Structural and Functional Relationships between Pasteurella multocida and Enterobacterial Adenylate Cyclases

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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.

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 μg·ml⁻¹), and different carbon sources (0.4% each). Transductions using P1vir 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 Sau3A, the DNA fragments, in the 2- to 10-kb range, were cloned into the unique BamHI 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 dideoxyxynucleotide chain termination method (22) when using Polk or by a modified dideoxyxynucleotide chain termination method when using Sequenase (23). Restriction enzymes, T4 DNA ligase, and Polk were from Boehringer-Mannheim. Modified T7 DNA polymerase (Sequenase) was from USB. Oligodeoxynucleotide primers 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 [³⁵S]methionine were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions (13).
TABLE 1. E. coli strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP610</td>
<td>F&quot; thi-l thr-l leuB6 pro lacY1 tonA21 supE44 hsdR hsdM recBC lop-11 lig&quot; cya-510</td>
<td>12</td>
</tr>
<tr>
<td>TP9500</td>
<td>F&quot; xyl argH1 ΔcyA</td>
<td>4</td>
</tr>
<tr>
<td>TP2339</td>
<td>F&quot; xyl argH1 ΔcyA Δcrp-39 ΔlacX74</td>
<td>21</td>
</tr>
<tr>
<td>TP9510</td>
<td>F&quot; xyl argH1 lva Δcrp-39 Δacr Km</td>
<td>4</td>
</tr>
<tr>
<td>TP9512</td>
<td>F&quot; xyl argH1 ΔcyA Δcrp-39 Δacr Km</td>
<td>TP9510 × P1 (TP9500 lvi+)</td>
</tr>
</tbody>
</table>

* Bacterial strain derived during this work.

**RESULTS AND DISCUSSION**

Cloning of *P. multocida* AC (cya) gene. We transformed an *E. coli ΔcyA* 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 *HindIII-EcoRI* fragment (Fig. 1).

**Nucleotide sequence of the cya gene and deduced amino acid sequence of its gene product.** The 0.7-kb *HindIII-EcoRI* 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 *HindIII* 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 *HindIII* 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 mini-cells 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 NH2 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 mg). The nucleotide sequence of 2.8 kb of the 4-kb *HindIII* 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 mini-cells 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).

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![FIG. 1. Partial restriction map of the HindIII 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).]
assay evaluation was used to analyze the production of cAMP by *P. multocida* AC in the *E. coli* crp strain.

The 4-kb HindIII fragment (Fig. 1) was inserted into HindIII-digested pSA206, a low-copy-number plasmid (3), to give pDIA1955. This plasmid was used to transform a Δcyd strain (TP5900) and a Δcrp strain (TP2339). The secretion of cAMP by the Δcrp strain containing the Δcrp plasmid (pDIA1955) was analyzed on maltose MacConkey plates and compared with that of the same strain containing the Δcyd plasmid (pDIA1900), which carries the cyd 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 Δcyd plasmid (pDIA1955). No halo was observed when the same experiment was carried out with a Δcyd Δcrp Δcrr strain (TP9512) deficient in AC, CAP, and enzyme III-glucose and containing the Δcyd 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 Δcyd Δcrp Δcrr strain containing either the Δcyd plasmid or the Δcyd 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 Δcyd strain containing the same plasmids (Table 2). When the Δcyd Δcrp strain was used as the host cell, higher levels of cAMP were observed with both plasmids. The level of cAMP obtained with the Δcyd Δcrp strain containing the Δcyd plasmid was lower than that obtained with the same strain containing the Δcyd plasmid, a result in agreement with the cAMP secretion level observed on MacConkey plates. With the Δcyd Δcrp Δcrr strain containing pDIA1956, a derivative of pSa206T containing the *P. multocida* cyd 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 cyd gene of *E. coli* has been shown to be negatively regulated by CAP-cAMP). However, the fact that the Δcyd Δcrp and Δcyd Δcrp Δcrr strains expressing the *P. multocida* cyd 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 Δcyd strain containing the Δcyd 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 Δcyd strain containing the Δcyd plasmid (400 pmol/mg). Thus, *P. multocida* AC activity (like *E. coli* AC) is 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 Δcyd Δcrp strain containing either pPM140 or pPM150 carrying truncated genes (Fig. 1). When the minicell-producing cell AR1062 was transformed with plasmid pPM140, a polypeptide of the ex-
expected 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, \textit{Bordetella pertussis} and \textit{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

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
<th>Total cAMP [pmol/mg (dry wt) of bacteria] with plasmid:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pDIA1900 (E. coli)</td>
</tr>
<tr>
<td>TP9500 (( \Delta cyt ))</td>
<td>1,000</td>
</tr>
<tr>
<td>TP2339 (( \Delta cyt \Delta cpr ))</td>
<td>32,000</td>
</tr>
<tr>
<td>TP9512 (( \Delta cyt \Delta cpr \Delta crr ))</td>
<td>400</td>
</tr>
</tbody>
</table>

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REFERENCES


