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The *Rhizobium etli cyaC* Product: Characterization of a Novel Adenylate Cyclase Class

Juan Téllez-Sosa,[1] Nora Soberón,[1] Alicia Vega-Segura,[2] María E. Torres-Márquez,[2] and Miguel A. Cevallos[1]*

Programa de Evolución Molecular, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, C.P. 62210, Cuernavaca, Morelos,[1] and Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, C.P. 04510, Distrito Federal,[2] México

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Adenylate cyclases (ACs) catalyze the formation of 3′,5′-cyclic AMP (cAMP) from ATP. A novel AC-encoding gene, *cyaC*, was isolated from *Rhizobium etli* by phenotypic complementation of an *Escherichia coli cya* mutant. The functionality of the *cyaC* gene was corroborated by its ability to restore cAMP accumulation in an *E. coli cya* mutant. Further, overexpression of a *malE*::*cyaC* fusion protein allowed the detection of significant AC activity levels in cell extracts of an *E. coli cya* mutant. CyaC is unrelated to any known AC or to any other protein exhibiting a currently known function. Thus, CyaC represents the first member of a novel class of ACs (class VI). Hypothetical genes of unknown function similar to *cyaC* have been identified in the genomes of the related bacterial species *Mesorhizobium loti*, *Sinorhizobium meliloti*, and *Agrobacterium tumefaciens*. The *cyaC* gene is cotranscribed with a gene similar to *ohr* of *Xanthomonas campestris* and is expressed only in the presence of organic hydroperoxides. The physiological performance of an *R. etli cyaC* mutant was indistinguishable from that of the wild-type parent strain both under free-living conditions and during symbiosis.

Cyclic AMP (cAMP) is widely distributed in prokaryotic and eukaryotic organisms and plays a central role in the regulation of diverse cellular functions. In eukaryotic cells, increased levels of cAMP regulate enzyme activities, channel activities, and gene expression, mainly via cAMP-dependent protein kinases (22). In some prokaryotes, such as *Escherichia coli* and other enterobacteria, cAMP regulates gene expression when bound to its receptor protein (CRP) (for a review, see reference 27). In other bacteria, however, the mechanism through which cAMP acts remains unclear.

Adenylate cyclases (ACs) are the enzymes responsible for the synthesis of cAMP from ATP and have been classified into five classes according to their evolutionary relationships. Class I embraces the ACs of enterobacteria, the enzyme from *E. coli* being the best-known member of this class. Class II includes the calmodulin-activated toxins from *Bacillus anthracis* and *Bordetella pertussis*. Class III (the universal class) contains ACs or guanylate cyclases (GCs) from eukaryotic and prokaryotic organisms, and class IV encompasses AC2 of *Aeromonas hydrophila* encoded by the *cyaB* gene, which has close sequence similarities to proteins of hyperthermophilic archaebacteria (13, 46). The recently identified class V is comprised of one AC from the strict anaerobic bacterium *Prevotella ruminicola* (11). All five classes of enzymes are present in bacteria, while in eukaryotes, only enzymes belonging to class III have been described. Recently, the crystal structures of the catalytic domains of mammalian class III ACs have been solved (50, 60). Based on these structures, on modeling studies, and on other mutational analyses, essential residues required for substrate binding (ATP) and catalysis have been identified (32, 51).

In rhizobia, cyclic nucleotides such as cAMP and cyclic GMP (cGMP) have been implicated in some metabolic functions. In *Bradyrhizobium japonicum*, it has been suggested that cAMP participates in regulating ammonia assimilation, since the addition of this compound to cultures decreased the specific activities of glutamate synthase and glutamine synthetase (54). In the same organism, malate and other tricarboxylic acid cycle intermediates repressed hydrogen uptake and glutamate utilization when glutamate is utilized as the sole source of carbon and nitrogen. The addition of exogenous cAMP alleviates this inhibition (30). In *B. japonicum,* growth and ex planta nitrogenase activity were inhibited when 100 μM cGMP was added to the culture media (23, 31). In *Sinorhizobium meliloti,* an apparent catabolite repression-like phenomenon has been described in which the addition of succinate (the preferred carbon source) causes the immediate repression of β-galactosidase activity in cells growing in lactose. Unexpectedly, cAMP is not involved in this repression (53).

The elucidation of the physiological role of cAMP and cGMP in *Rhizobium* has been difficult, since multiple ACs have been identified: two in *B. japonicum* (10, 19) and three in *S. meliloti* (5, 6, 44). However, analysis of the recently sequenced genomes of *S. meliloti* and *Mesorhizobium loti* has shown that these organisms contain genes coding for 26 and 12 different ACs/GCs, respectively. All of these predicted proteins present catalytic domains similar to those found in class III enzymes (18, 24).

With the aim of reaching a deeper insight into the role of cAMP in *Rhizobium*, we have isolated several AC (*cya*) genes from *Rhizobium etli* by functional complementation of an *E. coli cya* mutant strain with a genomic library. In this study, we present the isolation, sequencing, functional characterization,

* Corresponding author. Mailing address: Programa de Evolución Molecular, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, A. P. 565-A, C. P. 62210, Cuernavaca, Morelos, México. Phone: 52(777)3114663. Fax: 52(777)3175581. E-mail: mac@cifn.unam.mx.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Strains		
Escherichia coli		
W3110	Wild type	C. Gómez
CON1	W3110, Δ(*cya-1400*)::Km	This work
SP850	*relA1 spoT* Δ(*cya-1400*)::Km *thi-1* e14$^-$ λ$^-$	43
HB101	F$^-$ *hsdS20 leuB6 proA2 thi-1 lacY1 supE44 ara14 galK2 xyl5 rpsl20 recA13*	8
DH5α	F$^-$ *hsdR17 thi-1 gyrA* Δ(*lacZYA-argF*) *supE44 recA1* (ϕ80dΔ*lacZ*M15) *relA*	21
S17-1	*thi-1 proA2 recA hsdR hsdM* RP4.2-Tc::Mu-Km::Tn*7*	45
Rhizobium etli		
CE3	Wild-type Sm^r derivative of CFN42	37
CON31	CE3 *cyaC*::*lacZ*	This work
CON32	CE3 *cyaC*::*lacZ*	This work
CON40	CE3 *ohr*ΩKm	This work
Plasmids		
pLAFR1	Broad-host-range IncP2, cosmid, Tc^r	17
pRK2073	Helper plasmid, Sp^r	15
pRK415	Broad-host-range IncP cloning vector, Tc^r	25
pBluescriptIISK+	ColE1 replicon, cloning vector, Ap^r	Stratagene
pWS233	Mobilizable replicon ColE1, Gm^r Tc^r *sacRB*	42
pKK233-3	ColE1 replicon, expression vector, Ap^r	Stratagene
pMAL-c2X	ColE1 replicon, expression vector, Ap^r	New England Biolabs
pKOK6	*lacZ*-Km^r interposon in pKOK4, Km^r Cb^r	26
pBLS128	ΩKm donor	1
pRKOhr	pRK415 derivative containing PCR product with *ohr* gene and its upstream region	This work
p3310	pBluescriptIISK+derivative carrying 2.9-kb *Sal*I fragment containing the *R. etli cyaC* gene	This work
p3310m	pBluescriptIISK+derivative carrying 2.9-kb *Sal*I fragment containing *R. etli cyaC* gene, but with single base mutation (G2316C)	This work
p3310CyaCKm	p3310 derivative, but with *R. etli cyaC* gene interrupted by a ΩKm	This work
p3310OhrKm	p3310 derivative, but with *R. etli ohr* gene interrupted by ΩKm	This work
pKKCyaC	pKK233-3 derivative containing PCR product with *R. etli cyaC* gene	This work
pMALCyaC	pMAL-c2X derivative containing PCR product with *R. etli cyaC* gene fused to *E. coli malE* gene	This work
pRKACEc	pRK415 derivative carrying 2.8-kb *Eco*RI fragment containing *E. coli* W3110 *cya* gene	This work
pWSACEcKm	pWS233 derivative carrying genomic fragment of 8 kb from SP850 strain with Δ(*cya-1400*)::Km	This work
pWSCyaCLac1	pWS233 derivative carrying same insert as p3310m, but with *cyaC* gene fused to *lacZ* in direct orientation	This work
pWSCyaCLac2	pWS233 derivative carrying same insert as p3310m, but with *cyaC* gene fused to *lacZ* in inverse orientation	This work

and expression analysis of the *cyaC* gene of *R. etli* CE3. The deduced protein product of *cyaC* gene does not contain canonical motives or signatures resembling those of any other ACs reported, indicating that it defines a new class of ACs, class VI.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The plasmids and strains used in this study are described in Table 1. *E. coli* strains were grown at 37°C in Luria-Bertani, MacConkey's, or M9 minimal medium with different sugars (lactose, maltose, galactose, or arabinose at 1%). *Rhizobium* strains were grown at 30°C in PY (37) or minimal medium (MM) (16) containing 10 mM NH_4Cl as a nitrogen source and the sole carbon source at a concentration of 10 mM. Batch cultures of *R. etli* cells were grown according to the method of Encarnación and coworkers (16). Culture growth was estimated as the total protein content by using the Lowry method (33). Swarming soft-agar plates for *E. coli* contained 1% yeast extract, 0.8% NaCl, and 0.3% agar. Swarming soft-agar plates for *R. etli* contained MM plus 0.005% yeast extract, 0.028% agar, and 10 mM succinate as the carbon source. When required, antibiotics were added in the following final concentrations: tetracycline, 10 μg/ml; carbenicillin, 100 μg/ml; kanamycin, 30 μg/ml; gentamicin, 30 μg/ml; fosfomycin, 25 μg/ml; spectinomycin, 100 μg/ml; streptomycin, 100 μg/ml.

DNA manipulations. Standard DNA manipulations were carried out as described previously (41). Restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase were purchased from Amersham-Pharmacia Biotech. All enzymes were used according to the supplier's recommendations. Cosmids were introduced into *E. coli* by electroporation as reported by Kubicka and Kramaric (28).

Construction of an *E. coli* W3110 *cya* mutant. In the *E. coli* SP850 strain, most of the *cya* coding sequence has been replaced by a kanamycin resistance inter-

poson (Δ*cya-1400*::Km) (43). To create the *E. coli* W3110 *cya* mutant, the genomic DNA of SP850 was digested with *Eco*RI, and the mutation (Δ*cya*-1400::Km) was subcloned into the conditionally lethal plasmid pWS233 (42), exploiting the kanamycin resistance. The resulting plasmid, pWSACEcKm, which harbors a genomic fragment of 8 kb, was transformed into *E. coli* strain W3110, and one-step double recombinants were selected by the method described by Selbitschka and coworkers (42). The *cya* mutants were selected as golden colonies able to grow in MacConkey medium plates supplemented with lactose as the carbon source. The genotype of the resultant strain, CON1, was verified by PCR and Southern analyses. Strain CON1 showed the expected phenotype for a *cya* mutant (data not shown).

The *cya* gene of *E. coli* strain W3110 was isolated by PCR amplification with specific primers derived from the published *E. coli cya* nucleotide sequence (GenBank accession no. K02969) (5′-GTCATTATCATCCGTGGT-3′ and 5′-AGTTTCCGCTAAGATT-3). The amplification product of 2,957 bp, harboring the whole *cya* gene and its promoter region, was cloned into the medium-copy-number plasmid pRK415 (15) to generate plasmid pRKACEc.

Complementation analyses. Individual clones from an *R. etli* strain CE3 genomic library cloned in pLARF1 were conjugally mated into *E. coli cya* strain SP850, with plasmid pRK2073 as a helper. A mixture of overnight cultures of *E. coli* SP850 and HB101(pRK2073) strains (50 μl each) was spread onto Luria-Bertani agar plates. Approximately 1,200 clones from the library were then individually streaked onto these plates and incubated overnight at 37°C. The mated clones were subsequently replicated onto selective agar plates (M9 plus lactose as carbon source) and incubated at 37°C until colony development. Positive transconjugants were retested in the *E. coli cya* mutant strain CON1.

DNA sequencing and bioinformatics. Double-stranded templates were sequenced manually by the dideoxy method with the Thermosequenase kit from Amersham-Pharmacia Biotech. Sequencing reaction mixtures were electrophoresed in 6% polyacrylamide–8 M urea gels in a Bio-Rad Sequi-gen sequencing apparatus. Routine sequence analysis was accomplished with the Genetics Computer Group suite (version 8) and the GeneWorks suite (release 2.5; IntelliGenetics). Nucleotide and deduced amino acid sequences were compared to those deposited in the GenBank nonredundant database by using the Blast 2.1 algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) (4) The assessment of transmembrane regions in the predicted CyaC protein was carried out with the programs PSORT (36) (http://psort.nibb.ac.jp/), TMHMMM (47) (http://www.cbs.dtu.dk/services/TMHMM-1.0/), HMMTOP (52) (http://www.enzim.hu/hmmtop/), DAS (12) (http://www.biokemi.su.se/~server/DAS/), and TopPred (56) (http://www.biokemi.su.se/~server/toppred2/toppredServer.cgi).

Localization of the *cyaC* gene. Genomic DNA from *R. etli* strains cured of each one of the endogenous megaplasmids was digested with the appropriate restriction enzymes, electrophoresed in 1% agarose, blotted onto nitrocellulose, and hybridized against a *cyaC* internal probe with the Rediprime kit and Rapid-hyb buffer from Amersham-Pharmacia Biotech. *R. etli* plasmid profiles were obtained by the in-gel lysis method of Wheatcroft and coworkers (57), blotted onto nitrocellulose, and hybridized as described above.

cAMP determination and estimation of AC activity. The cAMP content in cell extracts was determined by using the cyclic AMP ^{3}H Assay System kit from Amersham-Pharmacia Biotech (48). To determine the cAMP content in *E. coli*, cells were grown in M9 MM supplemented with 1% lactose and 0.1% Casamino Acids. To determine the cAMP content in *R. etli*, cells were grown in MM supplemented with succinate. In both cases, 10-ml samples from exponentially growing cultures were boiled for 10 min. Extracts were then centrifuged to remove cell debris and lyophilized. Dried residues were resuspended in 0.5 ml of assay buffer and assayed for cAMP according to the supplier's protocol.

The AC activity of CyaC was measured in cell extracts of the *E. coli* CON1 strain overexpressing MBP-CyaC, a fusion protein formed by the maltose binding protein and CyaC. The *cyaC* coding sequence was amplified by PCR with the oligonucleotides 5′-GGAATTCATGAGCAACAGCCAG-3′ and 5′-GGAAGCTTGATCCAGCGCGCGCTGCCG-3′, which contain the restriction sites *Eco*RI and *Hin*dIII, respectively. The PCR product was digested and then cloned into the pMAL-c2X expression vector (New England BioLabs), yielding the plasmid pMALCyaC, which contained the intact *cyaC* gene fused to *malE*, thus encoding the MBP-CyaC fusion protein. CON1 cells harboring pMAL-c2X or pMALCyaC were grown in 100 ml of LB medium supplemented with 0.2% glucose to an optical density at 600 nm (OD_{600}) of 0.5, and then 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) was added to the medium. After 2 h, the bacterial cells were harvested by centrifugation and washed with 20 mM Tris-HCl (pH 7.4) buffer containing 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and 1 mM dithiothreitol and disrupted by sonication in 10 ml of the same buffer. The cell extract was centrifuged for 20 min at 14,000 rpm with a F0630 rotor in a Beckman GS-1R centrifuge (Beckman Instruments), and the supernatant was kept frozen for further AC assay.

AC was estimated by the two-column method described by Salomon and coworkers (40) by incubating aliquots of 50 μg of protein for 20 min at 30°C. For the optimum pH determination, combinations of buffers containing either 40 mM Tris, 20 mM morpholineethanesulfonic acid (MES), or 20 mM morpholinepropanesulfonic acid (MOPS) were adjusted to the desired pH and used during the incubation.

Construction of *cyaC*::*lacZ* transcriptional fusions. A promoterless *lacZ*-Km interposon (26) was inserted in both orientations into the coding regions of the putative *R. etli cyaC* gene harbored in plasmid p3310. Because this plasmid lacked a suitable single restriction site for the insertion of the interposon, a *Pst*I site was introduced by oligonucleotide-driven mutagenesis near the termination codon of *cyaC* according to the method of Ali and Steinkasserer (2). In the oligonucleotides used (5′-GGTTGCCTGCAGTTCGG-3′ and 5′-CCGAACTGCAGGCAACC-3′), the Ser 250 TCC codon was replaced with a Cys TGC codon. The resulting gene was sequenced to ensure that only the desired change had been introduced. Finally, the *lacZ*-Km interposon was inserted in this site in both orientations. These constructions were then subcloned into the mobilizable suicide vector pWS233. The resulting plasmids (pWSCyaCLac1 and pWSCyaCLac2) were conjugately transferred from the broad-host-range-mobilizing *E. coli* strain S17-1 (45) into *R. etli*. Double recombinant derivatives were obtained as described by Selbitschka and coworkers (42). The correct genotype of the resultant mutant strains was verified by Southern analysis.

Estimation of β-galactosidase activity. The β-galactosidase activity from samples (1 ml) of bacterial cultures was estimated as previously recommended (55). Specific activities are reported as nanomoles of *O*-nitrophenol produced per minute per microgram of culture protein. Activity values are the mean of three independent samples.

Expression of the *cyaC*::*lacZ* fusions in the presence of cumene hydroperoxide. The MIC of cumene hydroperoxide was determined by evaluating the growth of the wild-type CE3 strain at concentrations of this compound ranging from 50 to 600 μM. An inhibitory effect was observed when cumene hydroperoxide concentrations above 300 μM were used (data not shown). Cultures of *R. etli* strains growing exponentially in MM succinate plus ammonium chloride (150 ml) were challenged with 300 μM cumene hydroperoxide (Sigma). Samples of the cultures (1 ml) were taken at different times after the challenge, their β-galactosidase activity and protein content were estimated as previously described, and protein content was estimated as previously described.

Nodulation and nitrogen fixation assays. Sterile *Phaseolus vulgaris* cv. Negro Jamapa seedlings inoculated with the *R. etli* strains were grown in pots with vermiculite-Fahraeus medium, free of combined nitrogen. *R. etli* strains used as inocula were grown for 18 h in PY medium, washed twice with 100 mM $MgSO_4$, and diluted to an OD_{540} of 0.05. Groups of four plants were examined to determine nitrogenase activity by the acetylene reduction method at 24 days after inoculation (9).

Nucleotide sequence accession number. The nucleotide sequence of the plasmid p3310 insert was determined and deposited in GenBank under accession no. AF299113.

RESULTS

Isolation of the *R. etli cya* genes. To isolate the *cya* genes of *R. etli*, a cosmid library was introduced by conjugation in to an *E. coli* SP850 strain (Δ*cya*-1400::Km) (43). Four transconjugant colonies were selected based on their ability to grow on M9-lactose agar plates (*cya*$^{+}$ phenotype). To confirm the *cya*$^{+}$ phenotype conferred by the cosmids, they were isolated and introduced into another *E. coli cya* mutant strain, CON1. Transformants were tested for three unrelated cAMP-dependent traits: growth on MacConkey plates supplemented with different sugars, motility in swarm plates, and sensitivity to fosfomycin and serine (3, 14). All four cosmids were able to rescue the *cya* phenotype of the *E. coli* CON1 strain (data not shown), indicating that all of them encode at least one protein with AC activity. Restriction analyses confirmed that all cosmids were different (data not shown). In this communication, we present the characterization of one of them, which we named c934.

To localize the *cya* gene encoded in clone c934, pure cosmid was fragmented with different endonucleases, and all fragments were individually cloned into the pBluescriptIISK+ vector. Recombinant plasmids were transformed into the *E. coli* CON1 *cya* strain and spread onto MacConkey-lactose agar plates. Several red clones were selected, and one of them, p3310, which harbored the smallest insert (a 2.8-kb *Sal*I fragment), was selected for further analysis. Moreover, the complemented strain with p3310 was able to produce up to 40% of the cAMP level detected in the W3110 wild-type strain, demonstrating that this plasmid encodes a *cya* gene (Table 1). No cGMP accumulation could be detected in the complemented strain (data not shown). Hybridization experiments indicated that this gene is maintained as a single copy in the chromosome.

ORFC encodes an AC gene. Figure 1A shows a scheme of the three (ORFs) identified within the nucleotide sequence of the plasmid p3310 insert fragment: two of them are on the same DNA strand (ORFB and ORFC), while the third one was encoded in the opposite strand (ORFA). ORFA, which lays truncated at the 5′ end of the p3310 insert, encodes a polypeptide that shows weak similarity to proteins having methyltransferase activity. ORFB encodes a protein of 131 amino acid residues. The ORF was named *ohr*, given its significant similarity to the gene coding for the organic hydroperoxide resistance Ohr protein of *Xanthomonas campestris* (34). Similarity searches with the predicted product of ORFC against translated GenBank sequences revealed a high similarity to hypothetical genes that code for proteins of unknown function in the recently published genome sequences of the related bacteria *M. loti*, *S. meliloti*, and *Agrobacterium tumefaciens*. None of the three ORFs identified in p3310 showed similarity to any known AC.

To identify which of the ORFs in plasmid p3310 encodes an AC, an ΩKm cassette was inserted within ORFB (*ohr*) or within ORFC; no insertion into ORFA was performed, since it was naturally inactivated by truncation. Both insertions prevented the complementation of the *E. coli* CON1 strain by p3310, suggesting both ORFs are organized in a single transcriptional unit. A plasmid containing only *ohr* and its upstream region (pRKOhr) was unable to complement the *E. coli* *cya* phenotype, indicating that ORFC is the gene responsible for the complementation.

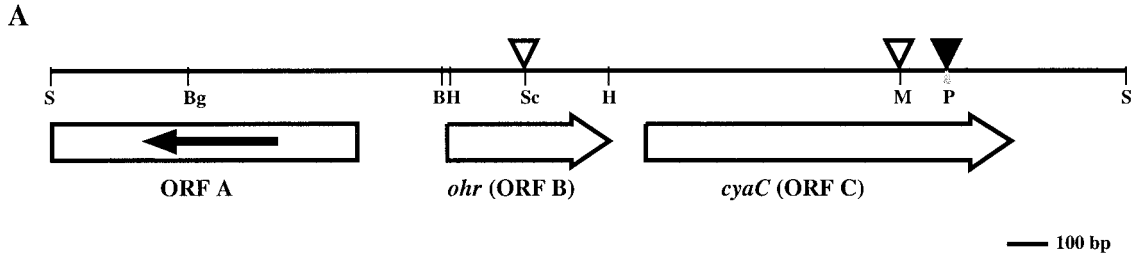
To further demonstrate that ORFC encodes an AC, a PCR product containing the coding region of ORFC downstream from a consensus *E. coli* ribosomal binding sequence was cloned into the expression vector pKK223-3 under the control of the P*tac* promoter, yielding plasmid pKKCyaC. This plasmid was able to complement the *E. coli* CON1 strain under both induced and noninduced conditions. This result indicates that ORFC encodes an AC and that the background expression level under noninduced conditions suffices to rescue the genetic defect of the *E. coli* CON1 strain. Accordingly, ORFC was designated *cyaC*.

The CyaC homologs were identified in the chromosome of *M. loti* (24), in the circular chromosome of *A. tumefaciens* (58), and in the *S. meliloti* symbiotic plasmid B (18). These sequences were highly similar to that of CyaC, as can be seen in Fig. 1B (42 to 45% identity). Conservation is especially evident over discrete regions, one of them being the sequence GEIDFGLHRQS (positions 288 to 298 in CyaC), which is almost identical to the ATP binding motif previously proposed by Peterkofsky and coworkers (39), suggesting that this region could be involved in ATP binding. None of five different computational prediction methods that were used to assess the existence of transmembrane elements in CyaC reported significant results, which suggests CyaC is cytoplasmic.

Catalytic properties of CyaC. To investigate the biochemical properties of CyaC, we overexpressed an MBP-CyaC fusion protein in the *E. coli* CON1 *cya* strain, and the AC activity was estimated in cell extracts (as described in Materials and Methods). Extract samples were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (8% polyacrylamide) and stained with Coomassie blue, and an induced band at the position corresponding to the predicted molecular weight of the MBP-CyaC fusion protein was visible (data not shown). No AC activity was detected in cell extracts from *E. coli* CON1 harboring the pMAL-c2X vector. The optimal pH for CyaC activity was 9.5. The initial velocity of cAMP production by MBP-CyaC was dependent on Mg-ATP concentration (data not shown), with an affinity (K_m) of 0.97 ± 0.08 mM and a maximal specific rate of 3.2 ± 0.14 nmol min^{-1} mg of $protein^{-1}$. GTP was found to be a competitive inhibitor for the ATP binding site. These data substantiate the identity of CyaC as an authentic AC.

Transcriptional analysis of *cyaC*. To determine the expression profile of *cyaC*, transcriptional fusions of this gene with *lacZ* were constructed and integrated into the *R. etli* CE3 chromosome by homologous marker exchange, as described in Materials and Methods, producing strain CON31 (*cyaC*::*lacZ*) and the control strain CON32, which harbors the fusion in inverse orientation. The β-galactosidase activities of strains CON31 and CON32 were estimated in MM supplemented with lactose, maltose, galactose, sucrose, glucose, pyruvate, succinate, acetate, or glutamine as the sole carbon source. The β-galactosidase activities of fusion strains CON31 and CON32 were low and indistinguishable from the endogenous activity of the wild-type strain, CE3, under all conditions tested. To further explore other conditions that might result in *cyaC* induction, strains CE3 and CON31 were cultured in MM supplemented with succinate and subjected to diverse stress conditions, such as heat shock (42°C for 2 min), osmotic shock (300 mM NaCl), or oxidative insult (200 μM hydrogen peroxide). The biomass yields and growth rates of both strains were similar in all treatments. The β-galactosidase activities of both strains were estimated at different times after exposure to stress conditions; no differences between the activities of cultures with treatment and those without treatment were found (data not shown).

We suggested above that *cyaC* forms an operon with *ohr*, the product of which has been shown to confer resistance to organic hydroperoxides in *X. campestris* (34). Furthermore, the expression of the *X. campestris ohr* gene is induced in the presence of organic hydroperoxides (34). In order to evaluate the expression profile of the *R. etli ohr-cyaC* operon, the β-galactosidase activities of mid-exponential cultures of the wild-type CE3 and the CON31 (*cyaC*::*lacZ*) strains were determined in the presence of 300 μM cumene hydroperoxide (Fig. 2). As expected, the β-galactosidase activity was induced in the CON31 strain culture, but not in the wild type. The response of



B

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M. loti          MPFGGRRRQRDDWVTSAARGRQFSSPDPIIGKPAQVNFEARSTSPSSSGM   50
S. meliloti      ---------------------------------------------------
A. tumefaciens   ---------------------------------------------------
R. etli          ---------------------------------------------------

M. loti          CYLLLMETAAASDPFVASLPVFAKFESVADIDNYRPLPDGWALATADIVG   100
S. meliloti      ------MVQPADREFYAGLPLFEAFEGVADEANYRPLPDGWWLAVADIVN   44
A. tumefaciens   -------MIAADEEFLTGLPVFRHFEDVADPALYRALPPGWGLAIADIVD   43
R. etli          ----------MSNSQPEPRRAYDEFSLVLDPDVVEPLPDDWLIGITDVVS   40

M. loti          STKAIEAGRYKTVNMAGASVISALLNALGRQDLPFVFGGDGALVAFPGSA   150
S. meliloti      STGAIAEGRYKSVNMAGASVISALMNGLDERNLAFVFGGDGALAAVPGTL   94
A. tumefaciens   STAAIGTGRYKAVNMAGAAVISGVSNSLGRHDLPFVFGGDGAAVAVPPHG   93
R. etli          STAAIRSGRYEDVNYAGASIIAALGNAWGSFDFPFVFRGDGAAFALPANG   90

M. loti          LEITRNALAAVQRWVADELDLTLRAAIVPIKDIRAQGLDVRVARFQASEA   200
S. meliloti      AAKARDVLAAAKTWVAEELGLELLRAAIVPVSDVRASGFDMRVARFKASEE   144
A. tumefaciens   LPVARTALSNVQRWVKDDLDLALMRVALVPVEDIRKNGFDIRVARFQASED   143
R. etli          IMAATSALRDVAGFAKAELHLDLLRVGLVTVGEIRATGRDVRIARYAASES   140

M. loti          VLYAMFAGGGGSWAEAEMKAGRYRIDPAPAGARPDLTGLSCRWNPIEARH   250
S. meliloti      VSYAMFSGGGASWAEAEMKAGRYQIEAAPPGTRPDLTGLSCRWNPIVSHH   194
A. tumefaciens   VSYAMFSGGGNSWAETRMKEGQYALPVAEAGERPDLTGLSCRWNPIPTNH   193
R. etli          ATYAMFAGGGLKWAEQQIKNGRFLVKPGRYAMRPDLTGLSCDWAPFPSQR   190

M. loti          GEIVSIIAIPGVSRDVRGFQFLASDIIALAGRQERDGHPVPVDGPRYSLL   300
S. meliloti      GAIVSIIAVPGERGIGPEYQALIGDIVALAEGEERGGHPVPEKGPEPHLS   244
A. tumefaciens   GKVVSIIAVPGPSRDMSAFRQLVIDLVDLAEQDARHGHPVPEDGPKLGFV   243
R. etli          GETLSLLVEPRDHTRPEVFAALARRVLAVFDAGPRRSHPLS----------   231

M. loti          PAGLDIEARAMAPVGRRW---LSKLWIVFLMTLTAVTDRYGWTIGRFDPK   347
S. meliloti      VRGITAESRAVAPRGRRF---LTWSFVAAQSLALFLCFRLGINFGPFDVK   291
A. tumefaciens   REGLGLEARAGAAYHDVWGKTRRSLRILGENLLVNFLGVTGLSLGRFSAA   293
R. etli          --GRTAMAREKQVSAKRW----------------------------------   247

M. loti          IYKRDVASNSDFRKFDDGLKMTIDVDADVLQRIENRLKQAEEAGICSYGL   397
S. meliloti      RYARDIASNSDFRKFDDALKMTIDVSLDRLGRIEERLKQGAAAGICRYGL   341
A. tumefaciens   HYRRSVASNTDFRKFDDGLKMTVDIDITRLEKIRSRLEAGRLSGSCYYGL   343
R. etli          -SK--VASSSDFRKFDDGLRLTLDDCAPEQIDSVEAMLVAARARGEIDFGL   294
                              *

M. loti          HRQKSALMTCLVASPLQRDHIHFIDGAAGGYAMAAASLKAKAPV------   441
S. meliloti      HRQDSALMTCIVPTPMSRDHMHFIDGAAGGYAVAARNLKAPLSGEDLLRT   391
A. tumefaciens   HEQDAALMTCIVPSPLSKDHMHFVDGADGGYAAAASRLKAQMQAAAAN--   391
R. etli          HRQSHALMTCLVPSGRPDSHLHFLDGMGGGYAKAAEMMEEGALEEVRQRA   344

M. loti          ---------                                            441
S. meliloti      GDISPAVKP                                            400
A. tumefaciens   ---------                                            391
R. etli          L--------                                            345
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FIG. 1. (A) Physical and genetic map of the insert in p3310. Open arrows indicate the positions of complete ORFs. The position of the incomplete ORF is indicated by an open rectangle, and the arrow located within indicates the direction of transcription. Open triangles indicate the positions at which Ω interposons were inserted. A black triangle indicates the position at which *lacZ*-Km cassettes were inserted. A small gray black triangle marked with letter P indicates the position where *Pst*I was generated. (B) Protein sequence alignment of the *R. etli* CyaC and the three homologous proteins found in the databases: *M. loti*, protein identification no. BAB50193.1; *S. meliloti*, protein identification no. CAC49159.1; *A. tumefaciens,* protein identification no. AAK88018.1; and *R. etli*, accession no. AF299113. Amino acid residues identical in at least three of the sequences are shown in open boxes, and conservative substitutions are shaded in gray. The asterisk indicates the position of a serine in CyaC, which was changed to cysteine by site-specific mutagenesis. The amino acid residues that potentially confirm an ATP binding site, as described by Peterkofsky and coworkers (35), are underlined.

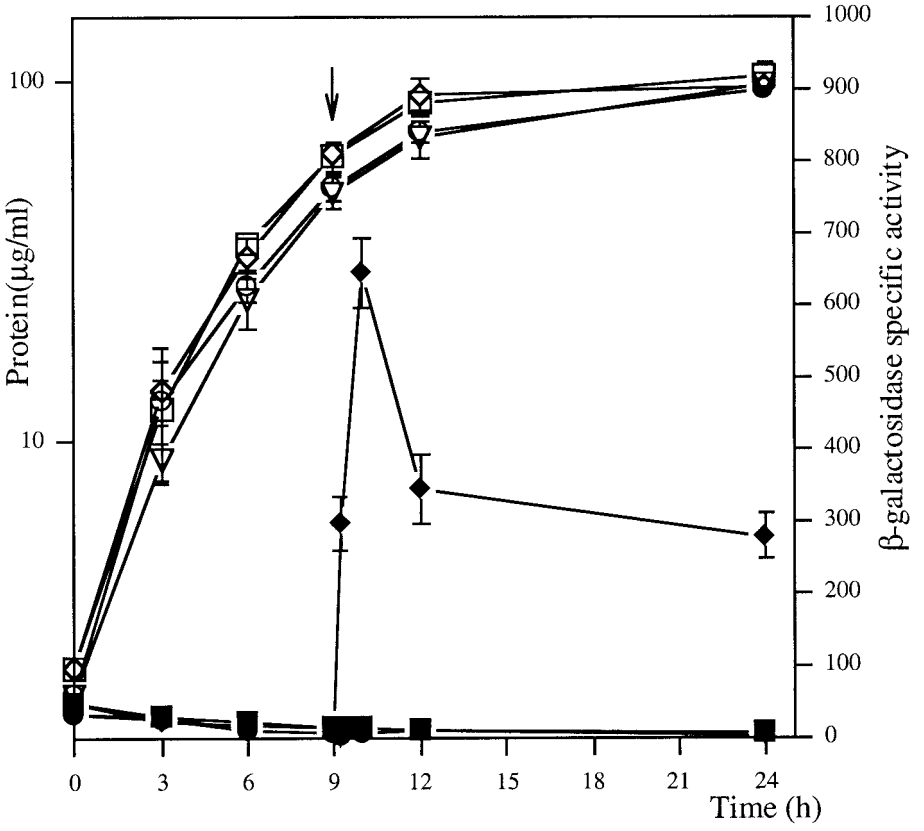

FIG. 2. Induction of the *cyaC-lacZ* fusion in the presence of 300 μM cumene hydroperoxide (CHP). Open symbols represent protein yields as a measure of growth: □, CE3; ○, CE3 with CHP; ▽, CON31; ◇, CON31 with CHP. Solid symbols represent specific β-galactosidase activities (nanomoles per minute per milligram of protein): ■, CE3; ●, CE3 with CHP; ▼, CON31; ◆, CON31 with CHP. The arrow marks the addition of CHP.

the *cyaC*::*lacZ* fusion to cumene hydroperoxide was very fast; 10 min after exposure, the β-galactosidase activity increased 5-fold and within 1 h had risen up to 10-fold. There was no significant difference between the growth rates of the cumene hydroperoxide-treated and control cultures.

Taken together, these data suggest that while *cyaC* is induced by cumene hydroperoxide, it does not seem to participate in the resistance phenotype against this compound. To assess the physiological role of Ohr-CyaC in *R. etli*, we evaluated the effect of different cumene hydroperoxide concentrations upon the growth rates of the wild-type CE3, CON31, and CON40, an *ohr* ΩKm mutant strain. No differences were found among the biomass yields and growth rates of all three strains when subjected to the same treatment (data not shown). Exposure to organic hydroperoxides during the *R. etli* life cycle might be limited, with the exception of nodule establishment, when the bacteria get contact with plant roots, which excrete hydrogen peroxide and organic hydroperoxides as a defense strategy against pathogens (7). The effect of bean plant root exudates upon *ohr-cyaC* operon expression was evaluated. Unexpectedly, no difference was found between the β-galactosidase activities of strain CON31 whether in the presence or absence of bean root exudates (data not shown).

Physiological role of *R. etli* CyaC. To assess the physiological role of CyaC in free-living *R. etli* cells, several approaches were undertaken. In the first place, the requirement of CyaC for the utilization of glycolytic (maltose, glucose, lactose, galactose, and sucrose) or nonglycolytic (pyruvate, succinate, acetate, and glutamine) carbon sources was evaluated. Strain CON31 grew in all of these carbon sources at the same rate as the wild-type strain, suggesting CyaC is dispensable for carbon source utilization, in contrast to *E. coli*, in which Cya is necessary for the utilization of carbon sources other than the preferred source, glucose (38; data not shown). In *E. coli*, Cya is also required for motility; however, when strain CON31 was tested in swarm plates, no differences were observed in comparison to the wild-type strain.

To assess if the symbiotic abilities of the *cyaC* mutant were affected, bean plant seedlings were inoculated with strain CON31 or the wild-type strain. After 24 days, nodule morphology and nitrogen-fixing activity were evaluated. No significant differences were found between the nodules induced by the mutant strains and those elicited by the wild-type strain nor between the nitrogenase activities of root systems inoculated with either strain (data not shown).

Succinate is the preferred carbon source for rhizobia and exerts catabolic repression over other carbon sources, including glucose (36). In the wild-type *R. etli* strain CE3, cAMP accumulation is similar, whether grown with succinate or glucose as the carbon source, suggesting cAMP levels are not involved in catabolic repression, in contrast to *E. coli*, in which cAMP levels mediate the catabolic repression of glucose (the

TABLE 2. cAMP levels in different strains of *E. coli* and *R. etli*

Strain[a]	Carbon source	cAMP levels (pmol/mg of protein)
E. coli		
W3110 (wild type)	Lactose	318.18 ± 32.3
CON1 (*cya* mutant)	Lactose	ND[b]
CON1(pRKACEc)	Lactose	650.25 ± 42.8
CON1(p3310)	Lactose	125.91 ± 13.1
R. etli		
CE3 (wild type)	Succinate	17.63 ± 1.9
	Glucose	22.14 ± 1.7
CON31 (*cyaC* mutant)	Succinate	17.15 ± 2.2
	Glucose	19.99 ± 1.5

[a] *E. coli* strains were grown in mm M9 supplemented with 1% lactose and 0.1% Casamino Acids. *R. etli* strains were grown in mm supplemented with succinate or glucose as carbon sources. The cAMP content was determined from samples of exponential growing cultures. Each value is the average of three independent experiments.
[b] ND, not detected.

preferred carbon source) over other carbon sources (Table 2). To estimate the role of CyaC on cAMP accumulation, strain CON31 was grown on MM supplemented either with succinate or glucose as the carbon source, and the cAMP content was determined. No differences were found between both carbon sources or between strain CON31 and the wild-type cAMP values, suggesting that CyaC is not required for cAMP synthesis under the conditions tested (Table 2).

DISCUSSION

ACs make up a large and complex family of proteins that can be classified into five classes according to their evolutionary relationships (11, 13, 46). Representatives of all five classes have been identified in bacteria. In this work, we present the isolation and characterization of a new class of AC, class VI, encoded by *cyaC* of *R. etli*. The *cyaC* gene was isolated by functional complementation of an *E. coli cya* mutant strain with a cosmid library of *R. etli* CE3. Several cosmids were isolated by this method: one of them encodes three ORFs. ORFA, which lays truncated at the 5′ end of the p3310 insert, encodes a polypeptide that shows weak similarity to proteins having methyltransferase activity. The other two ORFs are located in the same strand. The upstream ORF, named ORFB, encodes a protein that is 36% identical to Ohr of *X. campestris*, which is involved in the resistance to organic hydroperoxides (34). The downstream ORF, named ORFC, encodes a protein that has no significant similarity to other ACs or to signatures related to this broad family of proteins. In this work, we present evidence that ORFC encodes a novel AC. First, ORFC was able to rescue different nonrelated phenotypes of two different *E. coli cya* mutant strains. This ability was abolished when ORFC was disrupted by insertion. Second, ORFC restored the synthesis of cAMP in an *E. coli cya* mutant strain. Third, cell extracts of an *E. coli cya* mutant strain complemented with ORFC presented AC activity. For these reasons, ORFC was named "*cyaC*," which allowed us to propose its product, CyaC, as the first member of a new class of ACs, class VI.

The catalytic properties of class I AC from *E. coli* (59), class II AC from *Bordetella pertusis* (29), class III AC from *Mycobacterium tuberculosis* (20), and class IV AC from *A. hydrophila* (46) have been published. Here we report the preliminary characterization of the novel class VI AC activity as an MBP-CyaC fusion protein in cell extracts. Compared to members of other AC classes, MBP-CyaC showed a reduced specific activity, although this might be due to the lack of purity of our preparations. MBP-CyaC shares with the class IV AC from *A. hydrophila* the peculiarity of having an alkaline optimum pH of 9.5 (46), but not its thermophilic properties, since incubation at 40°C sharply decreased the MBP-CyaC activity (data not shown). GTP was found to be a competitive inhibitor for the MBP-CyaC AC activity; whether it is a substrate, it is yet to be characterized.

The carboxy-terminal region of CyaC is probably involved in its catalytic function. An S252C missense mutation abolished the CyaC complementation ability (data not shown). This position is invariant in the four homologous proteins mentioned above. Also, a region highly similar to the ATP binding site proposed by Peterkofsky and coworkers (39) is conserved in the carboxy-terminal region of CyaC and its homologs (Fig. 1). To date, six different classes of ACs have been described. The complexity of proteins with nucleotide cyclase activity suggests that they arose by convergent evolution rather than by divergence from an ancestral protein, as was previously described (13, 46).

The physiological role of cAMP in members of the family *Rhizobiaceae* remains elusive, despite the continuous efforts of several research groups. Genetic approaches have been hampered by the presence of multiple *cya* genes. The recently published genomic sequences of *M. loti* and *S. meliloti* allowed the identification of at least 12 and 26 different ACs/GCs, respectively (18, 24). The physiological relevance of harboring multiple nucleotide cyclases is poorly understood; however, it is possible to argue that the activity and expression of each one these cyclases might be regulated in response to specific external signals or to particular growth conditions, probably as a consequence of belonging to different signal transduction pathways. This complexity might be the reason we were not able to find a discernible phenotype for our *cyaC* mutant under any condition tested.

The expression of *cyaC* could only be detected in the presence of cumene hydroperoxide, which triggers a significant induction (approximately 10-fold) (Fig. 2). This result was not totally unexpected, since *cyaC* is cotranscribed with a gene named *ohr,* the product of which is highly similar to the Ohr protein of *X. campestris,* which is induced in the presence of organic hydroperoxide (34). Plants increase the production of oxygen-reactive species as part of their defense strategy against pathogens (7). Early nodule establishment stages can be considered similar to early phytopathogenesis. Although this assumption suggests that the transcription of the *ohr-cyaC* operon might be activated by bean root exudates, we found no evidence to support this assumption. Alternatively, we suggest that *ohr-cyaC* operon expression can be induced in response to the accumulation of oxygen-reactive species as a consequence of the higher respiratory rates required to sustain bacteroid metabolism. However, the symbiotic capacities of an *R. etli cyaC* mutant strain were not altered.

We were not able to find any discernible phenotype, either

for the *ohr* or for the *cyaC* mutant strains in the presence of cumene hydroperoxide. This might be due to the existence in *R. etli* of alternative detoxification mechanisms, as has been observed for *X. campestris* (34).

It has been shown recently that the *X. campestris ohr* gene is negatively regulated by the *ohrR* product (49). As for the *X. campestris ohr* gene, the *R. etli ohr-cyaC* operon did not respond to hydrogen peroxide, indicating that the transcription of these genes is independent of OxyR. We suggest that *R. etli* contains a specific regulator functionally similar to *ohrR* mediating the transcriptional response to organic peroxides. In fact, sequences highly similar to *ohrR* were found in the genome sequence of other members of the family *Rhizobiaceae* (data not shown).

The analysis of the whole genome sequences of three members of the family *Rhizobiaceae*: *M. loti* (24), *S. meliloti* (18), and *A. tumefaciens* (58) revealed the presence of genes of unknown function coding for CyaC homologs. In none of these cases is the *R. etli ohr-cyaC* genetic organization conserved. The presence of *cyaC* seems to be an exclusive trait of *Rhizobiaceae*, since no homologs could be identified in the genome sequences of other alpha proteobacteria, such as *Rhodobacter sphaeroides* and *Caulobacter crescentus.*

In enteric bacteria, such as *E. coli*, cAMP exerts its function while bound to the regulatory protein CRP. The CRP-cAMP complex is able to bind to specific DNA sequences and by this means positively or negatively regulate gene transcription of operons involved in functions such as catabolite repression (27). However, no CRP homologs can be detected in the genomes of *Rhizobiaceae*, and for this reason, the mechanism of how cAMP acts in this group of organisms remains unknown.

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