Rhizobium meliloti Adenylate Cyclase Is Related to Eucaryotic Adenylate and Guanylate Cyclases

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A gene from Rhizobium meliloti coding for an adenylate cyclase was sequenced, and the deduced protein sequence was compared with those of other known adenylate cyclases. No similarity could be detected with the procaryotic counterparts. However, striking similarity was found with the catalytic region of Saccharomyces cerevisiae adenylate cyclase, the cytoplasmic domains of bovine adenylate cyclase, and two mammalian guanylate cyclases. The gene was fused to the enteric β-galactosidase, and the chimeric protein was purified by affinity chromatography. This fusion protein was found to direct the synthesis of cyclic AMP in vitro. This activity was strongly inhibited by the presence of GTP, but no cyclic GMP synthesis could be detected in conditions permitting cyclic AMP synthesis.

Rhizobia are gram-negative bacteria that are able to enter symbiotic association with legume plants, leading to the formation of N2-fixing root nodules. This process involves differential gene expression between the host plant and bacteria, but the signals involved in the modulation of gene expression are not well understood. Cyclic AMP (cAMP) might play a role in regulating metabolism of rhizobia. Indeed, in Bradyrhizobium japonicum, it has been shown that synthesis of glutamate synthetase and glutamate dehydrogenase is repressed by cAMP. Genes presumably encoding an adenylate cyclase were isolated from both B. japonicum (9) and Rhizobium meliloti (12). The gene cloned from R. meliloti only weakly complemented an Escherichia coli strain that was defective in cAMP production (12). It was therefore necessary to identify the putative cya gene product as an authentic adenylate cyclase. This prompted us to determine the nucleotide sequence of the gene and to purify the corresponding gene product for enzymological assessment. In particular, it was important to assay whether the protein could synthesize cyclic GMP (cGMP) in addition to cAMP.

Previous experiments with the maxicell technique had suggested that the region encompassing the R. meliloti cya gene could direct synthesis of two proteins (18). To identify the translational product that was endowed with adenylate cyclase activity, a gene fusion with the E. coli lacZ gene was performed, and the corresponding β-galactosidase hybrid protein was purified.

A new class of phylogenetically related eucaryotic enzymes directing synthesis of cAMP and cGMP was recently discovered (4, 11, 13, 14, 35, 37). It was therefore of interest to investigate whether the R. meliloti enzyme had any relationship with this new class. As demonstrated below, this was indeed the case; the R. meliloti enzyme, although able to recognize GTP, was not able to synthesize cGMP but could synthesize cAMP at the expense of ATP.

This indicates either horizontal gene exchange or, more probably, that synthesis of cyclic nucleotides was already present very early in evolution before the separation between eucaryotes and procaryotes.

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MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli strains and plasmid vectors are described in Table 1. Strain TP610A was selected as a derivative of strain TP610 on maltose plates in the presence of 50 μM cAMP. Plasmid pCU5 (18), which can complement E. coli strains that are defective in cAMP synthesis, was used as a source of the R. meliloti cya gene for DNA sequencing and for creating a gene fusion to the lacZ gene of E. coli. Plasmid pMC1403 (3) provided a cartridge containing the enteric β-galactosidase gene truncated at its 5' end. Plasmid pDIA7010 was created during our work by cloning the β-galactosidase gene from pMC1403 into pCU5.

Growth media. M63 minimal medium was prepared as described by Miller (25) and supplemented with the appropriate carbon source (0.4%) and with amino acids (40 μg/ml) as required. MacConkey medium supplemented with the appropriate carbon source (1%) was used as an indicator medium for sugar utilization. Ampicillin was used at 50 μg/ml (final concentration).

Genetic procedures. Plasmid preparations, restriction enzyme digestions, and ligations were performed as described by Maniatis et al. (21). Competent bacterial cells were prepared by the technique of Chung and Miller (5).

Nucleotide sequencing. Standard methods for DNA sequencing by using dideoxy sequencing (32) and M13 single-stranded phage (24) were employed. Sequential deletions of the M13 clones were generated by using DNase I as described by Lin et al. (19). The sequences of both strands were determined independently in our two laboratories. The DNA sequence information was analyzed in Cork by using the Microgenie sequence analysis program (Beckman Instruments, Inc., Fullerton, Calif.). The data bank was the Genbank compilation of DNA and protein sequences (National Institutes of Health, Bethesda, Md.). In Paris, sequences were analyzed by using the Institut Pasteur software designed by the Unité d’Informatique Scientifique.

Hybrid adenylate cyclase-β-galactosidase protein purification and β-galactosidase assays. Bacteria were disrupted by sonication in the presence of 2 M NaCl added to a 20 mM Tris hydrochloride-10 mM MgCl2 buffer (pH 7.4) (6). The purification of the hybrid protein by affinity chromatography on TPEG (thiophenylethyl-β-D-galactoside)-Sepharose was...
TABLE 1. Bacteria and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant features</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli TP610</td>
<td>F- thi-l thr-l leuB6 pro lacY1 tonA2I supE44 hsdR hsdM recBC lop-11</td>
<td>10</td>
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<tr>
<td></td>
<td>in lacY id-60</td>
<td></td>
</tr>
<tr>
<td>TP610A</td>
<td>As TP610 carrying a mutation</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>permitting growth on maltose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>in the presence of 50 μM cAMP</td>
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</tr>
<tr>
<td>TP2010</td>
<td>F- xyl Δcya argHI ΔlacX4 recA</td>
<td>28</td>
</tr>
<tr>
<td>TP2339</td>
<td>F- xyl Δcya Δcya argHI ΔlacX4</td>
<td>31</td>
</tr>
<tr>
<td>MC1060</td>
<td>ΔlacX4 galU galK rpsL hsdR</td>
<td>3</td>
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<tr>
<td>Y1083</td>
<td>ΔlacIPOZYA) U169 proAΔ Δlon</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>aroD139 rpsL thi</td>
<td></td>
</tr>
</tbody>
</table>

Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMC1403</td>
<td>Contains the lacZYA gene</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>truncated at its 5'-end, Amp'</td>
<td></td>
</tr>
<tr>
<td>pCU5</td>
<td>pBR322 containing the cya gene of R. meliloti cloned at the BamHI restriction site</td>
<td>18</td>
</tr>
<tr>
<td>pDIA7010</td>
<td>Constructed by cloning the lacZYA gene of pMC1403 downstream from the TGA stop codon of the cya gene of pCU5</td>
<td>This work</td>
</tr>
<tr>
<td>pDIA7010A</td>
<td>Derivative of pDIA7010 containing an in-phase cya-lacZ gene fusion obtained by a spontaneous deletion</td>
<td>This work</td>
</tr>
<tr>
<td>pDIA7020</td>
<td>Derivative of pDIA7010A with a deletion of the 0.15-kb XhoI restriction fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pDIA7030</td>
<td>Derivative of pDIA7010A with a deletion of the 0.298-kb NatI restriction fragment</td>
<td>This work</td>
</tr>
</tbody>
</table>

Protein electrophoresis and immunoblotting. Polyclonal antibody gel electrophoresis of proteins in sodium dodecyl sulfate and electrophoretic blotting of these proteins onto nitrocellulose were carried out by the procedures of Laemmli (17) and Towbin et al. (38), respectively. The fusion protein was visualized by Western immunoblotting with anti-β-galactosidase antibodies (diluted 1:200) by using a reaction with peroxidase-conjugated antibodies provided by Diagnostic Pasteur.

Adenylate cyclase and guanylate cyclase assays. In vitro cyclase reactions were performed at 28°C in 10 mM MgCl₂, 2.5 mM dithiothreitol, 60 mM Tris hydrochloride buffer at various pHs and ATP and GTP concentrations. ATP and GTP were obtained from Sigma Chemical Co. (St. Louis, Mo.). Reactions were stopped with 50 mM sodium acetate buffer (pH 6.2) and incubated at 65°C for 5 min. Measurements of adenylate cyclase activity were performed by monitoring cAMP synthesis with a radioimmunoassay assay with 125I-labeled cAMP from Institut Pasteur Production and anti-cAMP antibodies kindly provided by A. Ullmann. A cGMP radioimmunoassay assay kit from Amersham was used to assay for guanylate cyclase activity (36).

RESULTS

Nucleotide sequence of the R. meliloti cyaA gene. The R. meliloti adenylate cyclase gene, originally located on a 5.4-kilobase (kb) BglII fragment, was previously subcloned into various high-copy-number vectors, and a 0.8-kb DNA segment was found to be able to weakly complement cya-defective E. coli strains (18). The nucleotide sequence of the 1.5-kb BamHI-EcoRI fragment cloned in pBR322 (pCU5) and containing the 0.8-kb PstI-EcoRI core fragment of the cya gene is presented in Fig. 1.

Two long open reading frames (ORF1 and ORF2) that overlap, as well as a third one (ORF3) in the opposite strand and coinciding with ORF1, are prominent. ORF1 spans almost the entire fragment and stops 39 base pairs (bp) upstream from the EcoRI site. At its 5’ end it is in frame with the tet gene of pBR322 present in pCU5 and pDIA7010, as well as with the cat gene of pACYC184 present in pCU11, and used by Lathigra et al. (18).

Translated ORFs are enriched in codons starting with a purine and ending with a pyrimidine (34). Analysis of the RNY triplets of ORF1 and its third base G+C content strongly suggested that it might code for a protein (34). Since most of the known adenylate cyclase proteins comprise a catalytic domain coupled to a regulatory domain (11, 16, 22, 30), it seemed important to identify the catalytic domain of the R. meliloti protein. The DNA sequence revealed, internal to ORF1 and downstream from the second PstI restriction site, a perfect consensus ribosome-binding site at position 875 followed by an ATG start codon 7 nucleotides downstream. As discussed below, it appeared that in E. coli this start codon serves as an initiator of translation and directs synthesis of a protein endowed with adenylate cyclase activity. ORF2 extended from the TGA at position 134 to the TGA at position 154. There was no obvious signal for initiation of translation, and this ORF did not score highly with the RNY analysis. Furthermore, it featured an unlikely high number of arginine residues. Because of the high G+C content in the third base of ORF1 and the fact that this third-base position of ORF1 coincides with the first base of ORF2 (66% in the present case), the probability of finding a termination codon (A+T rich) in ORF2 is low, thus explaining the presence of such a long noncoding ORF. Similarly, ORF3 on the opposite strand, extending over the entire 1.5-kb fragment, also shared its first base of the triplet code with the third base of ORF1. Although fitting with the RNY rule (because it is in frame with ORF1), ORF3 did not reveal a high third-base G+C content as expected. It is therefore unlikely that it codes for a protein.

Upstream from the ribosome-binding site and the ATG start codon located at position 875 in ORF1, several G+C-rich palindromic sequences and inverted repeats were present, located at positions 325 through 345, 360 through 430, and 791 through 912. This suggested that a corresponding transcript might be folded in stable hairpin structures, possibly preventing translation of the corresponding region (15). This puzzling organization, together with the two translational products of 65 and 28 kilodaltons (kDa) previously found in maxicells (18), prompted us to identify the corresponding protein.

Construction of cyaA-lacZ fusion. To purify the R. meliloti adenylate cyclase protein, a fusion gene that expressed a hybrid protein with both adenylate cyclase and β-galactosidase activities was constructed. First, the SmaI-Sall fragment of pMC1403 was inserted in pCU5 between the SmaI and Sall sites (Fig. 2). The plasmid pDIA7010 thus obtained was still able to complement the cya-deficient strain E. coli TP610A, but no β-galactosidase activity could be detected. This was as expected, because the stop codon of the putative cya gene was located 79 bp upstream from the SmaI site.

performed as described by Ullmann (39). The eluted protein was dialyzed against 50 mM phosphate buffer (pH 7.4). β-Galactosidase activities were measured as described by Fardee et al. (27). The amount of protein was determined as described by Bradford (2).
FIG. 1. Nucleotide sequence of the R. mellii cya gene. The nucleotide sequence of a 1.5-kb BamHI-EcoRI fragment encompassing the cya gene is presented. The deduced amino acid sequence of the adenylate cyclase protein is also given (letters in lowercase type indicate amino acid residues translated from ORF1) but presumably absent from the adenylate cyclase protein expressed in E. coli. Key restriction enzyme sites are indicated. The intragenic RBS at position 871 through 879, the ATG at position 885, and the TGA stop codon at position 1464 are underlined. The regions of dyad symmetry are overlined.

corresponding to the truncated lacZ gene start. Strain TP2339 (ΔlacX74) was then transformed with plasmid pDIA7010. To obtain a spontaneous cya-lacZ fusion, mutants that restored growth on minimal medium supplemented with 0.4% lactose were then selected, and it was determined that the mutation permitting growth on lactose was carried by the plasmids thus obtained. Strain TP2010 was subsequently transformed with the mutant plasmids. One such plasmid (pDIA7010A), shown to be cya and lacZ positive, was chosen for further study. Strain TP2010 transformed with pDIA7010A was able to grow on minimal medium supplemented with 0.4% lactose or 0.4% maltose. The 0.28-kb Xhol-BamHI fragment of pDIA7010A that included the junction of the gene fusion was sequenced. It showed a deletion of 34 bp that had removed the TGA stop codon and resulted in an in-phase fusion of the cya and lacZ reading frames. The resulting gene fusion also confirmed that all or part of ORF1 was expressed in E. coli under the present conditions.
conditions. This deletion appeared to be created by a crossover event involving an 11-bp direct repeat (Fig. 3). To substantiate the hypothesis that it was generated by homologous recombination, the experiment designed to provide Lac⁺ colonies in minimal plates was repeated in a recA-deficient background (strain TP2010); no Lac⁺ clones were recovered after strain TP2010 harboring plasmid pDIA7010 was plated under conditions similar to those of the original experiment (data not shown).

**Biochemical analysis of the adenylate cyclase-β-galactosidase hybrid protein.** Bacteria harboring plasmid pDIA7010A were grown to the stationary phase in minimal medium supplemented with lactose, and their β-galactosidase content was purified as described in Materials and Methods. The eluted protein was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotting analysis was performed with anti-β-galactosidase antibodies.

Two main bands were observed (Fig. 4, lane A). The higher band of 135 kDa corresponded to the adenylate cyclase-β-galactosidase hybrid protein. The second band of 116 kDa, corresponding to the size of the β-galactosidase, was presumably a degraded form of the first protein. This latter form was predominant under our growth conditions. Other conditions of purification (cells disrupted with alumina, elution at lower pH) were tried to increase the amount of the hybrid protein; they did not permit significant improvement of the hybrid protein yield. Similar experiments were also carried out with a protease-deficient strain (Δlon) (41) with no further improvement (data not shown).

The Mr of the hybrid protein corresponded to that expected from the synthesis of a protein translated from the ATG at position 885 and was in agreement with the smaller product previously found in maxicells (18). The protein was blotted on a poly(vinylidene difluoride) membrane, which

**FIG. 2.** Construction of plasmid pDIA7010. Plasmid pDIA7010 was constructed by cloning the SmaI-SalI fragment, containing the enteric β-galactosidase genes truncated at the NH₂ terminus, into pCU5, downstream from the *R. meliloti cya* gene. lacZYA, *E. coli* lactose operon genes; ptt, tetracycline resistance promoter; AmpR, ampicillin resistance gene; cyaA, *R. meliloti* adenylate cyclase gene.

**FIG. 3.** Nucleotide sequence of the spontaneous deletion in pDIA7010 that resulted in a gene fusion of the *R. meliloti cya* gene and the lac gene. The deletion is boxed, and the two direct repeats involved in homologous recombination are underlined. The TGA stop codon is boxed. The lacZ gene starts from the SmaI restriction site.
could be directly used in a gas-phase sequencer (23). Determination of the NH₂-terminal residues of the hybrid protein was then attempted but failed to yield significant results, suggesting that under our conditions the N terminus was blocked (data not shown). The purified preparation was used to assay adenylate cyclase activity as described in Materials and Methods. The reactions were carried out at pH 7.5, which was found to be the optimum pH for the adenylate cyclase activity. The hybrid protein synthesized cAMP, and the Kₘ for ATP was determined and estimated to be 4 mM (i.e., the usual concentration of ATP inside living cells) (Fig. 5). The enzyme activity was inhibited by a concentration of substrate of 10 and 20 mM. GTP was a strong inhibitor of the reaction (Fig. 6). However, no guanylate cyclase activity was detected under the assay conditions. Furthermore, measurements on cultures overexpressing the unfused protein showed no synthesis of cGMP (data not shown).

To check that the hybrid protein of 135 kDa was the main significant translational product endowed with an adenylate cyclase activity, a small deletion was introduced upstream from the ATG codon present at position 885 (see below). In this latter case, Western blotting analyses indicated synthesis of a hybrid protein with an Mₘ similar to that of the protein synthesized from the native plasmid pDIA7010A (Fig. 4, lane B).

**Genetic analysis of plasmid pDIA7010A.** Deletion of the 0.15-kb XhoI fragment preserving the translation frame (Fig. 1) in plasmid pDIA7010A led to the construction of plasmid pDIA7020. Such a deletion maintained β-galactosidase activity, as expected, but abolished the adenylate cyclase activity. This confirmed that the catalytic center of the protein was, at least in part, coded for by this region of the ORF.

To prove that the translation of the fusion protein is initiated at the ribosome-binding site at position 875, a deletion was created that modified the reading frame upstream from this translational start. Plasmid pDIA7010A was digested by the restriction enzyme NotI (Fig. 1), generating plasmid pDIA7030, which harbored a deletion of a 298-bp fragment. This resulted in alteration of the ORF upstream from the ribosome-binding site and ATG located at positions 875 and 885, respectively. Strains TP2339 and TP610A were transformed with plasmid pDIA7030. They displayed both β-galactosidase and adenylate cyclase activities. This confirmed that initiation of translation of the fusion protein could occur at the more downstream ATG codon (located at position 885) and that the corresponding protein could synthesize cAMP.

**Analysis of the protein sequence of the cya gene.** Analysis of the polypeptide sequence translated from the cya gene (from the ATG at position 885 to the TGA at position 1464) revealed no significant similarity with other procaryotic adenylate cyclases either from enterobacteria (1) or from *Bordetella pertussis* (8) or any other protein translated from the DNA sequences present in the EMBL data bank (release 18) when the algorithm of Lipman and Pearson (20) was used. However, a more thorough comparison of the *Rhizobium* cyclase sequence with all other cyclases revealed blocks of identical amino acid residues (Fig. 7). This appeared to be highly significant, since the regions of identity were located in regions whose integrity is necessary for cAMP synthesis (*Saccharomyces cerevisiae* [11, 22], *R. meliloti*, XhoI deletion). A further substantiation of this interpretation stems from comparison with guanylate and

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**FIG. 4.** Western blotting analysis of the hybrid protein encoded by the plasmids pDIA7010A and pDIA7030. The anti-β-galactosidase antibodies were used at a dilution of 1:200, and the blotting was performed as described by Towbin et al. (38). Lanes: A, purified *E. coli* extract from cells harboring plasmid pDIA7010A; B, purified *E. coli* extract from cells harboring plasmid pDIA7030. Molecular size markers were from Sigma.

**FIG. 5.** In vitro activity of the *R. meliloti* adenylate cyclase protein. Initial velocities were measured as described in the text. Lineweaver-Burk plots allowed us to estimate the Kₘ of the reaction to be 4 mM at pH 7.5 and 28°C.

**FIG. 6.** GTP inhibition of the adenylate cyclase reaction. Reactions were carried out at 4 mM ATP in the presence of different concentrations of GTP.
FIG. 7. Alignment of catalytic domains from *S. cerevisiae* adenylate cyclase (SCCYA), *R. meliloti* adenylate cyclase (RMCYA), *Rattus norvegicus* guanylate cyclase (RNCYG), *Bos taurus* guanylate cyclase (BTCYG), and bovine brain adenylate cyclase (BTCYA). Identical residues are marked by asterisks, residues of the same class (33) are indicated by plus signs, and gaps are indicated by dashes.

adenylate cyclases from higher eucaryotes (4, 13, 14, 37); the alignments performed by Chinkers et al. (4) and by Krupinski et al. (14) highlight the same blocks of identity (Fig. 7). It can be therefore inferred that the procaroytic *Rhizobium* adenylate cyclase shares a common ancestor with adenylate cyclases from *S. cerevisiae* and bovine brain and also with guanylate cyclases from higher eucaryotes.

**DISCUSSION**

Determination of the nucleotide sequence of a gene from *Rhizobium meliloti* complementing cya strains of *E. coli* allowed characterization of a *Rhizobium* adenylate cyclase. The status of this *cya* gene, which could be expressed in *E. coli*, was not clear in *R. meliloti* until recently, when it was possible to inactivate it in *R. meliloti* F34 (26). It could be demonstrated that in these latter mutants the level of cAMP was decreased as compared with the level found in the wild-type parental strain. However, a significant level of cAMP was still present in the mutant strain, leading O'Regan et al. to propose that two *cya* genes exist in *R. meliloti* (26). The presence of two adenylate cyclases might permit fine tuning of cAMP synthesis according to growth or symbiotic conditions. It was therefore important to characterize as precisely as possible the protein coded for by the isolated
cyclizing enzyme gene. It seems important at this stage to emphasize that the $M_r$ of the protein as expressed in *E. coli* was about 20,000, a very low value when compared with those of other adenylate cyclases (7–9, 11, 14, 22, 29), and therefore presumably constituted nothing more than a catalytic domain. In this respect it is worth noting that plasmid pDIA7030 directed synthesis of a protein that was able to synthesize cAMP. Indeed, in this construction (NotI deletion), the only start codon is ATG$_{N32}$ encoding a 193-residue protein. Analysis of the DNA sequence located upstream from the translation start site revealed the presence of a long ORF. However, this ORF encompasses a region rich in potential secondary structures. This suggests that a complex regulatory pattern involving translation of the upstream region and coupling to transcription might be operating in *R. meliloti*. This is reminiscent of the situation in *S. cerevisiae*, in which adenylate cyclase seems to be produced from transcripts of different lengths, leading to different polypeptides depending on growth conditions (11, 22). It cannot be completely ruled out at present, however, that other, longer translation products can be synthesized from this ORF. In particular a protein with 41 extra NH$_2$-terminal residues might have been synthesized, starting at ATG$_{N32}$. In this respect it seems worth noting that Lathigra et al. found two polypeptides of 26 and 64 kDa in maxicells when the cya gene was placed under the control of the chloramphenicol acetyltransferase promoter (18). In our case the cya-lacZ gene fusion was cloned downstream from the Tet promoter, and the resulting 135-kDa protein correspond to the fusion of the 26-kDa polypeptide with $\beta$-galactosidase. The 64-kDa product might derive from translation initiated at an ATG codon located upstream from the BamHI cloning site and going through the secondary structures present in the region at positions 791 through 912, to be fused to the catalytic center of the protein. Thus, adenylate cyclase might be (as seems to be the case in *S. cerevisiae* [11]) present in two forms, one composed of a catalytic center alone and another fused to a regulatory domain. The $K_m$ of the enzyme for ATP was 4 mM, which is somewhat higher than that of its *E. coli* counterpart (ca 0.6 mM) (6). Another important feature of the adenylate cyclase activity was its inhibition by GTP. Activity measurements of cGMP were performed on purified preparations of a cyclase–$\beta$-galactosidase fusion protein and on cultures overexpressing the unfused protein. In both cases we failed to detect cGMP.

The polypeptide sequence comparison indicated significant identities between *R. meliloti* and *S. cerevisiae* catalytic domains. This strongly suggests that the corresponding domain in bovine adenylate cyclase (14) is the catalytic domain of the protein. Since two homologous domains are prominent in the latter, one may wonder whether the enzyme from the higher eucaryote does not comprise two catalytic domains. A further consequence of these observations is that they indicate a common descent between eucaryotes and procaryotes. Another possibility, although it is unlikely because the overall base composition of the gene is typical of *R. meliloti*, would be horizontal transfer of the gene. Thus cAMP synthesis may have been present at the origin of the separation between the two kingdoms. In addition, similarity with guanylate cyclase proteins from higher eucaryotes, still visible in *R. meliloti* through the inhibitory potential of GTP, indicates that a nucleotide-cyclizing enzyme of broad specificity was present very early. In this respect, unequivocal confirmation of the presence of cAMP or cGMP in archaebacteria would be of unusual interest.

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