cytoplasmic domain of mutant ChiS protein into pQE30, the region encoding cytoplasmic domain was PCR amplified using primers ChiSCDF and ChiSCDR, with the template pChiSH144A. Obtained PCR amplicon was eluted from the agarose gel, digested with *Bam*HI and *Hind*III and ligated to pQE30 to obtain pQEChiS$_{CD}$H144A. The frame of ligation and the sequence of the insert were confirmed by sequencing of both the strands.

2.6. Expression of ChiS, ChiS$_{CD}$ and ChiS$_{CD}$H144A in E. coli

Recombinant pQEChiS was used to transform expression hosts M15pREP4 and Rosetta2. pQEChiS$_{CD}$ and pQEChiS$_{CD}$H144A were used to transform M15pREP4. For protein expression, the cultures were prepared and induced as per Qiagen and Rosetta manual of instructions. The cells were recovered by centrifugation and suspended in SDS-Lysis buffer (0.025 M Tris-HCl pH 7.4 and 0.3% SDS), placed in a boiling water bath for 5 min and the clarified lysate was analyzed for expression of the proteins on 12.5% SDS-polyacrylamide gel (PAGE).

2.7. Purification and refolding of ChiS$_{CD}$ and ChiS$_{CD}$H144A

M15pREP4 transformants expressing ChiS$_{CD}$ and ChiSH$_{CD}$144A were cultured in 200 ml Luria broth (LB) containing kanamycin (25 μg/ml) and ampicillin (50 μg/ml). Purification was performed under denaturing conditions as specified in Qiagen manual of instructions. Briefly, cell pellets were suspended in denaturing buffer (8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris pH 8.0) by vortexing for 5 min and incubated at 30 °C for 30 min. Proteins loaded on the column were eluted using an acidic buffer (pH 4.5), fractions of one ml were collected and the purity of the purified protein was checked on 12.5% SDS-PAGE. Fractions containing the purified proteins were pooled and dialyzed in refolding buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10% glycerol, 500 mM L-arginine) for 20 h (Arora and Khanna 1996).

2.8. In vitro phosphorylation of ChiS$_{CD}$ and ChiS$_{CD}$H144A

Autophosphorylation assay was performed as described previously by Hutchings et al. (2006) with minor modifications. The autophosphorylation reaction mixture (50 μL) contained kinase buffer, 100 pm refolded ChiS$_{CD}$ or ChiS$_{CD}$H144A, 10 μCi [γ ^{32}P]ATP. The reaction mixtures were incubated at 30 °C for 5, 15, 30, 60 min and stopped by adding 0.25 volume of 5X SDS-PAGE sample buffer (0.25 M Tris-HCl pH 6.8, 10% glycerol, 0.02% bromophenol blue, 1% sodium dodecyl sulfate). The samples were resolved in a 12.5% SDS-PAGE and then the gels were dried under vacuum for 90 min. The dried gels were exposed to X-ray film (Kodak India Ltd., Bangalore, India) for 12 h and developed.

2.9. Heat and chemical stability of phosphorylated protein

Heat, acid and alkali stability of phosphorylated ChiS$_{CD}$ were examined as described earlier by Pollack and Singer (Pollack and Singer 2001).