JOURNAL OF BACTERIOLOGY, May 2005, p. 3593–3598 Vol. 187, No. 10
0021-9193/05/$08.00+0 doi:10.1128/JB.187.10.3593–3598.2005

An Adenylyl Cyclase, CyaB, Acts as an Osmosensor in *Myxococcus xanthus*

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Received 1 November 2004/Accepted 4 February 2005

We have previously reported that a receptor-type adenylyl cyclase (CyaA) of *Myxococcus xanthus* undergoes an osmosensor mainly during spore germination (Y. Kimura et al., J. Bacteriol. 184:3578–3585, 2002). In the present study, we cloned another receptor-type adenylyl cyclase gene (*cyaB*) and characterized the function of the *cyaB*-encoded protein. Disruption of *cyaB* generates a mutant that showed growth retardation at high ionic (NaCl) or high nonionic (sucrose) osmolarity. When vegetative cells were stimulated with 0.15 M NaCl, the increases in intracellular cyclic AMP levels of *cyaB* mutant cells were lower than those of wild-type cells. Under nonionic osmostress, the *cyaB* mutant exhibited reduced spore germination; however, the germination rate of the *cyaB* mutant was significantly higher than that of the *cyaA* mutant.

The ability to adapt to changes in environmental osmolarity is fundamental to survival of organisms (20, 24). *Myxococcus xanthus* is a gram-negative soil bacterium that undergoes multicellular development when starved for essential nutrients (23). During development, high densities of cells (10^5 to 10^6) cooperate to produce a mound in which cells differentiate into spores. At least five extracellular signals, known as the A, B, C, D, and E signals, are required for this multicellular development process (7, 8, 13). When spores are exposed to a nutrient-rich environment, they germinate and become vegetative rod-shaped cells. In *M. xanthus*, we demonstrated that MokA and CyaA are required for osmotic tolerance and involved in sensing extracellular osmolarity (10, 11). MokA consists of a sensor domain in its amino-terminal half and a histidine kinase domain and a response regulator domain in its carboxy-terminal half, making it a typical transmembrane hybrid-type histidine kinase. *mokA* mutant showed growth retardation at high osmolarity and reduced sporulation under starvation conditions. CyaA is a receptor- or sensor-type adenylyl cyclase that is composed of an amino-terminal sensor domain and a carboxy-terminal catalytic domain of adenylyl cyclase. The *cyaA* mutant exhibited a marked reduction in spore formation and spore germination under conditions of osmostress (10).

In this study, we cloned another receptor-type adenylyl cyclase gene (*cyaB*) and characterized the function of the *cyaB*-encoded protein. The predicted *cyaB* gene product had structural similarity to *M. xanthus* CyaA, and CyaB acted as an osmotic sensor during the growth phase, suggesting that two receptor-type adenylyl cyclases, CyaA and CyaB, function as osmotic sensors at different phases of the *M. xanthus* life cycle.

Cloning of *M. xanthus cyaB*. We attempted to clone adenylyl cyclase genes from an *M. xanthus* FB (5) (IFO [Institute for Fermentation, Osaka] 13542) genomic library (11) by using three oligonucleotide probes. The three oligonucleotides (Cya1: GACAAGTA/TCATCGGC/GGACG/TCC/GVTC/GATG; Cya2: GTC/GAAC/GCTC/GGCC/GTCC/GCGC/GCTC/GGAG; Cya3: TACACC/GGTC/GA/CTC/GGGC/GGACGC/GC/GGT, where V is A, C, or G) were deduced from the conserved catalytic domain of the adenylyl cyclase and were labeled with digoxigenin-11-dUTP using an oligonucleotide tailing kit (Roche Diagnostics GmbH). Three positive clones were obtained by plaque hybridization, and various DNA restriction fragments of the clones were sequenced. The sequence analysis revealed that two clones contained *cyaA* genes and one clone contained another adenylyl cyclase gene (*cyaB*). A 7.1-kb SalI fragment of the clone containing *cyaB* was found to contain six open reading frames (ORFs) (Fig. 1). *cyaB* encoded 765 amino acid residues deduced from its nucleotide sequence with a molecular mass of 82.6 kDa. A computer search using the BLAST program revealed that the C-terminal region of CyaB shares significant sequence homology to the catalytic domains of adenylyl cyclases. The catalytic domain of CyaB showed 30% identity to the CyaA of *M. xanthus* (10), 37% identity to the putative adenylyl cyclase of *Bdellovibrio bacteriovorus* (22), and 41% identity to the putative adenylyl cyclase of *Leptospira interrogans* (21) (Fig. 2A). The amino-terminal region of CyaB showed similarity to the amino-terminal region of the CyaA of *M. xanthus* (27% identity) (10), the putative adenylyl/guanylyl cyclase of *Bradyrhizobium japonicum* USDA 110 (25% identity) (9), and the putative adenylyl/guanylyl cyclase of *Rhodopseudomonas palustris* (27% identity) (14) (Fig. 2B). Hydropathy analysis of the *cyaB* gene product suggested that CyaB possesses a signal peptide (Met-1 to Asp-19) and three transmembrane regions (Ala-261 to Gly-283, Ala-390 to Thr-412, and Val-445 to Phe-464) (Fig. 1). These results suggested that *M. xanthus* CyaB is a receptor-type adenylyl cyclase that belongs to the class III adenylyl cyclases as described by Danchin (4). An adenylyl cyclase, AC1, of the myxobacterium *Stigmatella aurantiaca* contains a 17-amino-acid motif, which is a signature of G-protein-coupled receptors, in membrane domain (3), but the motif did not exist in CyaB.

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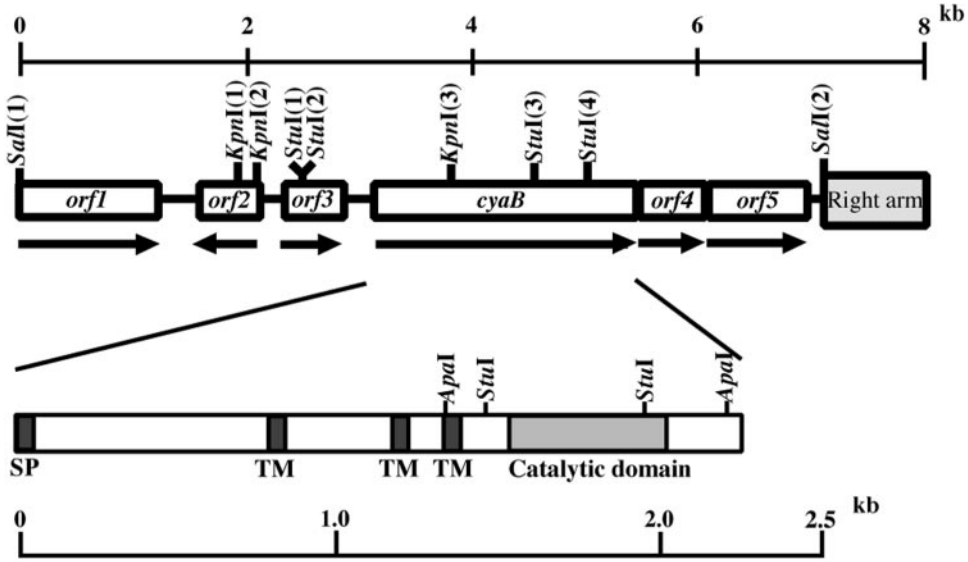

FIG. 1. Restriction map of the *cyaB* gene of *M. xanthus*. Lines with arrows indicate orientation. The 0.5-kb StuI fragment was replaced by the Km^r gene. The 7.1-kb SalI fragment contains a partial right arm of λEMBL3 vector. SP, signal peptide; TM, transmembrane domain.

The *orf4* gene was located 27 base pairs downstream from the stop codon of the *cyaB* coding region, and the start codon of the *orf5* gene overlapped with the stop codon of *orf4*. The predicted Orf4 and Orf5 proteins were 192 and 328 amino acids, respectively; however, these predicted gene products showed no extensive homology with other proteins or low homology with unknown proteins in the GenBank database.

Expression of *cyaB*. We investigated the expression of the *cyaB* gene in *M. xanthus* cells during growth and development by a reverse transcription-PCR (RT-PCR) (Fig. 3A). Total RNA was isolated from *M. xanthus* at the exponential growth phase and during development as described previously (15), and 0.1 μg of RNA was used for cDNA synthesis with *Bca*BEST polymerase in accordance with the manufacturer's protocol (Takara shuzo, Kyoto). PCR was performed with *Bca*-Optimized *Taq* polymerase, a 5′ gene-specific primer (5′-CTCGGGCCATCGTGTTCG-3′), a 3′ gene-specific primer (5′-AGGTAGAAG GGGATGGACG-3′), and the synthesized cDNA. The expected 105–bp RT-PCR product was amplified from RNA of vegetative cells. The *cyaB* gene was also expressed at similar levels in developing cells at the mound formation stage (18 h) and the early stage of fruiting body formation (36 h). 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).

Phenotypes of *cyaB* mutant. To investigate the biological function of CyaB, we constructed a *cyaB* deletion-insertion mutant. A plasmid containing a 3.3-kb KpnI-SalI fragment was digested with StuI, and 1.2 kb of kanamycin-resistant (Kmr) cassette digested with SmaI was ligated into the StuI sites of the plasmid (Fig. 1). *cyaB*::Km^r was amplified by PCR using a pair of primers (5′-CCATTCCCGTGCTGGTGG-3′ and 5′-TGGGTGCCATTCCCTTGC-3′) and introduced into *M. xanthus* cells by electroporation (19). We confirmed the replacement by PCR analysis and Southern hybridization (Fig. 3B). PCR of genomic DNA from wild type and *cyaB* mutant with the above primers had a 2.0-kb band and 2.7-kb band, respectively. Southern analysis of ApaI-digested genomic DNA with a probe (0.9-kb ApaI fragment of *cyaB* gene) revealed bands of 0.9 kb for the wild type and of 1.6 kb for the mutant strain. These results indicated that the 0.5-kb StuI fragment of *cyaB* gene was replaced by the 1.2-kb fragment containing a Km^r cassette. Using an RT-PCR, we also confirmed that *orf4*, which is located downstream of *cyaB*, was transcribed in the *cyaB* mutant. A primer set (5′-ATGCGAAGCACAAGCGATG G-3′ and 5′-ACTTCTTCGGATCCACCTGC-3′) was used for PCR analysis. The expected 144-bp product containing part of *orf4* was amplified from total RNA of the *cyaB* mutant, suggesting that the phenotypes of *cyaB* mutant would not be due to polar effects (Fig. 3C).

The *cyaB* mutant showed normal growth in Casitone-yeast extract (CYE) medium (1). However, when 0.15 M NaCl or sucrose was added to early exponentially growing cultures, *cyaB* mutant showed almost twofold longer lag times than the wild type (Fig. 4A and B). No significant differences were found between the final cell densities and maximum growth rates of the wild type and *cyaB* mutant under osmotic stress. These results indicate that the deletion of *cyaB* did not affect the final biomass but extended the time needed for cells to adapt to growth under osmotic stress.

In contrast to the *mokA* mutant (6), the *cyaB* mutant developed normally on medium clone fruiting (CF). The wild-type and *cyaB* mutant strains formed fruiting bodies after 3 days of incubation on CF plates with 1.5% agar. Within the fruiting bodies of the wild type and mutant, vegetative cells were converted into spherical spores. The numbers of total and viable spores of *cyaB* mutant counted at 8 days of development were similar to those of the wild-type strain (Table 1). When the developing cells incubated on CF plates for 20 h were harvested and inoculated on CF plates containing up to 0.2 M NaCl or sucrose, the total spores, estimated by direct counts in

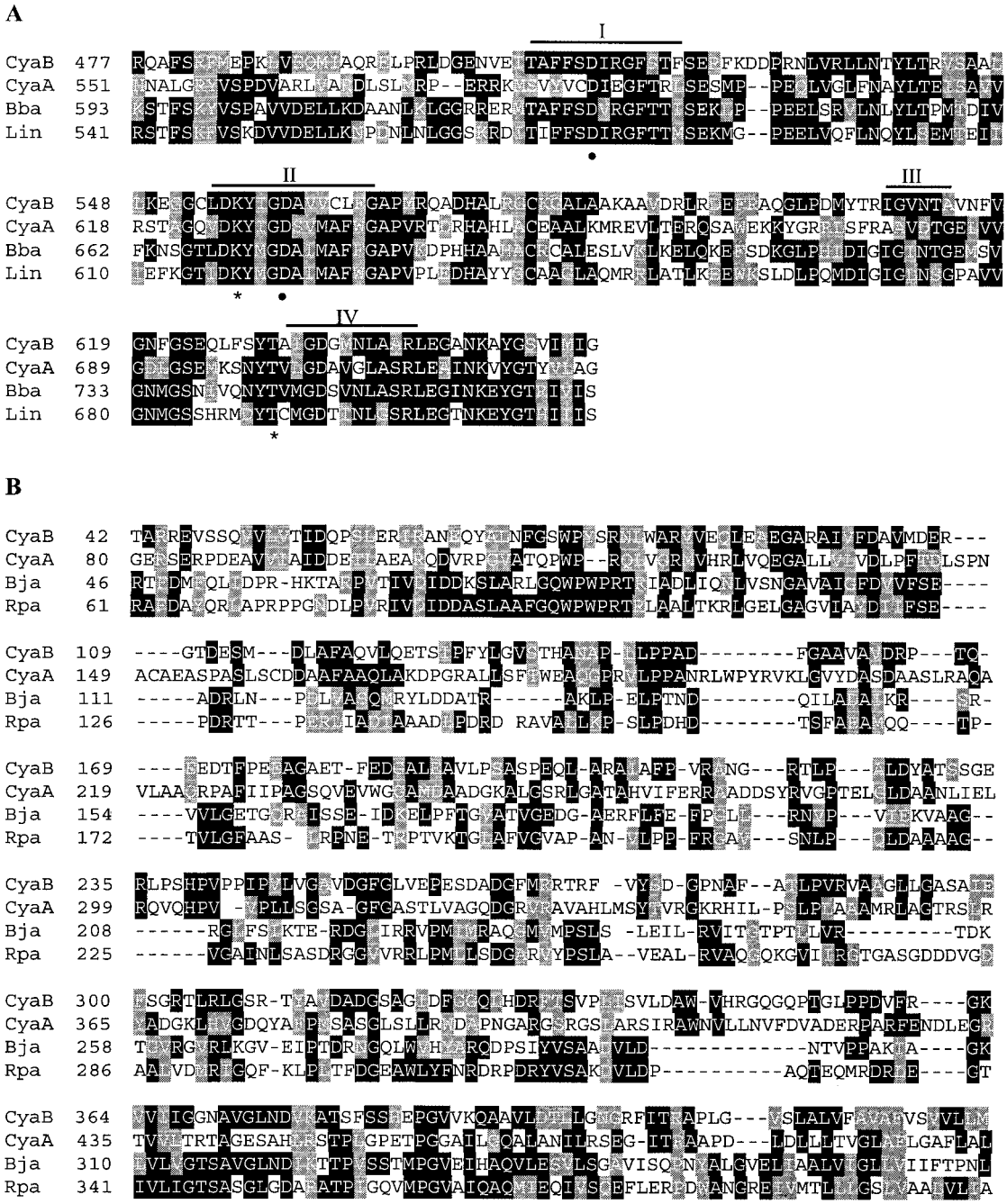

FIG. 2. Alignment of adenylyl cyclase sequences. (A) Alignment of the deduced catalytic domain of CyaB with the adenylyl cyclase CyaA of *M. xanthus* (CyaA), the putative adenylyl cyclase of *Bdellovibrio bacteriovorus* (Bba), and the putative adenylyl-guanylyl cyclase of *Leptospira interrogans* (Lin). Four conserved motifs (I to IV) in class III adenylyl cyclases are overlined (17). The asterisks and the closed circles mark amino acid residues that are involved in substrate definition and coordination of metal ions (Mg^{2+} or Mn^{2+}), respectively (2, 17). (B) Alignment of the deduced amino-terminal region of CyaB with the CyaA of *M. xanthus* (CyaA), the putative adenylyl/guanylyl cyclase of *Bradyrhizobium japonicum* (Bja), and the putative adenylyl/guanylyl cyclase of *Rhodopseudomonas palustris* (Rpa). Amino acid residues in agreement for more than two residues are indicated by filled boxes. Gray shading indicates degrees of similarity among amino acid residues. The sequence of the *M. xanthus cyaB* gene has been deposited in the DNA Data Bank of Japan sequence library under accession number AB188227.

microscopy, of the *cyaB* mutant strain on CF agar containing 0.15 M NaCl or sucrose at 8 days of development was approximately 70% or 90% of that of the wild-type strain, respectively. The *cyaB* mutant under osmotic stress showed higher spore yields compared to a previously reported *cyaA* mutant (10). On the other hand, the number of the viable spores of *cyaB* mutant on CF plates containing 0.15 M NaCl or sucrose was 40% or 78% of that of wild-type strain. There were no significant differences in spore formation on CF plates containing less than 0.1 M NaCl or sucrose between the wild-type and *cyaB* mutant cells.

When spores were harvested from CF plates and inoculated

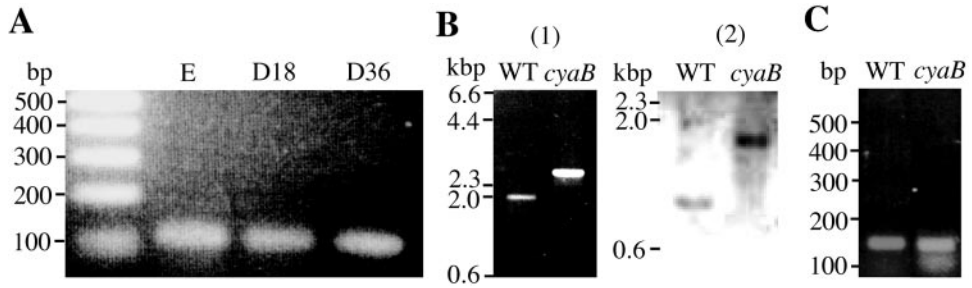

FIG. 3. RT-PCR analysis of *cyaB* gene expression in *M. xanthus* and characterization of the *cyaB* mutant. (A) RT-PCR analysis of *cyaB* gene expression in *M. xanthus*. Total RNA prepared from cultures at the exponential growth phase (E) and during development at 18 h (D18) and 36 h (D36) was used for RT-PCR analysis. Molecular sizes of DNA fragments are given in bases. (B) Confirmation of *cyaB* deletion-insertion by PCR amplification (1) and hybridization (2) (1). PCR was carried out using genomic DNA as templates and *cyaB*-specific primers (2). Genomic DNA was restricted with ApaI. Southern blots were probed with a 0.9-kb ApaI fragment of *cyaB*. (C) Detection of the *orf4* transcript in wild type (WT) and *cyaB* mutant strains. Total RNA prepared from vegetative cells was treated with reverse transcriptase. WT, wild type.

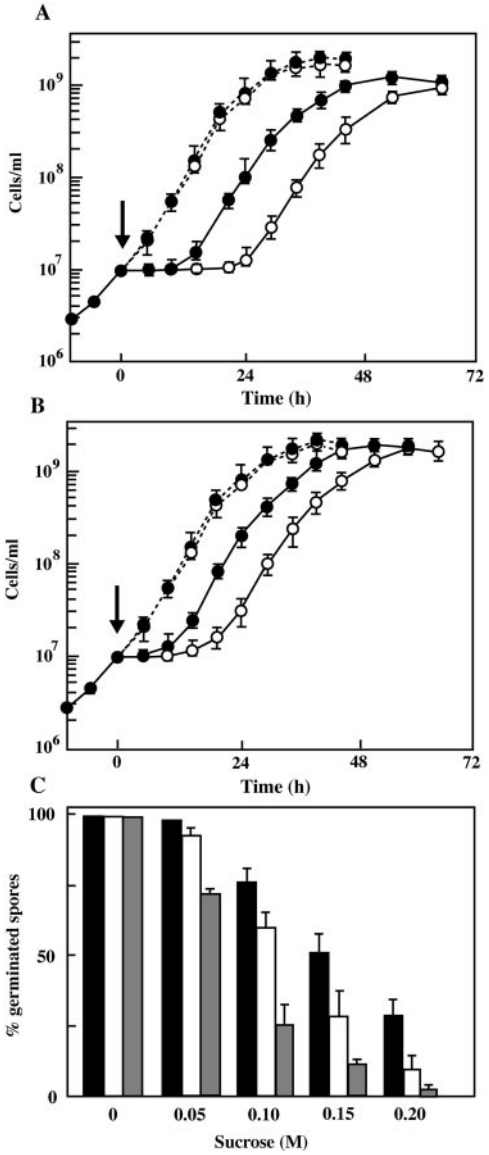

FIG. 4. Effect of the *cyaB* mutation on *M. xanthus* growth and spore germination under osmotic stress. Growth of *M. xanthus* wild-type (closed symbols) and *cyaB* mutant (open symbols) cells in CYE medium with 0.15 M NaCl (A) or 0.15 M sucrose (B) or without either (dashed lines). NaCl or sucrose was added at the point indicated by the arrow. The cells were cultured at 30°C with shaking, and the cell densities were determined with a hemacytometer. The data are expressed as the means for triplicate experiments. (C) Spore germination of *M. xanthus* wild-type (closed bars), *cyaB* mutant (open bars), and *cyaA* mutant (shaded bars) spores in CYE medium containing various concentrations of sucrose. Spores were harvested from 6- to 8-day-old fruiting bodies on CF plates, sonicated for 2 min, and treated with heat (60°C for 15 min). The spores were inoculated to 10^7 cells/ml in CYE medium containing up to 0.2 M sucrose and incubated at 30°C with continuous shaking until almost all spores in the medium without addition or sucrose were germinated, and then the number of ungerminated spores in each culture was counted with a hemacytometer. The percent germination (germinated spores per inoculated spores) is the mean of duplicate experiments. The standard deviations are shown by error bars.

in CYE medium containing 0 to 0.2 M NaCl, the *cyaB* mutant spores germinated at the same rate as wild-type spores (data not shown). Meanwhile the *cyaB* mutant spores in CYE medium containing more than 0.10 M sucrose exhibited a lower germination rate than the wild-type spores (Fig. 4C). However, the germination of the *cyaB* mutant spores was less inhibited by sucrose than that of the *cyaA* mutant spores. The *cyaB* mutant exhibited normal phenotypes with respect to stresses such as temperature shifts and pH changes.

Effects of osmotic stress on levels of cAMP. Figure 5 shows the changes in intracellular levels of cyclic AMP (cAMP) when vegetative cells were exposed to medium with 0.15 M NaCl for 10 min. The level of cAMP in wild-type cells increased immediately after exposure of the cells to medium with 0.15 M NaCl and reached a maximum level of 7.0 pmol/mg protein at 5 min. In the *cyaB* mutant, addition of 0.15 M NaCl resulted in a 1.3-fold increase in the cAMP level, with a peak at 1 min. The maximum level was 5.3 pmol/mg protein. Addition of 0.15 M sucrose to wild-type vegetative cells induced an increase in the accumulation of cAMP, but the increased levels (maximum of 1.2-fold) were lower than those after NaCl treatment. cAMP production by mutant vegetative cells was only weakly stimulated by addition of 0.15 M sucrose (data not shown).

We found no reports of increases in intracellular levels of cAMP in response to osmotic stress during the growth phase of bacteria. In *Zygosaccharomyces rouxii*, the cAMP levels increased 4.6-fold in response to osmotic stress (1 M NaCl) (18).

TABLE 1. Sporulation of the wild type and *cyaB* mutant under osmotic stress[a]

Addition	Total spores		Viable spores	
	Wild type	*cyaB* mutant	Wild type	*cyaB* mutant
No addition	3.7×10^8	3.6×10^8	2.9×10^7	2.8×10^7
0.10 M NaCl	1.8×10^8	1.7×10^8	7.2×10^5	7.0×10^5
0.15 M NaCl	6.5×10^7	4.6×10^7	2.0×10^4	7.9×10^3
0.20 M NaCl	5.9×10^6	3.5×10^6	9.1×10^2	1.4×10^2
0.10 M Sucrose	2.0×10^8	2.0×10^8	1.8×10^6	1.7×10^6
0.15 M Sucrose	7.4×10^7	6.5×10^7	3.3×10^5	2.6×10^5
0.20 M Sucrose	3.0×10^7	2.2×10^7	7.5×10^4	1.8×10^4

[a] The developing cells incubated on CF plates for 20 h were harvested and inoculated on CF plates containing 0.1 M to 0.2 M NaCl or sucrose. After 8 days, spots were scraped, resuspended in TM (10 mM Tris-HCl, pH 7.6, and 8 mM $MgSO_4$) buffer, sonicated for 1 min, and treated with heat (2 h at 55°C). The number of spores was counted with a hemacytometer (total spores); the number of viable spores was determined by plating out serial dilutions on CYE plates. Data are the means of duplicate experiments.

Compared with the increases in yeast, the increases in the intracellular cAMP levels of *M. xanthus* in response to osmotic stress were low, but the increase in cAMP may result in the induction of adaptation to osmotic stress in *M. xanthus*. Recently, we reported that an *M. xanthus* CbpB containing two cAMP-binding domains was involved in osmotic tolerance (12). CbpB is a hydrophilic protein, and its structural features were partially similar to those of PKA regulatory subunits. The *cbpB* mutant showed growth retardation under osmotic stress. These data suggest that the cAMP signaling plays an important role in osmotic adaptation in *M. xanthus*.

During 1 to 2 h of incubation with 0.15 M NaCl or sucrose, the intracellular cAMP levels of wild-type and *cyaB* mutant cells decreased by 20 to 30% compared with the 0 h value. The cAMP levels then returned to the initial values after 4 h of incubation (data not shown). This temporal decrease is possibly due to the damage of cells by osmotic stress.

We suggest that *M. xanthus* CyaB and MokA and CyaA function as osmosensors mainly during vegetative growth and germination, respectively. CyaA is also required for osmotic tolerance in fruiting formation and sporulation (10). Recently, we were able to run a BLAST search on the Institute for Genomic Research (TIGR) database. We searched for other receptor-type adenylyl cyclase genes in the TIGR database and found one. The gene encoded a membrane-spanning protein with five to six transmembrane domains and consisting of 375 amino acids, but the encoded adenylyl cyclase has only two short periplasmic regions (13 and 6 amino acids). On the other hand, it is reported that four histidine kinases, Hik16, Hik33, Hik34, and Hik41, act as sensors in the perception of salt stress in *Synechocystis* sp. strain PCC 6803 (16). We estimate that *M. xanthus* has about 100 ORFs that encode proteins containing domains homologous to the histidine protein kinase region of MokA. *M. xanthus* may have other osmosensor histidine kinases and have the ability to adapt to changes in environmental osmolarity using complicated signal transduction pathways.

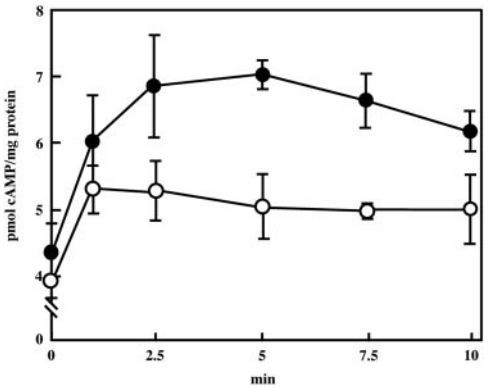

FIG. 5. Changes in intracellular levels of cAMP during osmotic stress in *M. xanthus*. Wild-type (closed circles) and *cyaB* mutant cells (open circles) were incubated in CYE medium with 0.15 M NaCl. cAMP assays were performed as described previously (10). Experiments were repeated three times. The standard deviations are shown by error bars.

Nucleotide sequence accession numbers. The sequence of the *M. xanthus cyaB* gene has been deposited in the DNA Data Bank of Japan sequence library under accession number AB188227.

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