An Adenylyl Cyclase, CyaA, of *Myxococcus xanthus* Functions in Signal Transduction during Osmotic Stress

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An adenylyl cyclase gene (*cyaA*) present upstream of an osmosensor protein gene (*mokA*) was isolated from *Myxococcus xanthus*. *cyaA* encoded a polypeptide of 843 amino acids with a predicted molecular mass of 91,187 Da. The predicted *cyaA* gene product had structural similarity to the receptor-type adenylyl cyclases that are composed of an amino-terminal sensor domain and a carboxy-terminal catalytic domain of adenylyl cyclase. In reverse transcriptase PCR experiments, the transcript of the *cyaA* gene was detected mainly during development and spore germination. A *cyaA* mutant, generated by gene disruption, showed normal growth, development, and germination. However, a *cyaA* mutant placed under conditions of ionic (NaCl) or nonionic (sucrose) osmolarity exhibited a marked reduction in spore formation and spore germination. When wild-type and *cyaA* mutant cells at developmental stages were stimulated with 0.2 M NaCl or sucrose, the mutant cells increased cyclic AMP accumulation at levels similar to those of the wild-type cells. In contrast, the mutant cells during spore germination had mainly lost the ability to respond to high-ionic osmolarity. In vegetative cells, the *cyaA* mutant responded normally to osmotic stress. These results suggested that *M. xanthus* CyaA functions mainly as an ionic osmosensor during spore germination and that CyaA is also required for osmotic tolerance in fruiting formation and sporulation.

*Myxococcus xanthus* is a gram-negative bacterium which migrates on semisolid surfaces by gliding. *M. xanthus* lives in the soil, where it moves in a social unit called a swarm and feeds on other bacteria. Under certain nutritionally limiting conditions, the vegetative cells undergo a developmental cycle involving cell-cell interactions. More than 10^5 cells migrate to an aggregation center and form a fruiting body, within which cells differentiate into myxospores. Since *M. xanthus* demonstrates complex social behavior, signal transduction systems in this bacterium have been studied widely (19, 24).

The environmental changes are transmitted to the cell by signal-transducing proteins. Cyclic AMP (cAMP) is one of the most common signaling molecules and is widely distributed from prokaryotes to eukaryotes. In mammalian cells, CAMP has significant roles in a variety of fundamental physiological regulatory mechanisms, e.g., cell differentiation, transcriptional regulation, and myocardial contraction. In the prokaryotes and in eukaryotic microorganisms, intracellular cAMP levels change in response to changes in environmental conditions. In *Escherichia coli*, there is an apparent interaction between concentrations of CAMP in cells and those of glucose in medium, and cAMP receptor protein-cAMP complex is essential for transcription of the glucose permease gene (20).

Under conditions of nitrogen starvation, the pseudohyphal growth of *Saccharomyces cerevisiae* is controlled by CAMP signal transduction pathways (3). Upon nutrient starvation, the eukaryotic slime mold *Dictyostelium discoideum* undergoes a spectacular development cycle, in which extracellular CAMP acts as a chemoattractant controlling cell aggregation and also induces the differentiation of spore cells (14). In this organism, sporulation is also controlled by cAMP signal transduction pathways (10, 33).

The synthesis of cAMP is catalyzed by the enzyme adenylyl cyclase, and the adenylyl cyclases are found in all animals, plants, and bacteria. There are at least nine closely related isoforms of adenylyl cyclases for which the genes have been cloned and characterized in mammals (9). These adenylyl cyclases are membrane proteins of more than 1,000 amino acids that commonly have two sets of six transmembrane spans and two sets of conserved catalytic domains. *E. coli* and some euobacteria have hydrophilic adenylyl cyclases, which possess an N-terminal catalytic domain and a C-terminal regulatory domain (1). There is no sequence similarity between these proteins and mammalian adenylyl cyclases. Receptor-type adenylyl cyclases are composed of an extracellular domain, a transmembrane domain, and a cytoplasmic catalytic domain. The receptor-type adenylyl cyclases are thought to have the ability to sense an extracellular signal and to control adenylyl cyclase activity of the catalytic domain. These enzymes were initially found in protozoans and have since been identified in euobacteria, although how the activity of these prokaryotic enzymes is regulated is unknown (23).

In *M. xanthus*, the cellular concentration of cAMP increases rapidly during early starvation-induced and glycerol-induced development (17, 34). Campos and Zusman also observed that the formation of fruiting bodies was stimulated by the addition of cAMP to agar containing a low level of nutrients (5). However, the adenylyl cyclase gene of *M. xanthus* has not been cloned. In a previous study, we cloned the *mokA* gene encoding an osmosensor histidine kinase from an *M. xanthus* genomic library (21). An open reading frame is present in the region of about 6 kb upstream of the *mokA* gene, and its deduced gene product (CyaA) showed a high degree of similarity with recep-

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tor-type adenyl cyclase. In this paper, we report characterization of the cyaA gene and the finding that cyaA encodes a protein that senses osmolarity and regulates cAMP synthesis during spore germination.

MATERIALS AND METHODS

Bacterial strains and culture conditions. M. xanthus IFO3542 (ATCC 25232) was used as the wild-type strain in this study. The M. xanthus wild-type or mutant strain was grown at 30°C in Casitone-yeast extract (CYE) medium (6), and kanamycin (70 μg/ml) was added when necessary.

DNA manipulation and sequencing. A positive clone which contains the mokA gene (21) was used in this experiment. Phage DNA was prepared from the clone and digested with Kpnl, Sall, and Smal. Sall (7.6 kb) and Smal (2.3 kb) fragments were extracted from an 0.7% agarose gel. The DNA fragments were ligated into pBluescript SK and sequenced by the primer walking method using the ABI PRISM 310 genetic analyzer (Perkin-Elmer Co., Norwalk, Conn.) with the BigDye terminator kit (Perkin-Elmer Co.).

Construction of cyaA disruption mutant. To investigate the biological function of CyaA, we constructed a cyaA deletion-insertion mutant. First, a 2.2-kb fragment containing the cyaA gene was amplified by PCR with the primers 5'-GAATTTGGAACATGGGG-3' and 5'-TACGGCTTCTGCCACATGGG-3'. The PCR product was ligated into vector pT7 Blue1. This plasmid was designated pT7CyaA. Plasmid pT7CyaA was digested with BglII and a 4.9-kb fragment was recovered from an agarose gel. A 1.2-kb DNA fragment containing a kanamycin resistance (Km') gene was amplified by PCR with Tn1 as a template and a pair of primers (15). The resulting DNA fragment was inserted into the BglII site of pT7CyaA. The disrupted gene constructed as described above was amplified by PCR with the above oligonucleotides. The PCR products thus obtained were introduced into M. xanthus by electroporation (28).

Developmental assays. M. xanthus wild-type and cyaA mutant strains were grown on CYE medium up to 5 × 10^9 cells/ml and washed in TM buffer (10 mM Tris-HCl [pH 7.5] and 8 mM MgSO_4). Aliquots (10 μl) of cell suspension (2 × 10^9 cells) were spotted on the surface of a clone fruiting (CF) agar plate or a CF agar plate containing up to 0.25 M NaCl or sucrose (16). The plates were incubated at 32°C. For glyceral induction of spore formation, cells were harvested at the mid-logarithmic phase of growth and washed with 1% Casitone-8 mM MgSO_4, pH 7.6. Glyceral was added to a final concentration of 0.5 M, and cells were shaken at 30°C for 10 h (13).

Spore germination. M. xanthus spores were harvested from 7-day-old fruiting bodies. Undifferentiated cells were killed by five 30-s treatments with a Branson sonifier and by incubation at 60°C for 15 min. Spores were inoculated to 2 × 10^9 cells/ml in CYE medium containing up to 0.25 M NaCl or sucrose. The cultures were incubated at 30°C with continuous shaking until the germination in the medium without addition of NaCl or of sucrose was about 50%. The numbers of spores/ml in CYE medium containing up to 0.25 M NaCl or sucrose. The cultures were incubated at 30°C for 6 or 22 h at 32°C. Cells were harvested and washed with 10 mM sodium phosphate buffer (pH 7.0). The cells were resuspended in the buffer and then stimulated with 0.2 M NaCl or sucrose. Aliquots (0.1 ml) containing 3 × 10^6 cells were taken at the respective time points and heated to 95°C for 10 min.

To obtain spores, vegetative cells were spotted on CF agar and cultured for 7 days. Spores were harvested and washed with TM buffer. After sonication and heat treatment, spores were cultured in CYE medium for 12 to 14 h, and then NaCl or sucrose was added to a final concentration of 0.2 M. Aliquots (1 ml) containing 2 × 10^6 spores were taken at various times, and the spores were washed with 10 mM sodium phosphate buffer (pH 7.0), resuspended in 0.25 ml of the buffer, and then immediately sonicated with 0.2 g of acid-washed, 0.4-mm diameter glass beads for 4 min at full power. Under this condition, approximately 95% of spores were disrupted. After centrifugation, a portion (80 μl) of the supernatant was used for assays with commercial immunoassay kit (Amersham Pharmacia).

Time courses of cAMP levels under ionic-osmotic stress. Intracellular cAMP levels were determined in wild-type and mutant cells during development and spore germination under ionic-osmotic stress. To measure cAMP production during development, vegetative cells were spotted on CF agar or CF agar containing 0.15 M NaCl. Cells were harvested at various times, washed once by centrifugation in 10 mM sodium phosphate buffer (pH 7.0), and suspended in 0.25 ml of the phosphate buffer. The cells were immediately sonicated with 0.2 g of glass beads for 4 min at full power. After centrifugation, a portion (80 μl) of the supernatant was mixed with perchloric acid and neutralized with KOH.

To estimate internal cAMP levels during germination, spores were inoculated in CYE medium or in CYE medium containing 0.2 M NaCl at 1.2 × 10^9 spores/ml and cultured at 30°C. Cells (1.8 × 10^9) were harvested by centrifugation at various times, washed once, and suspended in 250 μl of 10 mM sodium phosphate buffer (pH 7.0). Extraction and enzyme immunoassay of cAMP were performed by the above-mentioned method.

Protein concentrations were determined by the Bradford technique (4). Experiments were repeated at least twice with similar results, and results of typical assays are shown.

Transcript analysis. Total RNA was isolated from M. xanthus at exponential growth phase, at germination, and during development as described previously (22). Contaminating DNA was removed by digestion with DNase. For reverse transcriptase PCR (RT-PCR), 1 μg of RNA was used for cDNA synthesis with BcaBEST polymerase in accordance with the manufacturer's protocol (Takara Shuzo, Kyoto, Japan). PCR was performed with Bca-optimized Taq polymerase, a 5' gene-specific primer (5'ACACGGACATCTCTGGGCGG3'), and a 3' gene-specific primer (5'-GAAGCTGGGTGTCATCGAACCGC3'). Amplification products were separated by electrophoresis on 1.0% agarose gels and stained with ethidium bromide.

Nucleotide sequence accession number. The sequence of the M. xanthus cyaA gene has been deposited in the DDBJ sequence library under accession no. AB066996.

RESULTS

Cloning and sequencing of the cyaA gene from M. xanthus. Recently, we cloned a histidine kinase gene (mokA) from a phage library of M. xanthus genomic DNA (21). On DNA analysis, we found that a 10-kb KpnI fragment of the cloned phage DNA contained an open reading frame the predicted amino acid sequence of which is homologous to adenyl cyclases of prokaryotes and eukaryotes. We designated this gene cyaA. The restriction map of the cyaA gene and the positions of the mokA and cyaA genes are shown in Fig. 1. The nucleotide sequence of a 3.0-kb SalI-Smal fragment of the 10-kb KpnI fragment DNA that contained the complete transcription unit was determined. The cyaA gene sequence predicts a protein composed of 843 amino acids, with a calculated molecular mass of 91,187 Da.

Predicted protein structure and functional domains. The predicted amino acid sequence of M. xanthus CyaA was compared with those in the DDBJ sequence library by using the BLASTP program. The amino-terminal region of the CyaA protein shows high homology with two potential sensor histidine kinases (25, 30) and a target protein of serine/threonine kinase (31) (Fig. 2A). Analysis of the cyaA gene product with the PSORT or SOSUI program suggested that CyaA possesses a signal peptide (Met-1 to Val-21) and three transmembrane domains.

The carboxyl-terminal part of CyaA is a hydrophilic domain with a high degree of identity to the conserved catalytic domain of adenyl cyclases from eukaryotes and eubacteria. The four regions conserved in class III adenyl cyclases were found in the carboxyl-terminal region of CyaA. This region of CyaA shows 36.5% identity to the adenyl cyclase CyaB1 of Anabaena sp. strain PCC7120 (18), 34.5% identity to the adenyl cyclase CyaB of S. aureantiaca (11), 28.5% identity to the adenyl cyclase ACA of D. discoideum (27), and 35.5% identity to adenyl-γnyl cyclase GCY of Caeno-
rhodoides elegans (7). Sequence alignments of carboxy-terminal regions of these adenylyl cyclases are shown in Fig. 2B. No apparent sequence similarity with the E. coli adenylyl cyclase was found. These results suggested that the M. xanthus CyaA is a receptor-type adenylyl cyclase that belongs to the class III adenylyl cyclases as described by Danchin (12).

Stage-specific expression of CyaA. We investigated the expression of the cyaA gene in M. xanthus cells during growth, development, and sporulation by RT-PCR analysis (Fig. 3). The expected 1,190-bp RT-PCR products were mainly amplified from mRNA of developing and germinating cells. The cyaA gene was also expressed at a low level in vegetative cells. As a control, the expected product was not amplified without reverse transcription, indicating that there was no DNA contamination in the mRNA (data not shown).

Effect of disruption of cyaA on development. As described in Materials and Methods, the cyaA gene on the chromosome was disrupted by insertion of the kanamycin resistance gene from TnV. Using Southern hybridization and PCR analysis, we confirmed that the kanamycin resistance gene was inserted into the cyaA gene (data not shown). To study the function of CyaA in development, wild-type and cyaA mutant strains were cultured on starvation medium (CF agar). When wild-type cells were allowed to develop on CF agar, aggregations and fruiting bodies were formed at approximately 12 to 24 and 40 to 65 h, respectively. The cyaA mutant showed the normal developmental process, and the morphology of the fruiting body of the mutant strain was similar to that of the wild-type strain. The final yield of spores for the cyaA mutant was also identical to that of the wild-type strain. During development under nonosmotic conditions, there were no significant differences in cellular cAMP levels between the wild-type and mutant cells (see Fig. 7). In glycerol-containing medium, the cyaA mutant was also able to form spores. When cultured in CYE growth medium, the mutant grew as well as did the wild type.

Osmosensitivity of the cyaA mutant. Adenylyl cyclases of Trypanosoma brucei, Leishmania donovani, and D. discoideum, which have a large extracellular domain, are considered to function as receptors or as sensors (2, 29, 32). Since the structure of M. xanthus CyaA is similar to those of receptor- or sensor-type adenylyl cyclases, we investigated the phenotypic differences between wild-type and cyaA mutant strains under various stresses. The vegetative cells of the cyaA mutant were found to be reasonably normal with respect to stresses such as temperature shifts, pH changes, osmotic pressure, and anaerobic culture (data not shown). However, the cyaA mutant showed an osmosensitive phenotype during development and germination. Figure 4A shows fruiting bodies of the wild type and the cyaA mutant on CF agar plates containing 0.1 M NaCl. The wild-type strain formed normal fruiting bodies about 1 day later than did the wild-type strain in the nonosmotic medium. In contrast, fruiting bodies of the mutant strain were formed around the spot only after incubation for 3 to 4 days. As a result, the spore yield of the mutant strain was approximately 15% of that of the wild-type strain at 5 days after inoculation onto CF agar plates containing 0.1 M NaCl (Fig. 4B). Moreover, when the cyaA mutant cells were developed on CF agar containing 0.1 to 0.15 M sucrose, spore numbers of the mutant were one-third to one-fourth of those of the wild type.

When wild-type spores were incubated in CYE medium containing 0.2 M NaCl for 65 h, more than 90% of the spores were elongated into rod-shaped structures (Fig. 5A). Under this condition, only a minor population of mutant spores was converted into vegetative-cell-like cells. Figure 5B shows the rates of germination in wild-type and cyaA mutant spores at various NaCl concentrations. The rate of germination of wild-type spores decreased with increasing NaCl concentration, and almost all spores did not germinate in the presence of 0.3 M NaCl. In the presence of 0.1 and 0.15 M NaCl in CYE medium, the rate of germination of mutant spores showed nearly a 10-fold decrease compared with that of wild-type spores. The mutant showed little or no germination on CYE medium containing 0.2 or 0.25 M NaCl, respectively. In CYE medium containing more than 0.1 M sucrose, the cyaA mutant spores also germinated poorly compared with the wild-type spores, as shown in Fig. 5B. However, the spore germination of the cyaA mutant was more strongly inhibited by NaCl than by sucrose.
Assays for cAMP accumulation. cAMP production by spores was measured during a period of 10 min after osmotic stimulation (Fig. 6). In the response experiment, wild-type and mutant spores, in which germination programs progressed by incubation for 12 h in CYE medium, were used. In wild-type spores, addition of 0.2 M NaCl resulted in a rapid increase in cAMP level, which peaked at 1 min and slowly decreased thereafter. In contrast, cAMP production by mutant spores was only weakly stimulated by addition of 0.2 M NaCl. Addition of 0.2 M sucrose to wild-type spores induced an up-to-threefold increase in the accumulation of cAMP, but the accumulated cAMP levels were lower than those with NaCl stimulation.

We also measured whether CyaA activity during develop-
ment is regulated by high osmolarity. When 0.2 M NaCl or sucrose was added to developing cells, cAMP increased linearly for at least 60 min of incubation, resulting in accumulated cAMP levels of about 8 to 12 or 20 to 30 pmol/mg of protein, respectively. There was no significant difference in the amounts of cAMP accumulation between wild-type and cyaA mutant cells (data not shown).

**Time courses of cAMP levels during development and germination in high salt.** We next determined cAMP levels in wild-type and cyaA mutant cells during development and spore germination under ionic osmostress. Wild-type and cyaA mutant cells were cultured on CF agar or CF agar containing 0.15 M NaCl, and intracellular cAMP levels were determined over a time course of 120 h (Fig. 7A). In nonosmotic conditions, the intracellular cAMP levels of wild-type and cyaA mutant strains increased two- to threefold during the first 12 h of incubation. In the presence of NaCl accumulated cAMP levels of the wild-type strain were about twofold higher than those in its absence, and the cAMP accumulation stimulated by 0.15 M NaCl could be detected by 72 h of development. In contrast, the cyaA mutant developed under high osmolarity showed no increased intracellular cAMP levels during early stages of development, which was possibly due to damage of osmosensitive mutant cells.

Wild-type and cyaA mutant spores were germinated in CYE medium or in CYE medium containing 0.2 M NaCl, and the changes in intracellular cAMP levels were determined. As shown in Fig. 7B, the cAMP levels of wild-type spores under osmotic stress increased rapidly from 7 to 14 h of incubation and reached a maximum level at 20 h or approximately at the time of initiation of nonosmotic spore germination. The spores at the early stage of germination may not be susceptible to stimulation by osmolarity, because the spores are covered with thick spore coats. In contrast, adenyl cyclase activity of cyaA mutant spores was not stimulated by 0.2 M NaCl, and the changes in cAMP levels of mutant spores during germination were similar to those of nonosmotically stressed wild-type and mutant spores. There was a three- to fourfold difference in cAMP level at 20 h between wild-type and cyaA mutant spores under ionic osmostress. When wild-type and cyaA mutant spores were incubated in the absence of NaCl for 20 or 26 h, the percentages of rod-shaped cells in each culture were 0.25 and 0.28% and 5 and 7%, respectively (data not shown). By 26 h of incubation, no vegetative-cell-like cells of either strain were observed in the cultures with 0.2 M NaCl. As heat-treated spores were used in this experiment, germination proceeded slowly compared with that of non-heat-treated spores (26).

In the developmental or germination time course of cAMP levels, mutant cells under high-ionic osmolarity showed significantly lower levels of cAMP accumulation than did wild-type cells, indicating that cAMP accumulation plays a role in enhancement of osmotic tolerance during development and germination.

**DISCUSSION**

In the eukaryotic slime mold *D. discoideum*, cAMP coordinates the stages of the developmental program, and an adenyl cyclase, ACA, is expressed during early aggregation (27).
The proposed structure of ACA resembles those of typical mammalian adenyl cyclases, and the ACA-null mutant has little detectable adenyl cyclase activity during aggregation and fails to aggregate. The myxobacterium *M. xanthus* has a developmental cycle that resembles that of *D. discoideum*. During development, cAMP levels in *M. xanthus* increase by about 2- to 20-fold early in development, and cAMP stimulates fruiting body formation when added to low-nutrient or nonnutrient medium (5, 17, 34). Although activation of adenyl cyclase and accumulation of cAMP are likely to be required for the development of *M. xanthus* as well as of *D. discoideum*, it was difficult to demonstrate this assumption directly because the adenyl cyclase gene had not been cloned in *M. xanthus*. In this study, we cloned an adenyl cyclase (*cyaA*) gene from *M. xanthus*, constructed a *cyaA* mutant by gene disruption, and used the mutant for a developmental assay. *cyaA* was expressed during development, but the *cyaA* mutant was able to undergo normal development and exhibited cAMP production similar to that of the wild-type strain during development. These results indicated that *M. xanthus* CyaA is not involved in the production of cAMP during development and that another adenyl cyclase produces cAMP during development. Several prokaryotes have been reported elsewhere to possess multiple adenyl cyclase genes (8, 18).

Hydropathy profile analysis suggested that *M. xanthus* CyaA possesses three potential transmembrane regions that connect an extracellular domain in the amino-terminal region to an internal catalytic domain in the carboxyl-terminal region. This structure is often found in the receptor-type guanylyl cyclases of eukaryotes. As *M. xanthus* CyaA is thought to be a receptor-type adenyl cyclase, this implies that CyaA has the ability to sense an extracellular signal and control adenyl cyclase activity. Mutation of *cyaA* caused significant reduction of fruiting body formation, spore formation, and spore germination at high osmolarity. The phenotypes of the mutant would not be due to polar effects, because there was no open reading frame that formed an operon with the *cyaA* gene. We next investigated whether CyaA was a sensor for osmolarity and was regulated by high osmolarity. When spores cultured in growth media were incubated at high osmolarity, there was an apparent difference in cAMP accumulation between wild-type and *cyaA* mutant strains. In wild-type spores, high osmolarity caused a rapid cAMP increase which peaked at 1 min and then slowly declined. The cAMP accumulation in wild-type cells was much more strongly stimulated by NaCl than by sucrose. In contrast, the mutant cells showed only weak NaCl-stimulated adenyl cyclase activity. On the other hand, cAMP accumulation in *cyaA* mutant cells at developmental stages was stimulated by 0.2 M NaCl or sucrose, and the stimulated cAMP levels were similar to those of wild-type cells. Since the levels of cAMP accumulation were increased until at least 60 min after stimulation, the cAMP accumulation would be due to other adenyl cyclases. However, the *cyaA* gene was expressed during development, and the *cyaA* mutant cells at developmen-
far as we know, our results represent the first demonstration of an adenyl cyclase that acts as a sensor in a prokaryote. The signal transduction pathway for osmoregulation of germination in *D. discoideum* is assumed to be as follows: the increased intracellular cAMP by osmotic stress activates a CAMP-dependent protein kinase (protein kinase A [PKA]), and the activated PKA inhibits spore germination (32). The first prokaryote protein serine/threonine kinase was discovered in *M. xanthus*, and at least 11 serine/threonine kinase genes have been submitted to the DDBJ sequence library. We are currently investigating whether *M. xanthus* possesses a CAMP-PKA pathway for osmotic transduction of development and germination.

REFERENCES


