JOURNAL OF BACTERIOLOGY, Oct. 1991, p. 6265–6269
0021-9193/91/196265-05$02.00/0

Vol. 173, No. 19

Structural and Functional Relationships between *Pasteurella multocida* and Enterobacterial Adenylate Cyclases

MICHÈLE MOCK,[1] MARTINE CRASNIER,[2] EDITH DUFLOT,[1] VALÉRIE DUMAY,[2] AND ANTOINE DANCHIN[2]*

Unité des Antigènes Bactériens (Centre National de la Recherche Scientifique Unité Associée 557)[1] and Unité de Régulation de l'Expression Génétique (Centre National de la Recherche Scientifique Unité Associée 1129),[2] Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France

Received 6 May 1991/Accepted 19 July 1991

The *Pasteurella multocida* adenylate cyclase gene has been cloned and expressed in *Escherichia coli*. The primary structure of the protein (838 amino acids) deduced from the corresponding nucleotide sequence was compared with that of *E. coli*. The two enzymes have similar molecular sizes and, based on sequence conservation at the protein level, are likely to be organized in two functional domains: the amino-terminal catalytic domain and the carboxy-terminal regulatory domain. It was shown that *P. multocida* adenylate cyclase synthesizes increased levels of cyclic AMP in *E. coli* strains deficient in the catabolite gene activator protein compared with wild-type strains. This increase does not occur in strains deficient in both the catabolite gene activator protein and enzyme III-glucose, indicating that a protein similar to *E. coli* enzyme III-glucose is involved in the regulation of *P. multocida* adenylate cyclase. It also indicates that the underlying process leading to enterobacterial adenylate cyclase activation has been conserved through evolution.

Cyclic AMP (cAMP) in *Escherichia coli* is known to play a regulatory role in gene transcription via its receptor protein, catabolite gene activator protein (CAP) (25). Adenylate cyclase (AC) is the enzyme which converts ATP to cAMP. The AC structural gene (*cya*) of *E. coli* has been cloned and sequenced (1), and a model for the regulation of AC activity has been proposed. On the basis of genetic experiments, it was suggested that the phosphorylated form of enzyme III-glucose, a component of the phosphotransferase system, is an activator of AC (9, 17). When glucose transport takes place, the intracellular concentration of phosphorylated enzyme III-glucose decreases and correlates with a decrease in intracellular cAMP concentration. It has also been shown that the large increase of cAMP synthesis occurring in *crp* strains (deficient in CAP) (10) is dependent on the presence of enzyme III-glucose (4). In addition, gene deletion experiments have indicated that the carboxy-terminal domain of the protein is required for the regulation of AC activity by enzyme III-glucose (4, 20).

The *cya* gene of *Erwinia chrysanthemi*, another member of the family *Enterobacteriaceae*, has also been cloned and sequenced (5, 12). Comparison of the amino acid sequences of *E. coli* and *E. chrysanthemi* AC indicated that the proteins were very similar. The similarity was too large to permit significant identification of functional residues in the protein. This prompted us to sequence another gram-negative bacterium not closely related to *E. coli*.

In the present work, we have cloned and expressed in *E. coli* the *cya* gene of *Pasteurella multocida* from the family *Pasteurellaceae*, a gram-negative bacterium that is pathogenic for humans and animals (2). DNA sequencing data and genetic studies lead us to propose that *P. multocida* AC shares functional organization and regulatory properties with AC of the family *Enterobacteriaceae*.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The strains used in this work were *E. coli* K-12 derivatives (Table 1). Plasmid pSa206T was a derivative of plasmid pSa206, a low-copy-number plasmid (3). pSa206T was obtained by replacing the kanamycin resistance gene of pSa206 by the tetracycline resistance gene. The growth medium was either Luria broth or minimal medium M63 (14) supplemented with the required amino acids (1 mM each), thiamine (5 $\mu g \cdot ml^{-1}$), and different carbon sources (0.4% each). Transductions using P1*vir* were performed as described by Miller (14). When required, ampicillin, chloramphenicol, and tetracycline were added at 100, 40, and 2 μg/ml, respectively.

Cloning and nucleotide sequence analysis. Genomic DNA from *P. multocida* CNP1 (NCTC 10322) (6) was kindly provided by F. Escande. After partial digestion with *Sau*3A, the DNA fragments, in the 2- to 10-kb range, were cloned into the unique *Bam*HI site of plasmid pBR322. Nucleotide sequence analysis was performed by using subclones in the single-stranded phage vector M13mp19 (15). Unidirectional deletions were generated by using the Cyclone system (IBI) as recommended by the manufacturer. Nucleotide sequence was determined by the dideoxynucleotide chain termination method (22) when using *Pol*Ik or by a modified dideoxynucleotide chain termination method when using Sequénase (23). Restriction enzymes, T4 DNA ligase, and *Pol*Ik were from Boehringer-Mannheim. Modified T7 DNA polymerase (Sequenase) was from USBC. Oligodeoxyribonucleotides used as primers in DNA sequencing were purchased from Pharmacia. Sequence analysis was performed using the facilities of the Unité d'Informatique Scientifique of the Pasteur Institute.

Analysis of plasmid-encoded proteins. Minicells of strain AR1062 were purified as described by Rambach and Hogness (18). Plasmid-encoded proteins labeled with [^{35}S]methionine were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions (13).

TABLE 1. *E. coli* strains

Strain	Genotype	Source or reference
TP610	F⁻ *thi-1 thr-1 leuB6 pro lacY1 tonA21 supE44 hsdR hsdM recBC lop-11 lig⁺ cya-610*	12
TP9500	F⁻ *xyl argH1 Δcya*	4
TP2339	F⁻ *xyl argH1 Δcya Δcrp-39 ΔlacX74*	21
TP9510	F⁻ *xyl argH1 ilvA Δcrp-39 Δcrr* Km^r	4
TP9512[a]	F⁻ *xyl argH1 Δcya Δcrp-39 Δcrr* Km^r	TP9510 × P1 (TP9500 *ilv*⁺)

[a] Bacterial strain derived during this work.

cAMP assays. The excretion of cAMP by bacteria was analyzed on maltose MacConkey plates by using strain TP610A as an indicator bacterium (4). Strain TP610A is a spontaneous mutant of strain TP610 producing red colonies on maltose MacConkey agar when supplemented with a small amount of cAMP. A drop of an overnight culture of the strain to be analyzed was plated on a lawn of TP610A and then incubated at 37°C overnight. Strains excreting significant levels of cAMP produced a red halo on the plate around the culture drop because of the fermentation of maltose by the indicator bacterium. cAMP production was quantified by a radioimmunological assay (11) with cultures grown in minimal medium M63. Anti-cAMP antibodies were kindly provided by Agnès Ullmann. The amount of cAMP, including intracellular cAMP and cAMP excreted into the culture medium, was expressed in picomoles per milligram (dry weight) of bacteria. The background level was <5 pmol/mg. Total cAMP per milligram (dry weight) of bacteria was constant during the exponential phase of growth. The values are the means of three determinations.

Nucleotide sequence accession number. The *P. multocida* *cya* sequence has been assigned GenBank accession number M68901.

RESULTS AND DISCUSSION

Cloning of *P. multocida* AC (*cya*) gene. We transformed an *E. coli* Δ*cya* mutant (TP610) with a plasmid library of *P. multocida* chromosomal DNA and screened for Cya⁺ clones on MacConkey maltose plates. Of several thousand transformants a few Mal⁺ clones were found in which the *cya* deficiency of the recipient was complemented. To confirm that the phenotype was due to cAMP production, the variations in the amount of cAMP per milligram (dry weight) of bacteria in cells grown with pyruvate were measured. The cAMP values ranged between 35 and 2,000 pmol/mg (dry weight) of bacteria. The plasmid DNAs of the recombinants were then analyzed. Restriction site analysis of four plasmids indicated that they all had in common a 0.7-kb *Hin*dIII-*Eco*RI fragment (Fig. 1).

Nucleotide sequence of the *cya* gene and deduced amino acid sequence of its gene product. The 0.7-kb *Hin*dIII-*Eco*RI fragments of two plasmids, pPMA140 and pPMA150 (Fig. 1), were sequenced and were shown to be identical. The nucleotide sequence contained an open reading frame which was incomplete in that it lacked a termination codon. A complete open reading frame sequence was obtained after cloning a 4-kb *Hin*dIII fragment (Fig. 1) in plasmid pBR322 yielding plasmid pPMA155. The pyruvate-grown cells carrying pPMA155 produced more cAMP (20,000 pmol/mg) than cells carrying pPMA150 (2,000 pmol/mg) or pPMA140 (35 pmol/mg). The nucleotide sequence of 2.8 kb of the 4-kb *Hin*dIII insert, together with its deduced amino acid sequence, are presented in Fig. 2. The coding region was 838 codons in length. The deduced molecular size of 92 kDa was in agreement with the size of the protein synthesized in minicells containing plasmid pPMA155 (data not shown). The presumed start codon is TTG, a feature in common with the AC structural genes of *E. coli* (21) and *E. chrysanthemi* (5).

Comparison of the primary structures of *P. multocida* and enterobacterial ACs. As shown in Fig. 3, the *P. multocida* protein shares several regions of identity with *E. coli* and *E. chrysanthemi* ACs. There are 325 identical amino acid residues in the three proteins, and an additional 139 residues are conservative replacements. The lengths of the proteins are very similar (838 residues for *P. multocida*, 848 for *E. coli*, and 851 for *E. chrysanthemi*), and very few insertions or deletions are necessary to produce the best alignment. In general, deletions and insertions are in regions that are likely to be folded into loops of variable length (as seen from the presence of proline and glycine residues in their immediate vicinity). The 23 C-terminal residues of the *E. coli* protein, which are completely different from those of the *E. chrysanthemi* counterpart, had been thought to be dispensable (5); they are replaced by a set of only 5 residues in *P. multocida*, thus substantiating this hypothesis. Regions of identity are clustered into four major and several minor groups, suggesting a modular organization of the protein.

The main regions of divergence are located near the NH_2 terminus of the protein and in the region of residues 510 to 550. The former region cannot at present be related to a specific function of the protein, whereas the latter is a region that can be considered a hinge joining the catalytic and the regulatory domains of the protein (20). Another feature of these proteins is the high amount of cysteine and histidine residues. This may be related to a metal requirement for activity or some yet uncovered regulatory process. *P. multocida* AC also shares with the enterobacterial enzymes a common regulatory pathway affecting gene expression. The start codon of the three genes is the unusual UUG codon. The same observation was also made in the case of the enterobacterial species *Salmonella typhimurium*, from which the nucleotide sequence of the *cya* gene transcription regulatory region has been determined (8, 24). In the case of the *E. coli* *cya* gene, replacement of the UUG initiation codon by GUG or AUG has shown that the UUG codon has the lowest efficiency of translation (19). It was therefore proposed that the UUG codon provides a mechanism for limiting *cya* expression.

Regulation of *P. multocida* AC activity in *E. coli*. The CAP-dependent activation of *E. coli* AC leading to the synthesis of a large amount of cAMP is easily visualized on MacConkey plates (see Materials and Methods). This cAMP

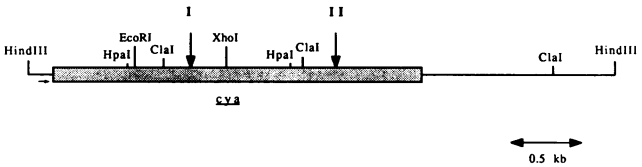
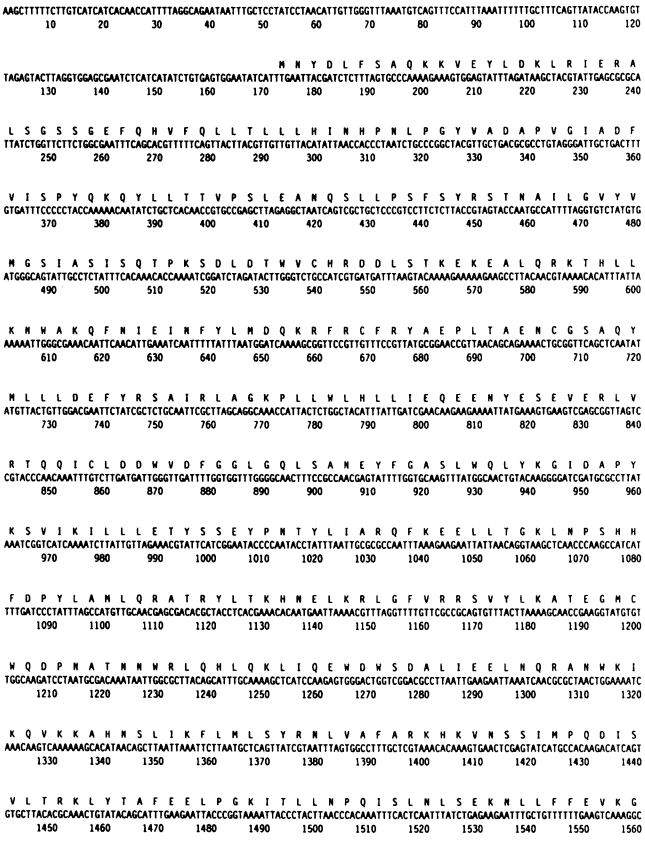


FIG. 1. Partial restriction map of the *Hin*dIII fragment encoding the *cya* gene of *P. multocida*. The filled box represents the coding region of the *cya* gene. The vertical arrows indicate the 3′ end of the truncated forms of the *cya* gene carried by plasmid pPMA140 (I) and plasmid pPMA150 (II).



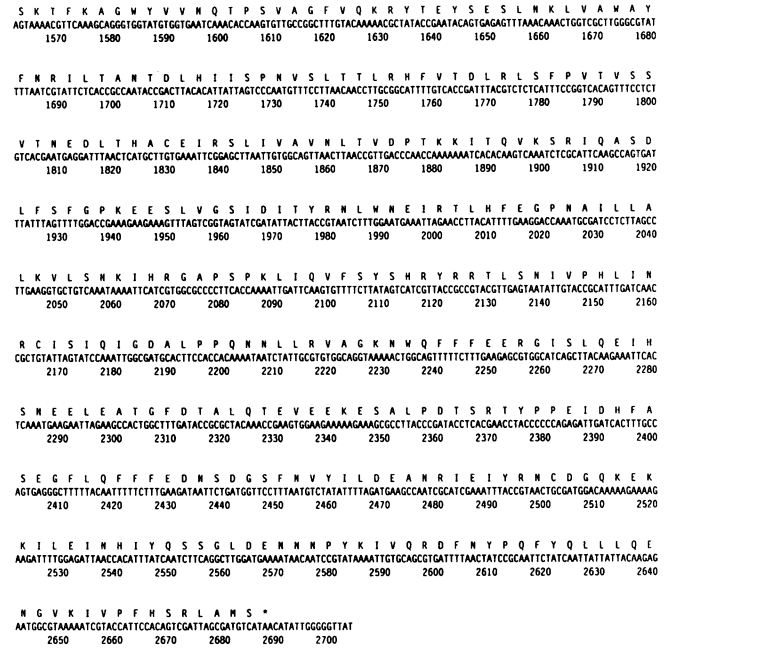


FIG. 2. Nucleotide sequence of the *P. multocida cya* gene and deduced amino acid sequence of the AC. The numbering of the nucleotides starts at the *Hin*dIII restriction site.

assay evaluation was used to analyze the production of cAMP by *P. multocida* AC in the *E. coli crp* strain.

The 4-kb *Hin*dIII fragment (Fig. 1) was inserted into *Hin*dIII-digested pSA206, a low-copy-number plasmid (3), to give pDIA1955. This plasmid was used to transform a Δ*cya* strain (TP9500) and a Δ*cya* Δ*crp* strain (TP2339). The secretion of cAMP by the Δ*cya* Δ*crp* strain containing the cya_{PM} plasmid (pDIA1955) was analyzed on maltose MacConkey plates and compared with that of the same strain containing the cya_{EC} plasmid (pDIA1900), which carries the *cya* gene of *E. coli* (4). cAMP was excreted by both strains, as visualized by the red halos, but a smaller halo was observed with the strain containing the cya_{PM} plasmid (pDIA1955). No halo was observed when the same experiment was carried out with a Δ*cya* Δ*crp* Δ*crr* strain (TP9512) deficient in AC, CAP, and enzyme III-glucose and containing the cya_{EC} or cya_{PM} plasmid. These results indicate that *P. multocida* AC activity in *E. coli crp* strains is dependent on the presence of *E. coli* enzyme III-glucose. In order to quantify cAMP production, the Δ*cya* Δ*crp* strain containing either the cya_{PM} plasmid or the cya_{EC} plasmid was grown on pyruvate as the sole carbon source and total cAMP production during exponential growth was measured. The levels of cAMP thus obtained were compared with those obtained with the Δ*cya* strain containing the same plasmids (Table 2). When the Δ*cya* Δ*crp* strain was used as the host cell, higher levels of cAMP were observed with both plasmids. The level of cAMP obtained with the Δ*cya* Δ*crp* strain containing the cya_{PM} plasmid was lower than that obtained with the same strain containing the cya_{EC} plasmid, a result in agreement with the cAMP secretion level observed on MacConkey plates. With the Δ*cya* Δ*crp* Δ*crr* strain containing pDIA1956, a derivative of pSa206T containing the *P. multocida cya* gene, the level of cAMP was very low. It may be argued that the increase of cAMP synthesis in the *crp* strain (about fourfold) could be accounted for by an elevated level of expression of AC (the transcription of the *cya* gene of *E. coli* has been shown to be negatively regulated by CAP-cAMP). However, the fact that the Δ*cya* Δ*crp* and Δ*cya* Δ*crp* Δ*crr* strains expressing the *P. multocida cya* gene produced different levels of cAMP demonstrated that enzyme III-glucose is involved in the activation of *P. multocida* AC.

When glucose was used as the carbon source, the level of cAMP of the Δ*cya* strain containing the cya_{PM} plasmid was lower (550 pmol/mg) than that obtained with pyruvate as the sole carbon source (about an eightfold increase) and very similar to that obtained with the Δ*cya* strain containing the cya_{EC} plasmid (400 pmol/mg). Thus, *P. multocida* AC activity (like *E. coli* AC) was inhibited during the glucose transport, as proposed by Postma (16), i.e., because of a decrease of the intracellular concentration of phosphorylated enzyme III-glucose.

Different C-terminal truncated forms of *E. coli* AC have been shown to retain enzymatic activity (20) but have lost the regulation leading to the high production of cAMP in *crp* strains (4). On the basis of these experiments, it has been proposed that *E. coli* AC is composed of two functional domains: the amino-terminal catalytic domain and the carboxy-terminal regulatory domain (20), the latter being required for both the glucose effect and the CAP-dependent activation process (4). In the case of *P. multocida* AC, both truncated forms were active (as shown by the complementation experiments) but no cAMP excretion was obtained on MacConkey plates with the Δ*cya* Δ*crp* strain containing either pPMA140 or pPMA150 carrying truncated genes (Fig. 1). When the minicell-producing cell AR1062 was transformed with plasmid pPMA140, a polypeptide of the ex-

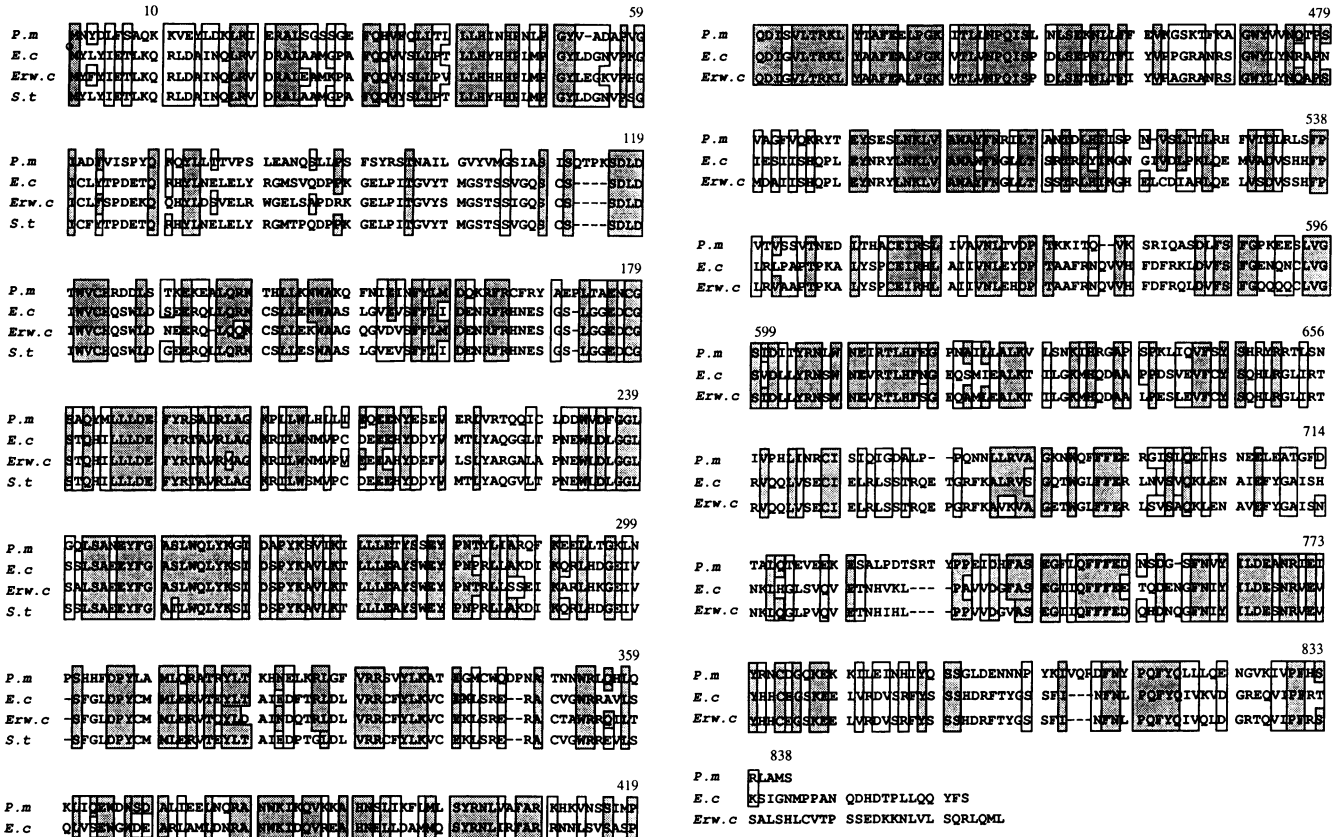


FIG. 3. Comparison of the amino acid sequences of *P. multocida* (P.m), *E. coli* (E.c), *E. chrysanthemi* (Erw.c) and *S. typhimirium* (S.t, first 412 residues only) adenylate cyclases. Identities are indicated in darkly shaded boxes and conservative amino acid changes are shown in lightly shaded boxes. Numbers refer to *P. multocida* AC residues.

pected size (35 kDa) was detected (data not shown). It therefore appears that the activity of the truncated 35-kDa polypeptide corresponding to the N-terminal part of *P. multocida* AC is not activated in the presence of enzyme III-glucose. It can then be proposed that *E. coli* and *P. multocida* AC belong to a class of proteins organized in functional domains. It is worth noting that different point mutations affecting the regulation of *E. coli* AC activity by enzyme III-glucose (4) were located in highly conserved regions of the COOH-terminal part of *P. multocida* AC.

Another class of bacterial AC includes the calmodulin-dependent AC toxins secreted by two pathogens, *Bordetella pertussis* and *Bacillus anthracis*. Although these ACs are quite different and are produced by taxonomically distinct organisms, they are antigenically related to each other and share regions of striking similarity (7). Since *P. multocida* is a toxigenic pathogen, we also investigated the possible effect of calmodulin on its AC. The enzymatic activity appeared to be insensitive to calmodulin. Therefore, the *B. pertussis* and *B. anthracis* ACs remain the only known examples of prokaryotic enzymes activated by eukaryotic protein. In conclusion, *P. multocida* AC clearly belongs to a class which was, until the present work, believed to be limited to enterobacterial ACs. Further experiments will be required to establish the presence of a phosphotransferase system in *P. multocida* as suggested by our data.

TABLE 2. cAMP levels in strains containing different plasmids

Strain (genotype)	Total cAMP [pmol/mg (dry wt) of bacteria] with plasmid:	
	pDIA1900 (*E. coli*)	pDIA1955 (or pDIA1956) (*P. multocida*)
TP9500 (Δ*cya*)	1,000	4,500
TP2339 (Δ*cya* Δ*crp*)	32,000	17,000
TP9512 (Δ*cya* Δ*crp* Δ*crr*)	400	300

ACKNOWLEDGMENTS

We are grateful to Agnès Ullmann for constructive suggestions and to Tony Pugsley for correcting the manuscript.

REFERENCES

1. **Aiba, H., K. Mori, M. Tanaka, T. Ooi, A. Roy, and A. Danchin.** 1984. The complete nucleotide sequence of the adenylate cyclase gene of *Escherichia coli*. Nucleic Acids Res. **12**:9427–9440.
2. **Carter, G. R.** 1984. Genus 1. *Pasteurella* Trevisan 1887, 94,[AL] Nom. cons. Opin. 13, Jud. Comm. 1954, 153, p. 552–557. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
3. **Close, T. J., D. Zaitlin, and C. Kado.** 1984. Design and development of amplifiable broad-host-range cloning vectors: analysis of the *vir* region of *Agrobacterium tumefaciens* plasmid pTIC58. Plasmid **12**:111–118.
4. **Crasnier, M., and A. Danchin.** 1990. Characterization of *Escherichia coli* adenylate cyclase mutants with modified regulation.

J. Gen. Microbiol. **136**:1825–1831.
5. **Danchin, A., and G. Lenzen.** 1988. Structure and evolution of bacterial adenylate cyclase: comparison between *Escherichia coli* and *Erwinia chrysanthemi*. Second Messengers and Phosphoproteins **12**:7–28.
6. **Escande, E., F. Grimont, P. A. D. Grimont, and H. Bercovier.** 1984. Deoxyribonucleic acid relatedness among strains of *Actinobacillus* spp. and *Pasteurella ureae*. Int. J. Syst. Bacteriol. **34**:309–315.
7. **Escuyer, V., E. Duflot, O. Sezer, A. Danchin, and M. Mock.** 1988. Structural homology between virulence-associated bacterial adenylate cyclases. Gene **71**:293–298.
8. **Fandl, J. P., L. K. Thorner, and S. W. Artz.** 1990. Mutations that affect transcription and cyclic AMP-CRP regulation of the adenylate cyclase gene (*cya*) of *Salmonella typhimurium*. Genetics **125**:719–727.
9. **Feucht, B. U., and M. H. Saier, Jr.** 1980. Fine control of adenylate cyclase by the phosphoenolpyruvate sugar phosphotransferase systems in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **141**:603–610.
10. **Fraser, A. D. E., and H. Yamazaki.** 1978. Construction of an *Escherichia coli* strain which excretes abnormally large amounts of adenosine 3′-5′ cyclic monophosphate. Can. J. Microbiol. **24**:1423–1425.
11. **Guidi-Rontani, C., A. Danchin, and A. Ullmann.** 1981. Isolation and characterization of an *Escherichia coli* mutant affected in the regulation of adenylate cyclase. J. Bacteriol. **148**:753–761.
12. **Hedegaard, L., and A. Danchin.** 1985. The *cya* gene region of *Erwinia chrysanthemi* B374: organisation and gene products. Mol. Gen. Genet. **201**:38–42.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Norrander, S., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26**:101–106.
16. **Postma, P. W.** 1982. Regulation of sugar transport in *Salmonella typhimurium*. Ann. Microbiol. **133A**:261–267.
17. **Postma, P. W., A. Schuitema, and C. Kwa.** 1981. Regulation of methyl-β-galactoside permease activity in *pts* and *crr* mutants of *Salmonella typhimurium*. Mol. Gen. Genet. **181**:448–453.
18. **Rambach, A., and D. S. Hogness.** 1977. Translation of *Drosophila melanogaster* sequences in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **74**:5041–5045.
19. **Reddy, P., A. Peterkofsky, and K. McKenney.** 1985. Translational efficiency of the *Escherichia coli* adenylate cyclase gene: mutating the UUG initiation codon to GUG or AUG results in increased gene expression. Proc. Natl. Acad. Sci. USA **82**: 5656–5660.
20. **Roy, A., A. Danchin, E. Joseph, and A. Ullmann.** 1983. Two functional domains in adenylate cyclase of *Escherichia coli*. J. Mol. Biol. **165**:197–202.
21. **Roy, A., C. Haziza, and A. Danchin.** 1983. Regulation of adenylate cyclase synthesis in *Escherichia coli*: nucleotide sequence of the control region. EMBO J. **2**:791–797.
22. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
23. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA **84**:4767–4771.
24. **Thorner, L. K., J. P. Fandl, and S. W. Artz.** 1990. Analysis of sequence elements important for expression and regulation of the adenylate cyclase gene of *Salmonella typhimurium*. Genetics **125**:709–717.
25. **Zubay, G., D. Schwartz, and J. Beckwith.** 1970. Mechanism of activation of catabolite-sensitive genes: a positive control system. Proc. Natl. Acad. Sci. USA **66**:104–110.