# EVIDENCE AGAINST THE PRESENCE OF CYCLIC AMP AND RELATED ENZYMES IN SELECTED STRAINS OF BACTEROIDES FRAGILIS

P. B. Hylemon and P. V. Phibbs, Jr.
Department of Microbiology
Health Sciences Division
Virginia Commonwealth University
Richmond, Virginia 23298

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SUMMARY: Cyclic AMP was not detected in whole cells, expended culture medium or culture supernatant fluid of selected strains of Bacteroides fragilis. Adenyl cyclase and c-AMP phosphodiesterase activities were also not detected in cell extracts of B. fragilis. The exogenous addition of dibutyryl-c-AMP or sodium cholate to cultures of B. fragilis growing on lactose did not significantly affect the specific activity of β-galactosidase measured in cell extracts of this organism. No diauxic growth pattern could be demonstrated in a chemically defined medium containing 5 mM glucose + 28 mM lactose.

INTRODUCTION: The role of 3',5'-cyclic adenosine monophosphate (c-AMP) in the regulation of synthesis of inducible catabolic enzymes has been described in several bacterial genera (1). All of these bacteria are members of the family Enterobacteriaceae. However, the distribution and function(s) of c-AMP in other, physiologically dissimilar bacteria has not been extensively investigated.

Members of the genus Bacteroides include strictly anaerobic, gram-negative, non-sporeforming bacteria (2). Bacteroides (primarily certain subspecies of B. fragilis) is predominant among the human fecal microflora and accounts for as much as 50% of all colonic bacteria (3). One gram weight of human fecal material has been reported to contain approximately $10^{10}$-$10^{11}$ viable Bacteroides cells (3, 4). However, there is a paucity of information on the mechanisms of metabolic regulation in this medically important anaerobic bacterium. In the studies described in this communication, we examined the c-AMP concentrations in cell cultures of selected subspecies of B. fragilis and adenyl cyclase and c-AMP phosphodiesterase activities in cell extracts of these organisms.

MATERIALS AND METHODS: Stock cultures of Escherichia coli K-12 were

maintained on nutrient agar slants incubated at room temperature (22-25 C). Stock cultures of Bacteroides fragilis were maintained in chopped meat medium under anaerobic conditions (2). E. coli was cultured in a basal salts medium containing the appropriate carbon source as described previously (5). A slightly modified chemically defined basal growth medium (Drs. V. H. Varel and M. P. Bryant, Abs. Ann. Meet. Amer. Soc. Microbiol. p. 116, 1973; personal communication) was used to culture strains of B. fragilis. The medium included: 6.6 mM $KH_2PO_4$, 15 mM NaCl, 3 mM $(NH_4)_2SO_4$, 0.5 mM L-methionine, 45 µM $CaCl_2.2H_2O$, 98 µM $MgCl_2.6H_2O$, 50 µM $MnCl_2.4H_2O$, 4 µM $CoCl_2.6H_2O$, 14 µM $Fe.SO_4.7H_2O$, 3 µM hemin, 28 mM fermentable sugar and following sterilization at 121 C (15 lb inch $^{-2}$ for 15 min), 6.3 mM L-cysteine and 29 mM $Na_2CO_3$ were added and the pH adjusted to 7.0 using sterile 6N HCl. A 2% (vol/vol) inoculum of cells of B. fragilis from a late exponential phase starter culture grown on homologous carbon source was used to initiate growth in all experiments with this organism. Cells were cultured in 2 liter flasks containing 1 liter of medium under anaerobic conditions. Growth of all bacteria was determined by measuring culture turbidity with a Klett-Summerson colorimeter equipped with a number 66 filter.

Measurement of intra- and extracellular levels of c-AMP: Bacteria cultured under specified conditions were centrifuged (22 C) at 12,000 g for 10 min and cells (0.4 - 0.5 g wet weight) were immediately suspended in 10 volumes of 0.1 N HCl and heated at 95 C for 10 min. Each sample was then centrifuged at 12,000 g for 10 min, and the supernatant fluid was adjusted to pH 7.0 with NaOH. The supernatant fluids were again centrifuged at 12,000 g for 10 min to remove precipitated material. The whole cell extracts were then concentrated 10-fold by lyophilization. A 0.15 ml portion of concentrated whole cell extract was added to a reaction mixture containing: 50 mM (Tris-HCl) (pH 8.0), 50 mM $MgSO_4.7H_2O$ and either 30 µg of purified beef heart c-AMP phosphodiesterase (Sigma Chemical Co.) or distilled $H_2O$ in a final volume of 0.175 ml. The reaction mixtures were incubated at 35 C for 60 min,

after which the reaction was terminated by heating at 95 C for 5 min under conditions which prevented evaporation. A sample of each of these reaction mixtures was then assayed for c-AMP by the Gilman procedure (6). The standard assay reaction mixture was conducted in a total volume of 200 µliter in 50 mM sodium acetate/acetic acid buffer (pH 4.0) containing 100 µl of the sample, 8 pmoles of [$^3$H] c-AMP (14.2 Ci/mM) (New England Nuclear, Boston, Mass.), and 20 µliter of partially purified protein kinase (1.5 mg/ml). The content of c-AMP in the original sample was calculated from the difference between the [$^3$H] c-AMP retained by Millipore filters (0.45 µm pore size; Millipore Corp.) with and without pretreatment of the extract sample with c-AMP phosphodiesterase using standard curves of known c-AMP concentrations.

Extracellular concentrations of c-AMP were measured as above after 20 ml of culture supernatant fluid was heated 3 min at 95 C to destroy possible c-AMP phosphodiesterase activity. Culture supernatant fluids of B. fragilis were lyophilized to dryness and suspended in 2 ml distilled $H_2O$. The c-AMP concentrations in total cell cultures were determined by boiling 20 ml of culture for 5 min, centrifugation for 10 min at 12,000 g, concentrating 10-fold by lyophilization in the case of B. fragilis and assaying for c-AMP concentrations by the standard procedure.

Enzyme assays: Cyclic-AMP phosphodiesterase (EC 3.1.4.c) was assayed as described by Thompson and Appleman (7) and the reaction mixture was modified as described by Nielson et al. (8). Heated (95 C for 5 min) cell extracts were used as background controls. The specific activities of c-AMP phosphodiesterase were determined at a protein concentration where the initial velocities were linear with respect to protein concentration and time. Adenyl cyclase activity was assayed as described by Khandelwal and Hamilton (9), using [$^3$H]-ATP (20-40 Ci/mmole) (New England Nuclear, Boston, Mass.). An ATP-regenerating system consisting of 5 mM P-enolpyruvate and 5 µg of pyruvate kinase was employed. The isolation of $^3$H-c-AMP was accomplished by the paper chromatographic method of Drummond and Duncan (10). Unlabeled c-AMP

(5 mM) was added as carrier to each reaction mixture. Protein estimations were determined by the method of Lowry et al. (11) and β-galactosidase activity was determined by the method of Pardee et al. (12).

RESULTS: The data presented in Figure 1 show the growth curves of

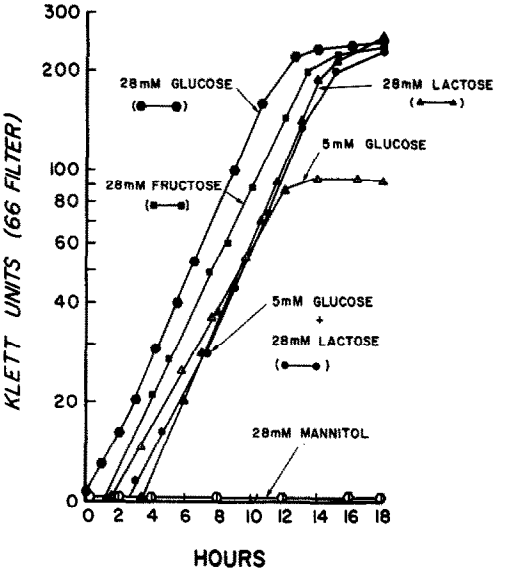


Fig. 1. Growth of B. fragilis ssp. fragilis ATCC 25285 in chemically defined basal medium containing the indicated carbon source(s). The growth is presented as optical density in Klett units.

B. fragilis ssp. fragilis on selected carbohydrates. Two points should be noted from the results presented in this figure: a) The growth rates on all substrates are nearly identical. b) No diauxic growth occurred in medium containing 5 mM glucose + 28 mM Lactose.

The results in Table 1 show that E. coli has high intracellular and culture medium concentrations of c-AMP. In contrast, c-AMP was not detected in whole cells, culture supernatant fluid, or total cultures of selected strains of B. fragilis.

Adenyl cyclase and c-AMP phosphodiesterase activities were readily detected in cell extracts of E. coli but not in strains of B. fragilis (Table 2).

Table 1. Cyclic 3',5'-adenosine monophosphate concentrations in Escherichia coli K-12, Bacteroides fragilis ssp. fragilis ATCC 25285 and ssp. thetaiotaomicron NCTC 10852[a]

| Species (Gram-negative bacilli) | Intracellular ($10^{-7}$M)[b] | Cells + Medium ($10^{-9}$M) | Medium ($10^{-9}$M) |
|---|---|---|---|
| E. coli K-12 | 1.5-8.0 | 200-500 | 200-500[c] |
| B. fragilis ssp. fragilis | <0.01 | 0 | 0 |
| B. fragilis ssp. thetaiotaomicron | <0.01 | 0 | 0 |

[a]Methods for c-AMP assay described in Materials and Methods

[b]Ranges of intracellular c-AMP concentrations determined irrespective of the carbon source. High values were obtained for E. coli under catabolite derepressed conditions and low concentrations were obtained under catabolite repressed conditions.

[c]Numbers in this column represent the range of c-AMP concentration found during the transition from early exponential to late exponential phase of growth.

Although adenyl cyclase activity was readily detected in broken cell suspensions of E. coli, it was difficult to obtain reliable values for specific activities. This may be in part due to the problem of competing enzymatic reactions for both substrate and product, e.g. ATPase and c-AMP phosphodiesterase activities, respectively. Moreover, the specific activities reported (13) for adenyl cyclase in cell extracts of E. coli are very low (picamolar amounts of substrate converted/min/mg protein) whereas, we have determined that the activity of competing enzymatic reactions are relatively very high. In additional experiments, 0.1 mM sodium c-AMP was added to growing cultures of B. fragilis and samples of the culture supernatant fluid was taken throughout the growth stages and assayed for c-AMP. No detectable degradation of c-AMP occurred.

Table 2. Adenyl cyclase and c-AMP phosphodiesterase activities of *E. coli* K-12, *Bacteroides fragilis* ssp. *fragilis* ATCC 25285 and ssp. thetaiotaomicron NCTC 10852.

| Organism | Carbon Source | c-AMP phosphodiesterase[a] (nmol/5 min/mg protein) | adenyl cyclase[b] |
|---|---|---|---|
| *E. coli* K-12 | glucose (30 mM) | 203 | + |
| *B. fragilis* ssp. *fragilis* | glucose (30 mM) | <1 | - |
| *B. fragilis* ssp. thetaiotaomicron | glucose (30 mM) | <1 | - |

[a]Cells of *E. coli* and *B. fragilis* were grown in chemically defined medium containing 30 mM glucose. The cells were harvested by centrifugation during the exponential-phase of growth and suspended in 0.1 M Tris-HCl (pH 7.0) containing 2.5 mM dithiothreitol. The cell suspensions were sonicated in a test tube using a Biosonik III (probe-type) sonicator for a total of three 15 s burst. The crude cell extracts were then centrifuged at 105,000 g for 2 h and the supernatant fluid was used to assay for c-AMP phosphodiesterase activity.

[b]Cells for this assay were harvested by centrifugation and suspended in 0.1 M Tris-HCl (pH 8.0) + 10 mM 2-mercaptoethonol. The cells were broken by sonication for a total of three 15 s burst and enzymatic activity determined directly on the broken cell suspensions.

The specific activity of β-galactosidase was not significantly affected by the exogenous addition of db-c-AMP, sodium cholate or a combination of these two compounds (Table 3). Moreover, in additional experiments, the exogenous addition of 1 mM sodium c-AMP did not alter the growth rate or specific activity of β-galactosidase in cultures of *B. fragilis*.

DISCUSSION: The inability to detect c-AMP in selected strains of *B. fragilis* was unexpected in view of the wide distribution of this compound in nature. Moreover, the apparent absence of adenyl cyclase and c-AMP phosphodiesterase activities in cell extracts of *B. fragilis* is consistent with the absence of c-AMP in cultures of this organism. The absence of diauxic growth patterns of *B. fragilis* in media containing combinations of

Table 3. Effects of exogenous addition of dibutyryl-cyclic-AMP and/or sodium cholate on the specific activity of β-galactosidase in Bacteroides fragilis ssp. thetaiotaomicron NCTC 10852[a]

| Organism | Growth Medium | β-galactosidase activity nmol/min/mg/protein |
|---|---|---|
| E. coli K-12 | glucose (30 mM) | 10 |
| E. coli K-12 | lactose (30 mM) | 839 |
| B. fragilis | glucose (30 mM) | 34 |
| B. fragilis | fructose (30 mM) | 28 |
| B. fragilis | lactose (30 mM) | 75 |
| B. fragilis | lactose (30 mM) + 0.5 mM db-c-AMP | 60 |
| B. fragilis | lactose (30 mM) + 0.5 mM cholate | 97 |
| B. fragilis | lactose (30 mM) + 0.5 mM db-c-AMP + 0.5 mM cholate | 90 |

[a] Cells of B. fragilis and E. coli were grown in chemically defined medium containing the appropriate carbon source. The cells were harvested by centrifugation during the exponential-phase of growth and suspended in 0.1 M Tris-HCl (pH 8.0) containing 10 mM 2-mercaptoethanol. The cell suspensions were broken by sonic oscillation in a test tube using a Biosonik III (probe-type) sonicator for a total of three 15 s burst. The crude cell extracts were then centrifuged at 105,000 g for 2 hr and the supernatant was used to assay β-galactosidase activity at 37 C.

two different sugars was also unexpected. These results are in contrast to the type of growth patterns reported for aerobic sporeforming Bacillus, E. coli and other members of the family Enterobacteriaceae, which preferentially use glucose to a number of other growth substrates (14). It appeared reasonable to assume that the c-AMP secreted by other microorganisms into the gastro-intestinal tract (our preliminary studies indicated that human fecal material

has approximately 700 nmoles c-AMP/g fecal material) may potentially serve as regulatory molecules for B. fragilis. However, the addition of db-c-AMP, sodium c-AMP or sodium cholate did not significantly affect the specific activity of β-galactosidase in cell extracts of this organism.

To our knowledge, this is the first reported evidence against c-AMP in a gram-negative anaerobic bacterium. However, it has been recently reported that the gram-positive bacteria Bacillus megaterium (15) and Lactobacillus plantarum (16) do not synthesize c-AMP or have detectable adenyl cyclase and c-AMP phosphodiesterase activities. Perhaps a large number of bacteria in nature do not synthesize or require this regulatory molecule.

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