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Myxococcus xanthus CbpC and CbpD containing two cAMP-binding domains are involved in temperature tolerance

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Abstract

Myxococcus xanthus CbpC and CbpD contained two cAMP-binding domains that have partial primary structural similarity to cAMP-dependent protein kinase（PKA）regulatory subunits. *cbpC* and *cbpD* mutants, generated by gene disruption, showed normal growth, fruiting body formation, spore formation, and spore germination. However, *cbpC* and *cbpD* mutants cultured under high-temperature conditions exhibited a marked reduction in growth. In contrast to *M.* xanthus *cbpB* mutant, *cbpC* and *cbpD* mutant cells were not shown to sensitive to osmotic stress.

Key words：cAMP signaling, cAMP-binding domain, PKA regulatory subunit, temperature stress, *Myxococcus xanthus*

Introduction

Information on environmental changes is transmitted to cells by signal transducing proteins. Cyclic AMP（cAMP）is one of the most common signaling molecules and exists in both prokaryotes and eukaryotes [1, 2]. Intracellular cAMP levels change in response to changes in environmental conditions. *Myxococcus xanthus* is a Gram-negative bacterium, which demonstrates complex social behavior [3, 4]. It is reported that the cellular concentration of cAMP in *M. xanthus* increases rapidly during starvation- and glycerol-induced development [5, 6]. Our previous study indicated that a receptor-type of adenylyl cyclase, CyaA, of *M. xanthus* is required for osmotic tolerance during spore germination, and the CyaA produces cAMP in response to osmotic pressure during spore germination [7]. Various signal transduction systems in *M. xanthus* have been reported [8–11]; however, the cAMP-mediated signal transduction pathway in this bacterium is unknown. In eukaryotic microorganisms, cAMP signaling controls cell growth, stress response, and developmental events [12, 13]. Intracellular cAMP acts as a second messenger through cAMP-dependent protein kinase A（PKA）. In most cases, the inactive form of PKA is a tetrameric protein composed of two regulatory and two catalytic subunits. The PKA regulatory subunit contains two tandem cAMP-binding domains（designated A domain and B domain）, and binding of cAMP to the regulatory subunit leads to the release and subsequent activation of a catalytic subunit of PKA [14–16]. On the other hand, there are no reports on PKA in bacteria.

In previous study, two genes, *cbpA* and *cbpB*（for cAMP-binding protein）, were isolated from an *M. xanthus* genomic library using oligonucleotide probes, designed from PKA regulatory subunits [17]. We also reported previously that a CbpB protein of *M. xanthus* contains a possible inhibitory（pseudosubstrate）site and two cAMP-binding domains, and indicated that CbpB has partial primary structural similarity to PKA regulatory subunits. A *cbpB* mutant cultured under high- or low-temperature conditions exhibited a marked reduction in growth. *cbpB* mutant cells were also more sensitive to osmotic stress than wild-type cells. The phenotype of the *cbpB* mutant was similar to those of PKA regulatory subunit mutants of some eukaryotic microorganisms.

In this study, we select two proteins, CbpC and CbpD, with homology to PKA regulatory subunits from the *M. xanthus* genome sequence database（TIGR database）, and describe the function in *M. xanthus* cells.

Materials and methods

Bacterial strains and development. The type strain of *M. xanthus* FB（ATCC 25232, IFO 13542）was used as the wild-type [18]. *M. xanthus* wild-type and mutant strains were grown in Casitone-yeast extract（CYE）medium [19], and kanamycin（70μg ml^{-1}）was added when necessary. For development, *M. xanthus* wild-type and mutant cells were harvested by centrifugation, washed in TM buffer（10 mM Tris-HCl, pH 7.5, and 8 mM $MgSO_4$）and spotted on the surface of CF agar [20]. The plates were incubated at 30°C for 7 days.

Construction of cbpC and cbpD disruption mutants by gene replacement. To investigate the biological function of CbpC and CbpD, we constructed *cbpC* and *cbpD* deletion-insertion mutants. First, 1.9- and 2.8-kb fragments containing the *cbpC* and *cbpD* genes, respectively, were amplified by PCR using as primers, 5' -AACACCATCGACGAACACG-3' and 5' ACGTCCTTCTTCGACCAGC-3' , and 5' -AAGCTGCTGGTGTTGGATGC-3' and 5' -CGTGAGACGGAGCTCACCC-3' , respectively. The PCR products from *cbpC* and *cbpD* were ligated into pT7Blue-T（Novagen）vector, and these plasmids were designated pCbpC and pCbpD, respectively. A 1.2-kb DNA fragment containing a kanamycin-resistance（Km^r）gene was amplified by PCR using TnV [21] as a template and a pair of primers. The resulting DNA fragment was inserted into the *Ehe*I-*Msc*I sites of pCbpC or *Pma*CI-*Aor*51HI sites of pCbpD. The disrupted gene constructed as described above was amplified by PCR using the above oligonucleotides. The PCR products thus obtained were introduced into *M. xanthus* cells by electroporation [22] .

Growth and stress conditions for M. xanthus cells. *M. xanthus* wild-type and mutant strains were precultured in CYE medium at 30°C, and then aliquots of 3 x 10^7 cells were inoculated into 3 ml of CYE medium. Cells were cultured at 17, 30 and 37 °C with shaking at 180 rpm for thermal stress experiments. For osmotic stress experiments, cells were inoculated to 1 x 10^7 cells ml^{-1}, and grown at 30°C in CYE medium containing various concentrations of NaCl or sucrose with shaking. The growth of cells was monitored by measuring optical density at 600 nm, and cell density was determined with a hemocytometer.

Transcript analysis. Total RNA was isolated from exponentially growing *M. xanthus* cells at 30°C [23] . Contaminating DNA was removed by digestion with DNaseI. For reverse transcription（RT）-PCR, RNA was used for cDNA synthesis with *Bca*BEST polymerase in accordance with the manufacturer' s directions（Takara Bio.）. PCR was performed with *Bca*-Optimized Taq polymerase, a 5' *cbpC* gene-specific primer（5' -ACTCCCTCTACGTCCTGGTCG-3'）, a 3' *cbpC* gene-specific primer（5' -ATCGTCTGGAACATGGGCG-3'）, or 5' *cbpD* gene-specific primer（5' -AAGCCGACTCCCTGTTGC-3'）, a 3' *cbpD* gene-specific primer（5' -GACGTAGAAAGCATCGCCG-3'）and the synthesized cDNA. The products were separated by electrophoresis on 1.5 % agarose gels, and stained with ethidium bromide.

Results

Predicted protein structures and functional domains. We searched the database of the Institute for Genomic Research（TIGR）with the tBLASTn program, and found two genes（*cbpC* and *cbpD*）that encode proteins with homology to *M. xanthus* CbpB. The predicted *cbpC* and *cbpD* gene products contain 443 and 715 amino acids, respectively. *cbpC* and *cbpD* genes encode a 443- and 715-amino acid protein with a calculated mass of 46.5 and 75.5kDa, respectively. Two near-duplicate cAMP-binding domains, designated A domain and B domain, were found in the C-terminal regions of CbpC and CbpD, and shared a high degree of identity with the conserved consensus sequence（FGE［L/I/V］AL［L/I/M/V］xxx［P/V］R［A/N/Q/V］A）, where x is any residue, of the cAMP-binding domain from PKA regulatory subunits [23] . CbpC and CbpD also contained a possible inhibitory（pseudosubstrate）site, which acts as an inhibitor of catalytic subunit kinase activity and also mediates interaction between the regulatory and catalytic subunits [24] . The amino acid sequences of the putative inhibitory sites of CbpC and CbpD（for example, RRLK, residues 91 to 94, and RRGT, residues 103 to 106, respectively）are similar, but not identical to the PKA regulatory subunit. The N-terminal region of CbpC or CbpD did not contain a dimerization domain. CbpC was 20% and 18% identical to the PKA regulatory subunits of *Neurospora crassa* [25] , and *Dictyostelium discoideum* [26] , respectively（Fig. 1）. Also, CbpD was 23% identical to the PKA regulatory subunits of N. *crassa* and D. *discoideum*（Fig. 1）.

Transcript analysis. It was conserved that whether *cbpC* and *cbpD* genes were transcripted in growing cells of *M. xanthus*. RNA was isolated from exponentially growing cells at 30°C and was used for cDNA synthesis with *Bca*BEST polymerase. The expected 298–bp and 286-bp RT-PCR products were amplified from RNA of growing cells（Fig. 2）. The expected product was not amplified without reverse transcription. The results indicate that the *cbpC* and *cbpD* genes are expressed during growth phase.

Temperature sensitivity of cbpC and cbpD mutants. To determine their biological functions, the *cbpC* or *cbpD* gene was disrupted by insertion of the kanamycin-resistance gene and homologous recombination（Fig. 3）. When developed on CF agar, both mutants showed a normal developmental process, and the final yields of spores for mutants were identi-

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CbpC   193  AVSREGEPADSLYVLVAGKAEVTRQME--------GEARTLGFLGGGSIFGEIALLTGAP
CbpD   475  RIIDQGSHGDAFYVICEGAVRVFRTEE--------GQRQDIATLEGGTFFGEMALLSGAA
Nco    156  KVITQGDAGDYFYVVEKGRFEVYVNSTGALQPGPDGMGQKVGEIAEGGSFGELALMYNAP
Dic     92  IIIKQGDEGDLFYVIDSGICDIYVCQN-------GGSPTLVMEVFEGGSFGELALIYGSP
CbpB   157  TVVEEGTPGASMFALVEGRADVVRALE-------DGQRRSVGTVTPGDFFGELALISEGP

            *
CbpC   245  PTATVS-AVSDTEVFEIRREHLNAVAKSHPAVPQVLADFAQQRMARNLMATSPMFQTMPE
CbpD   527  RTASVESASDDTQLLEISASVLAELSGSHPQVAQALKKFCRQRMLTNVMNTSELFRLFGR
Nco    216  RAATVVSAEPQCTLWALDRVTFRRILMES--------TFSRRRMYESFLEEVPILKTLTP
Dic    145  RAATVIART-DVRLWALNGATYRRILMDQ--------TIKKRKLYEEFLEKVSILRHIDK
CbpB   210  RLATVV-ATERAVLLELTRQHMEAVAARHPDVTAVVDAFYRRRMVENLLRSNPLFNQLSP

CbpC   304  SERGALLRRFTFRALQAREKVLVEGEHSPGLFLVLAGELVVQKEDPAG---GVVTLGMLR
CbpD   587  KDRRALVERFRSRDVERDTVIIRDGDPTDGLYVVLSGEVEVRKDG--------HLLTQLR
Nco    268  YERSKIADALESQKYPAGHEIILEGDPGHSFFLLEAG------EAAAFKRGNDSPVKNYK
Dic    196  YERVSLADALEPVNFQDGEVIVRQGDPGDRFYIIVEGKVVVTQETVPGDHSTSHVVSELH
CbpB   271  AQKAAVSRDFELRSFAAGEALMTQGQPGDAFYVLLRGRCTPWLEQPHG---RRVALSALR

                        *
CbpC   361  EGEVAGEISLLTGLRATATVAAARKTAAAFLERAAFHELVTAVPDIRTYLEQLSDRRLKQ
CbpD   639  EGDVFGEISLLQKTPATATVTATRHTTLLRLPRADFDALISSHPQILALISDLSDERLRR
Nco    322  KGDFFGELALLNDAPRAASVISQTEVKVARLGKNAFQRLLGPIESILRR-----TRYVEA
Dic    256  PSDYFGEIALLTDRPRAATVTSIGYTKCVELDRQRFNRLCGPIDQMLRRNMETYNQFLNR
CbpB   328  EGDVFGEISLLLDKPVSATVRADVAGVVLRLERDAFQKHLLSQPGLKGQLMRMGTERLQR

CbpC   421  IGEALRPAEIIDADELVLEPEAA
CbpD   699  TQRVLTEAGVEEDLILV------
Nco    377  EEVDPLQVS--------------
Dic    316  PPSSPNLTSQKS-----------
CbpB   388  TAQALASGRVLHDGDLRV-----
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Fig. 1 Sequence alignments of cAMP-binding domains. Alignment of the deduced cAMP-binding domains of CbpB, CbpC and CbpD with PKA regulatory subunits of *N. crassa* (Nco), and *D. discoideum* (Dic). cAMP-binding domains are indicated with underlines. Amino acid residues in agreement for more than three residues are indicated by filled boxes. Gray shading indicates degrees of similarity among amino acid residues. Asterisks show the Arg residue in the conserved consensus sequence of cAMP-binding domain.

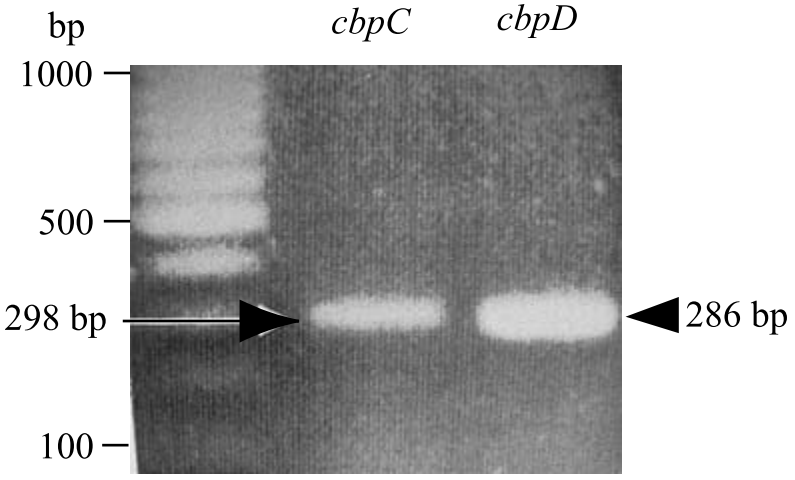

Fig. 2 RT-PCR analysis of *cbpC* and *cbpD* gene expressions in *M. xanthus* growing cells. Total RNA prepared from wild-type vegetative cells was treated with or without reverse transcriptase. RT-PCR was carried out by using primers that amplified a 298-bp fragment of *cbpC*, or a 286-bp fragment of *cbpD*. Molecular sizes of DNA fragments are given in bases.

cal to those of the wild-type strain（data not shown）. The spores of both mutants also germinated normally.

When cultured in CYE at optimal temperatures（30˚C）for *M. xanthus* growth, *cbpC* and *cbpD* mutants grew as well as the wild type. However, when cultured at 37˚C, the *cbpC* and *cbpD* mutants showed a significant reduction in growth compared to the wild type, where the cell density of the mutant in stationary phase decreased by 31% or 36%, respectively（Fig. 4）. The growth rate of the *cbpC* and *cbpD* mutants were approximately of the wild type. Incubation at low temperature（17˚C）of the *cbpC* or *cbpD* mutant showed a similar growth rate when compared to wild-type cells（data not shown）.

Osmosensitivity of *cbpC* and *cbpD* mutants. We next investigated the phenotypic differences between the wild-type and mutant strains under osmotic stress. When the cell densi-

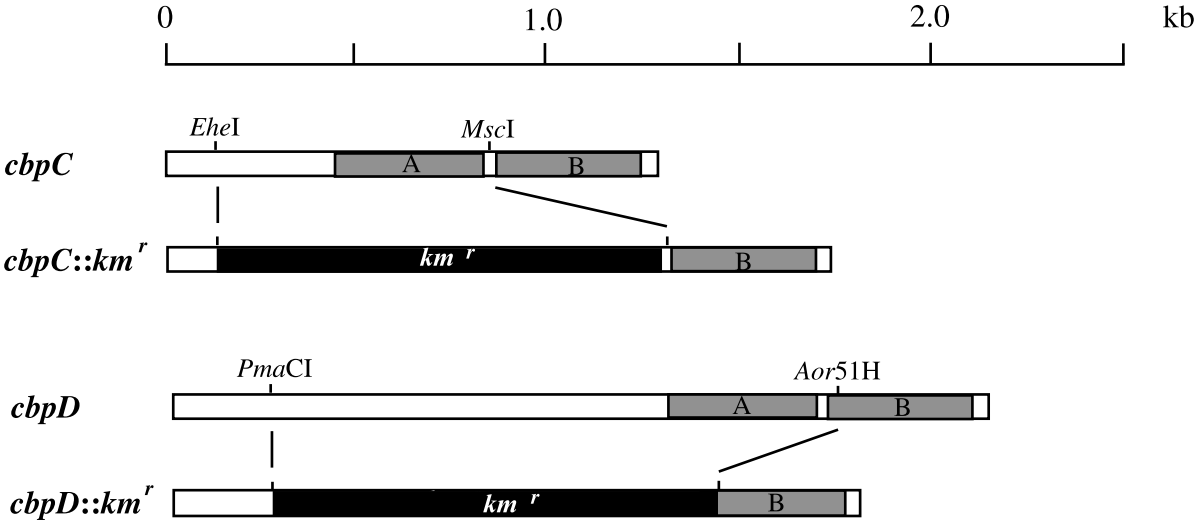

Fig. 3 Restriction map of the *cbpC* and *cbpD* genes of *M. xanthus*. Lines with arrows indicate orientations. The cAMP-binding domains are indicated with shadings of gray. A and B: cAMP binding-domains, A and B, of CbpC or CbpD, respectively. The *Ehe*I-*Msc*I site of *cbpC* and *Pma*CI-*Aor*51H site of *cbpD* were replaced by the Km^r gene.

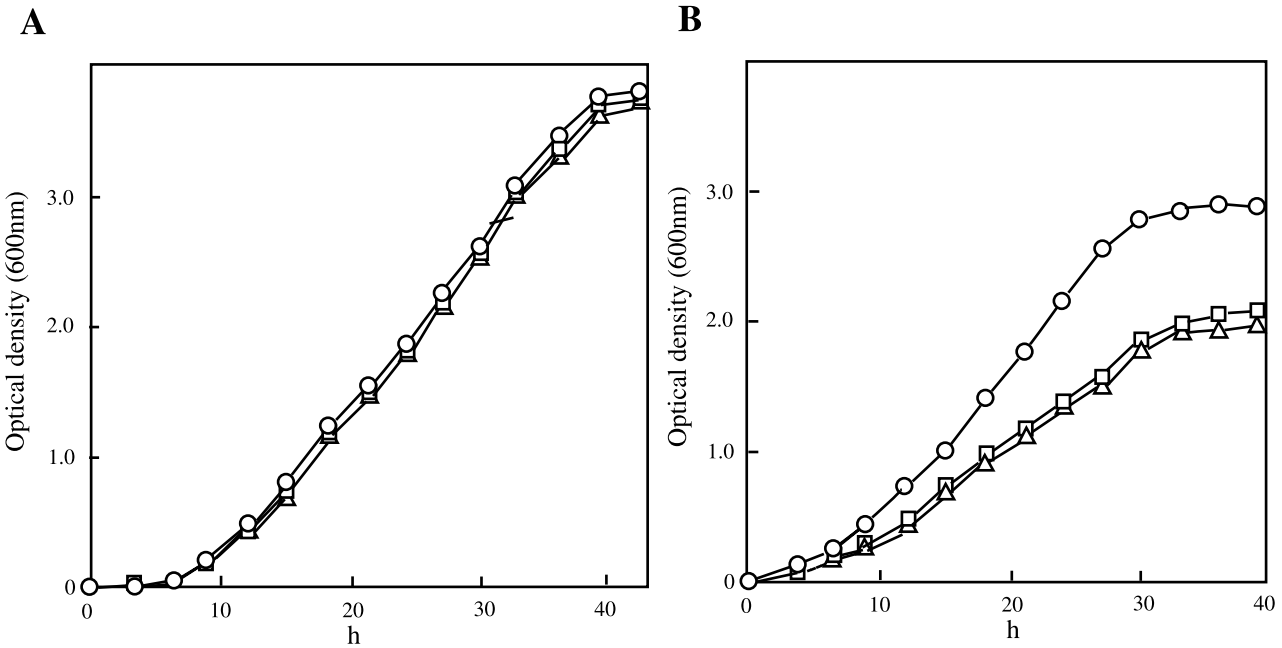

Fig. 4 Growth of wild-type and *cbpB* mutant cells at 30˚C and 37˚C. A. Wild-type（circles）, *cbpC* mutant（squares）and *cbpD* mutant（triangles）cells were grown in CYE medium at 30˚C（A）and 37˚C（B）.

ties of cultures in CYE medium with no addition reached 1.5 x 10^9 cells ml^{-1}, the growth was determined in all cultures by counting of cell density with a hemacytometer. There were no differences in growth between *cbpC*, *cbpD* mutant and wild cells, when they were grown in CYE medium containing 0.1 to 0.2 M NaCl or 0.1 to 0.3 M sucrose（data not shown）.

Discussion

In previously study, we indicated that CbpB is homologous to PKA regulatory subunit, and it involves in adaptation of heat and osmotic stresses in *M. xanthus* [17] . CbpC and CbpD also contained a putative inhibitory sequences and two-tandem cAMP-binding domains, and its structural features are partially similar to those of PKA regulatory subunits. Almost all PKA regulatory subunits possess a conserved domain structure comprising a dimerization/docking domain, an interconnecting linker region, and two-tandem cAMP-binding domains. An inhibitory sequence in the linker region is strictly conserved in almost all PKA regulatory subunits. PKA regulatory subunits are classified into two major types, RI and RII, and their inhibitory sequences are RRx［A/G］and RRxS, where x is any residue, respectively [23] . Putative inhibitory sequences, RERA, RRDY, RRLK or VRNA, exists in CbpC and RRGT, RRAD or FRTA, exists in CbpD, that are similar to those of the PKA regulatory subunits, but distinct from the consensus motif of eukaryotic regulatory subunits of PKA. The dimerization/docking domain that maintains the regulatory subunits as asymmetric dimers was not found in CbpC or CbpD. The C-terminal regions of about 250 residues containing two cAMP-binding domains in CbpC and CbpD were most similar to the PKA regulatory subunit homologous proteins of *Stigmatella aurantiaca*. The Arg residue in the conserved consensus sequence（FGE［L/I/V］AL［L/I/M/V］xxx［P/V］R［A/N/Q/V］A）of cAMP-binding domain is crucial for the binding of cAMP [23] . The conserved Arg residue was found only in cAMP-binding domain A of CbpD.

In contrast to *M. xanthus cbpB* mutant, *cbpC* and *cbpD* mutant cells were not shown to sensitive to osmotic stress. However, the *cbpC* and *cbpD* mutants showed a reduction in growth under temperature-stress condition. Similar phenotypes are observed in PKA regulatory mutants of *S. cerevisiae* and *N. crassa*. The BCY1 mutant in *S. cerevisiae* could not tolerate heat shock, starvation or osmotic stress [12, 13] . On the other hand, catalytic mutants of PKA in *Schizosaccharomyces pombe* and *D. discoideum* exhibited more resistance to osmotic or heat stress than wild-type cells [27, 28] . Given these findings, it is generally thought that the PKA pathway has a negative influence on the response to most types of stress in eukaryotic microorganisms.

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cAMP結合ドメインを２つ有する粘液細菌*Myxococcus xanthus* CbpCとCbpDは生育における温度耐性に関与する。

石橋向治、木村義雄

Myxococcus xanthus CbpCとCbpDは２つのcAMP結合ドメインを有し、cAMP依存性プロテインキナーゼ（PKA）調節サブユニットと部分的に類似した構造を有する。遺伝子破壊により作製された*cbpC*と*cbpD*変異株は正常な生育、分化（子実体及び胞子の形成）、胞子の発芽が見られた。しかしながら、*cbpC*と*cbpD*変異株が高温条件下で培養されると生育の低下が見られた。*M. xanthus cbpB*変異株と対照的に、*cbpC*と*cbpD*変異株細胞は浸透圧ストレスに感受性を示さなかった。