Identification of a novel adenylate cyclase in the ruminal anaerobe, Prevotella ruminicola D31d

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Abstract

Our previous evaluation of ruminal and other anaerobic bacteria showed only Prevotella ruminicola D31d produced detectable concentrations of cyclic AMP. In order to investigate the synthesis of this important metabolic regulator, the gene for adenylate cyclase (cya), which produces cyclic AMP, was cloned and expressed in a cyaA mutant of Escherichia coli. The cloned P. ruminicola D31d gene was able to complement the cyaA mutation and permitted fermentation of lactose on MacConkey Lactose agar plates. Analysis of the DNA sequence of the 2.5-kilobase pair insert revealed an open reading frame encoding for a 67-kDa protein. This protein was novel in that no amino acid similarity was observed with other procaryotic or eucaryotic adenylate cyclases in the GenBank database. Production of cyclic AMP in the E. coli clone was confirmed with a radioimmunoassay technique. This is the first example of an adenylate cyclase gene identified from an anaerobic bacterium. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Cyclic AMP (cAMP) is involved in the process of catabolite repressioin in a variety of aerobic and facultative anaerobic bacteria [1,2]. This molecule has also been demonstrated to be important for the development of competence in different bacterial species, including Haemophilus influenzae [3]. However, there is a paucity of evidence for the presence of cAMP, and the enzyme adenylate cyclase, in anaerobic bacteria. Hylemon and Phibbs [4] previously reported on the lack of cAMP and related enzymes in strains of the colonic anaerobe Bacteroides fragilis. Our previous work has demonstrated an absence of detectable cAMP from a variety of predominant anaerobes from the rumen and other sources [5,6]. The only exception to this was in the ruminal anaerobe, Prevotella ruminicola D31d, where cAMP was

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1 Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.
detected [6]. Cellular concentrations of cAMP in this bacterium were highest during early exponential and stationary phases of growth. Since this was the first observation that cAMP may be synthesized by an anaerobic bacterium, our studies on this bacterium were extended, and we now report on the cloning, expression, and sequence of an apparently novel adenylate cyclase gene from *P. ruminicola* D31d, the first such gene cloned from an anaerobic bacterium.

2. Materials and methods

2.1. Organisms, plasmids, and media

*P. ruminicola* D31d was obtained from B.A. Dehority, The Ohio State University [7]. Recent taxonomic changes for *Prevotella* species have been proposed [8]: strain D31d, on the basis of DNA-DNA relatedness to the type strain *P. ruminicola* 23 [9], is considered to be *P. ruminicola*. *Escherichia coli* strains DH5α-MCR and SPS50 were grown in Luria broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per l) unless otherwise indicated. For selection of plasmids, ampicillin (75 μg/ml), kanamycin (50 μg/ml), or tetracycline (15 μg/ml) were added to the medium when necessary. *E. coli* SPS50 (λ−, e14−, relA1, spoT1, cyaA1400(:,kan), thi-1) is a cya deletion mutant and thus lacks both adenylate cyclase and cAMP [10] and was obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT, USA). Growth was monitored spectrophotometrically (Spectronic 21, Milton-Roy, Rochester, NY, USA) by measuring optical densities of cultures at 600 nm. Plasmids pUC18 and pBR322 were used for cloning experiments.

2.2. Cloning protocols and DNA sequencing

Due to cyaA mutation, strain SP850 cannot utilize lactose (Lac−), produces colorless colonies on MacConkey Lactose agar, and is also kanamycin-resistant. The adenylate cyclase gene was cloned using genomic DNA isolated from *P. ruminicola* D31d as previously described [11]. A partial *Sau*3a digest was prepared from the DNA and ligated into *Bam*HI-digested and dephosphorylated pUC18. The ligated plasmid and pUC18 were introduced into *E. coli* SP850 by electroporation, and the complementation of the cyaA mutation was detected on Difco MacConkey Lactose agar by production of bright red colonies. Positive colonies were re-streaked to confirm the complementation. Plasmid DNA containing the putative adenylate cyclase gene was purified and used for DNA sequencing. DNA sequencing of both strands was carried out at the University of Illinois Genetic Engineering Facility, Urbana, IL. The DNA sequence has been submitted to GenBank and has been assigned accession number AF056932. Protein amino acid similarity analyses were carried out using BLAST similarity searches against the GenBank database.

2.3. Sample collection, extraction, and analyses

*E. coli* SP850, SP850/pUC18, SP850/pDAC2, and SP850/pDACSI were grown in 10 ml of LB-kanamycin or LB-kanamycin-ampicillin to an OD600 of about 1.0. Cells were harvested by centrifugation (6000× g, 20°C) and suspended in 1 ml of 0.1 N HCl. Samples were heated at 95°C for 10 min [12], then stored at −20°C until analyzed. A separate culture sample was collected for cell protein determination. The cAMP concentrations were determined in triplicate using a commercial competitive binding radioimmunoassay procedure according to the manufacturer's instructions (Biomedical Technologies Inc., Stoughton, MA, USA). Cell protein concentrations were determined by the Lowry method after hydrolysis of cells in alkali (0.1 N NaOH) at 70°C for 30 min. Horse heart cytochrome c was used as standard.

3. Results and discussion

Our previous results indicated that of the anaerobic bacteria tested, only *P. ruminicola* D31d produced detectable levels of cAMP, and intracellular cAMP concentrations varied with growth [6]. Therefore, we attempted to clone the gene for adenylate cyclase from this bacterium. *E. coli* SP850 clones were screened on MacConkey Lactose agar, and one colony was observed that produced a deep red color, indicative of lactose fermentation and complementation of the adenylate cyclase deletion. This col-
only was restreaked on MacConkey Lactose agar to confirm the complementation, along with SP850 containing the original pUC18 plasmid. The plasmid isolated from this clone, termed pDAC2, was found to contain a 2.5-kb genomic insert (Fig. 1). In order to confirm that the insert was complementing the mutation and lactose fermentation was not due to a secondary mutation of the SP850, purified pDAC2 was reintroduced into SP850. The results indicated all ampicillin-resistant clones were lactose-positive on MacConkey Lactose agar.

Restriction endonuclease analysis of pDAC2 indicated the presence of at least one SplI site approximately 500 bases downstream of the multiple cloning HindIII site. The pDAC2 was digested with SplI and re-ligated, forming plasmid pDACS1. This plasmid could not complement the cyaA mutation in SP850, indicating that expression of active adenylate cyclase had been interrupted.

The concentration of cAMP present in strains SP850, SP850/pUC18, SP850/pDAC2, and SP850/pDACS1 was determined from cells grown on Luria broth (Table 1). Increased concentrations of cAMP were detected in the SP850/pDAC2 extract compared with SP850 consistent with the observation that the cloned adenylate cyclase gene was expressed in strain SP850. The extracts from plasmid pDACS1 did not demonstrate an increase in cAMP concentration, indicating that removal of the 500-base pair SplI fragment interrupted the expression of the adenylate cyclase gene. When the insert from pDAC2 was removed from pUC18 and ligated into pBR322, this construct was also able to complement the cyaA mutation, indicating that the adenylate cyclase gene was being expressed in E. coli from an endogenous promoter.

DNA sequencing of the insert in pDAC2 revealed one open reading frame spanning the region from base 62–1843. This reading frame encodes for protein with a calculated molecular mass of 67411. This open reading frame also spans the SplI sites that interrupted the expression of the adenylate cyclase gene (Fig. 1). Similarity searches of the GenBank with the deduced amino acid sequence of the P. furinico/a adenylate cyclase gene revealed no significant similarity with known procaryotic or eucaryotic adenylate cyclase genes.

This is the first example of an adenylate cyclase gene being isolated from an anaerobic bacterium. The role of cAMP in the physiology of P. furinico/a is as yet unknown, but the molecule likely plays a role separate from catabolite repression. Our previous work indicated that cAMP concentrations did not alter in the organism under conditions where catabolite repression-like phenomena occurred [6]. Attempts are made to introduce the adenylate cyclase gene back into P. furinico/a D31d to determine if overexpression of the gene affects the physiology of the cell.

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References