

Brevibacterium liquefaciens Adenylate Cyclase and Its In Vivo Stimulation by Pyruvate

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Adenylate cyclase of *Brevibacterium liquefaciens* depends on pyruvate for activity. Growing in a simple medium containing glucose and DL-alanine, the microorganism excreted pyruvate, which reached 20 mM in the medium at stationary phase. Using [³H]adenosine to label the adenosine 5'-triphosphate pool, we showed that pyruvate in the medium stimulated adenylate cyclase of *B. liquefaciens* in vivo, in a manner similar to the stimulation observed in vitro. Adenylate cyclase in cells harvested at different phases of growth was equally responsive to exogenous pyruvate, indicating that the allosteric site for pyruvate was present in the enzyme throughout the various phases of cell growth. The specific activity of adenylate cyclase was highest in cells harvested at early log phase; thereafter it declined and was substantially lower at stationary phase. Although adenylate cyclase appears to be activated by pyruvate throughout the life span of the cell, the activity appears not to be critical to cell growth, which was comparable whether the medium contained high or low pyruvate.

Okabayashi et al. (12) first reported the excretion of large quantities of cyclic adenosine 3',5'-monophosphate (cAMP) into the culture broth of *Brevibacterium liquefaciens*, a gram-positive bacterium. In fact, this is the first observation that cAMP is present in microorganisms (15). Later, Hirata and Hayaishi (3) and Ide et al. (5) observed that adenylate cyclase prepared from this organism was greatly dependent on a metabolite, pyruvate, for activity. The enzyme has since been purified to homogeneity, and its biochemical and biophysical properties have been characterized (10, 17). Using [³H]adenosine to label the intracellular pool of adenosine 5'-triphosphate (ATP) (9), we describe experiments which suggest that pyruvate in the growth medium effectively stimulates adenylate cyclase in vivo, and that although adenylate cyclase appears activated throughout the growth cycle the functional state of the enzyme appears not to be critical to cell growth. A preliminary account of this research has appeared (T. J. Lynch and A. Tallant, Fed. Proc. 34:694, 1975).

MATERIALS AND METHODS

Chemicals. [³H]adenosine (21 Ci/mmol), [³H]-ATP (25 mCi/mmol), [¹⁴C]cAMP (49.2 μCi/mmol), and [³H]cAMP (23 Ci/mmol) were obtained from Schwarz/Mann. AG 1-X2 anion exchange resin was from Bio-Rad Laboratories and DL-sodium pyruvate and DL-lithium lactate were from Sigma Chemical Co. Florisil was purchased from Fisher Scientific

Co. and Glucostat was from Worthington Biochemicals Corp. All chemicals were reagent grade and were used without purification.

Bacteria. *B. liquefaciens* ATCC 14929 was grown in a 2-liter Erlenmeyer flask in shake culture (400 rpm) at about 22 C. One liter of medium contained 7 g of K₂HPO₄, 3 g of KH₂PO₄, 0.2 g of MgSO₄·7H₂O, 20 g of glucose, and 20 g of DL-alanine. The medium was inoculated with a 24-h culture to 0.4 optical density units measured at 650 nm. The cells were usually harvested in log phase after 40 to 50 h.

In vivo assay of adenylate cyclase. Routinely, 100 ml of a log-phase culture was withdrawn. The cells were sedimented by centrifugation, washed once with medium A (growth medium minus DL-alanine), and then resuspended in 50 ml of medium A. The incubation was started by adding 5 ml of the washed cell suspension to a 50-ml Erlenmeyer flask which contained 5 ml of medium A and 100 to 200 μCi of [³H]-adenosine (specific activity, 21 Ci/mmol) in the absence or presence of pyruvate. The flask was incubated at 37 C in a metabolic shaker. At appropriate times, 1-ml fractions were withdrawn and the cells were sedimented rapidly in an Eppendorf centrifuge 3200. The supernatant fluid was saved and the cells were quickly washed with 0.5 ml of cold medium A. The suspension was centrifuged as above and the supernatant fluid was removed and combined. The sedimented cells were then extracted twice, each time with 0.5 ml of 5% trichloroacetic acid, and the two extracts were pooled.

For prelabeling experiments (9), the washed cells were first incubated with [³H]adenosine to label the intracellular ATP pool. Typically, 15 ml of the washed cell suspension was added to an Erlenmeyer flask with 15 ml of medium A containing 600 μCi of [³H]adeno-

sine. After 10 min the cells were collected by centrifugation, washed in medium A, and then resuspended in 15 ml of medium A. Five milliliters of the cell suspension was transferred into another Erlenmeyer flask containing 5 ml of medium A with or without pyruvate (or lactate). One-milliliter samples were withdrawn at various times and processed as described above.

cAMP was isolated from both the medium and the trichloroacetic acid extract. The trichloroacetic acid sample was extracted twice, each with 3 volumes of ether, and then adjusted with 0.4 M tris (hydroxymethyl)aminomethane(Tris)-hydrochloride (pH 8) to neutrality. To 0.5 ml of the neutralized solution was added 0.05 ml each of 0.25 M Ba(OH)₂ and 0.25 M ZnSO₄. After centrifugation, the supernatant fluid containing cAMP was saved (8). The precipitate was washed once with 0.5 ml of water. The washing and the supernatant fluid were pooled and loaded onto an AG 1-X2 column, 0.5 by 7 cm (16). The column was eluted with 60 ml of 3 mM HCl, and the eluent was discarded. The column was next eluted with 50 ml of 10 mM HCl; this eluent contained cAMP. One milliliter of this eluent was counted in a Nuclear-Chicago Isocap 300 liquid scintillation spectrometer. The medium samples were loaded directly onto the column without the Ba(OH)₂, ZnSO₄ treatment. [8-¹⁴C]cAMP was added to all samples at the beginning of the isolation procedure to monitor recovery of the cyclic nucleotide, which was about 80% in the medium and 70% in the trichloroacetic acid extract. All data have been properly corrected for recovery.

In vitro assay of adenylate cyclase. One-tenth milliliter of a reaction mixture contained 100 mM Tris-hydrochloride (pH 9.0), 20 mM MgSO₄, 20 mM sodium pyruvate, 2 mM [¹⁴C]ATP (specific activity, 1.6 mCi/mmol), 2 mM cAMP, and an appropriate amount of enzyme under conditions that the activity was linear with respect to time of incubation and concentration of protein. The reaction was initiated with ATP, incubated at 30 C for 30 min, and then terminated by boiling for 1 min. Denatured protein was removed by centrifugation. A fraction of the supernatant fluid was spotted on Whatman no. 1 filter paper for descending chromatography in a solvent system containing 700 ml of 95% ethanol, 300 ml of 1 M ammonium acetate, and 476 mg of tetrasodium ethylenediaminetetraacetate. The solvent was adjusted to pH 5 with glacial acetic acid. Spots corresponding to cAMP were cut out, and the radioactivity was determined.

Assay of cyclic AMP phosphodiesterase. Phosphodiesterase was assayed by a two-stage procedure. The reaction mixture with a final volume of 0.1 ml contained 40 mM Tris-hydrochloride (pH 8.0), 10 mM MgCl₂, 1 mM β-mercaptoethanol, 0.01 mM [³H]cAMP, and enzyme. After incubation at 30 C for 10 to 30 min, the reaction was terminated by transferring the reaction tube to a boiling water bath for 1 min. The tube was cooled to 30 C, and 0.05 ml of snake venom (*Crotalus atrox*, 1 mg/ml for 5'-nucleotidase activity) was added to convert the product, 5'-AMP, to adenosine and Pi. Ten minutes later, 1 ml of an AG 1-X2 suspension (1 part of resin and 2

parts of water, vol/vol) was added to absorb the residual cAMP. An aliquot of the supernatant fluid which contained presumably all the adenosine was counted (18). It was found subsequently that this anion exchange resin binds 40% of the adenosine in the reaction mixture (11). The procedure therefore underestimated cAMP phosphodiesterase by 40%; the data were not corrected for the binding of adenosine by the resin.

Determination of medium and intracellular pyruvate. Pyruvate was measured using lactate dehydrogenase and reduced nicotinamide adenine dinucleotide with a PMQ II Zeiss spectrophotometer. This technique detects as little as 100 nmol of pyruvate.

Determination of medium glucose. Glucose in the medium was measured either chemically using o-toluidine (2) or enzymatically using Glucostat. Results from both procedures were comparable.

RESULTS AND DISCUSSION

Growth curve of *B. liquefaciens*. *B. liquefaciens* proliferated relatively slowly under our growth conditions. Figure 1 depicts a growth curve of the bacterium. After an initial lag lasting about 10 h, the organism entered into a logarithmic growth phase, which was followed by slower growth until the onset of a stationary phase some 200 h after inoculation.

Figure 1 also shows the disappearance of glucose and the accumulation of pyruvate in the growth medium. It is surprising that at stationary phase glucose was still plentiful, indicating that the carbon source was not limiting. Pyruvate accumulated throughout the growth period and reached 20 mM at stationary phase. Pyruvate was probably not primarily derived from glucose because pyruvate leveled off while glucose was still abundant in the medium. In fact, when the organism was grown in a medium containing glucose and arginine instead of glucose and DL-alanine, the level of pyruvate in the growth medium was less than 10 μM at a cell density of 4 optical density units. Umezawa et al. (19) showed that in *B. liquefaciens* pyruvate was formed predominately from D-alanine through the action of D-amino acid oxidase.

In vivo stimulation of adenylate cyclase by pyruvate. Hirata and Hayaishi first noted that pyruvate markedly stimulated adenylate cyclase prepared from *B. liquefaciens* (3). Since pyruvate in the growth medium reached 20 mM or higher at stationary phase, the question arose whether pyruvate stimulated the enzyme under physiological conditions. To answer this question we used an in vivo assay of adenylate cyclase by labeling intracellular ATP with [³H]-adenosine (9), a technique that has been shown to reflect closely the relative level of adenylate cyclase activity in both prokaryotes (14) and eukaryotes (7).

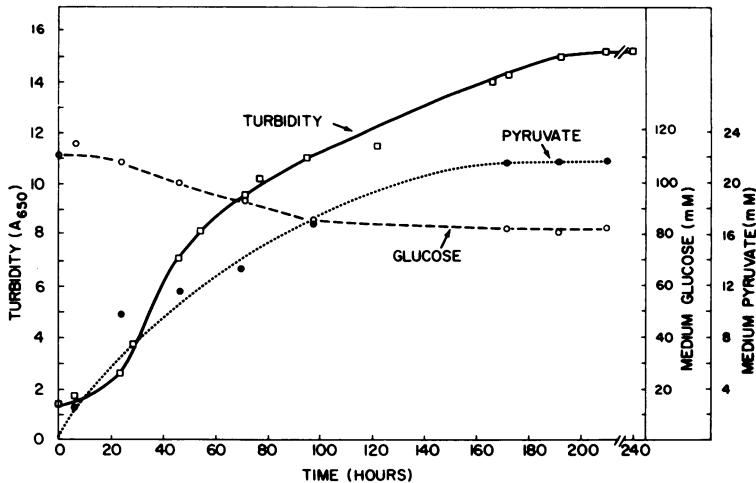


FIG. 1. Growth curve of *B. liquefaciens*. Optical density of the cell suspension was measured at 650 nm. Pyruvic acid was measured spectrophotometrically and glucose was determined chemically (o-toluidine) or enzymatically (Glucostat).

Figure 2 shows the effect of pyruvate on the *in vivo* synthesis of radioactive cAMP from exogenous [³H]adenosine. Intracellular [³H]cAMP increased rapidly in the presence of pyruvate (Fig. 2A). The fast response is reminiscent of the very rapid *in vitro* stimulation of adenylate cyclase by pyruvate. Although the level of cAMP diminished gradually after 5 min, it was still higher than the control at the end of 30 min. In the medium (Fig. 2B), on the other hand, the level of [³H]cAMP increased throughout the entire course of the experiment, indicating a continuous efflux of the nucleotide into the medium. To exclude the possibility that increased cAMP synthesis in the presence of pyruvate resulted from an enhanced transport of [³H]adenosine, we pre-labeled the cells with [³H]adenosine, washed them of extracellular [³H]adenosine, and then transferred them to separate flasks with or without pyruvate. Figure 3A shows that under this condition the level of intracellular [³H]cAMP increased as rapidly as in Fig. 2A, suggesting that the increase of [³H]cAMP synthesis was not a result of enhanced transport of [³H]adenosine. Figure 3B shows that the level of extracellular [³H]cAMP increased for about 20 min and then leveled off, presumably because of a depletion of the labeled ATP pool.

Analysis of the total radioactivity inside the cells at each time interval in Fig. 2 and 3 showed little difference between the control experiments and the experiments with pyruvate or lactate, thus further indicating that neither pyruvate nor lactate affected the transport of [³H]adenosine into the cells.

Because pyruvate stimulates adenylate cyclase *in vitro* (4), and because the rate of [³H]cAMP synthesis from prior labeling with [³H]adenosine has been demonstrated in both eukaryotes (7) and prokaryotes (14) to reflect closely the relative level of adenylate cyclase activity, the simplest explanation of these results is that pyruvate stimulates *Brevibacterium* adenylate cyclase *in vivo*. Although the increased accumulation of [³H]cAMP in the cells and in the medium might be explained also through an inhibition of phosphodiesterase by pyruvate, this possibility was considered unlikely because separate experiments showed that up to 20 mM pyruvate did not affect phosphodiesterase activity in a crude homogenate of *B. liquefaciens* (data not given).

During the preparation of this manuscript, Umezawa et al. (19) reported that in the presence of DL-alanine both the intracellular and extracellular levels of cAMP increased markedly in nonproliferating cells of *B. liquefaciens* within 4 h at 25 C. Concomitant with the increase of cAMP was the elevation of pyruvate in the medium, which reached 20 mM after 4 h of incubation. Because induction of adenylate cyclase was not observed during the 4-h incubation, they concluded that cAMP accumulation was due to *in situ* activation of adenylate cyclase by pyruvate derived from DL-alanine.

In the present experiments, the level of [³H]cAMP was measured by prelabeling the ATP pool with [³H]adenosine; thus the effect of pyruvate on the increase of [³H]cAMP could be observed within a minute (Fig. 2). Our results support the conclusion of Umezawa et al. (19)

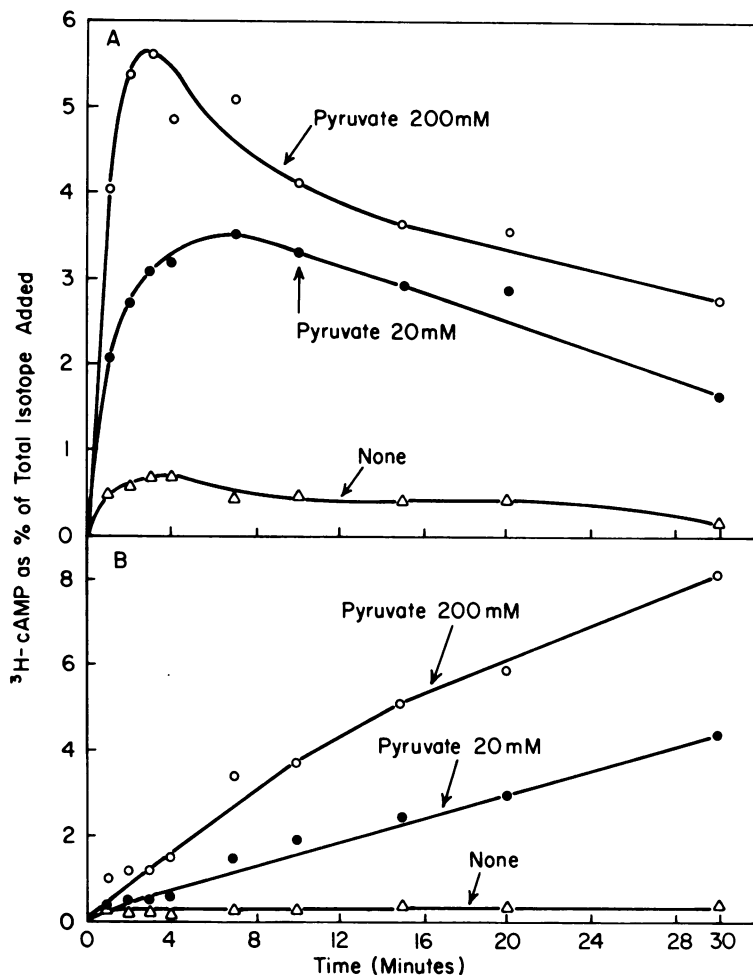


FIG. 2. Effect of pyruvate on the intracellular (A) and extracellular (B) accumulation of [³H]cAMP in *B. liquefaciens*. Five milliliters of a washed cell suspension was added to a 50-ml Erlenmeyer flask containing 5 ml of medium A, various concentrations of pyruvate, and 150 μ Ci of [³H]adenosine. At the times indicated, 1-ml samples were withdrawn and the [³H]cAMP was isolated from the medium and the trichloroacetic acid extract was determined.

that adenylate cyclase of *B. liquefaciens* was stimulated *in vivo* by pyruvate.

We have noted that DL-lactate stimulated adenylate cyclase *in vitro* (1). Figure 3 shows that 60 mM DL-lactate also stimulated the enzyme *in vivo*; the extent of stimulation was considerably lower than that produced by pyruvate, in accord with our *in vitro* studies (1).

Responsiveness of adenylate cyclase to pyruvate in cells harvested at different growth phases. All the cells that were used in the experiments described above were harvested at log phase. The question arose whether adenylate cyclase in other phases of cell growth also responded to pyruvate. Therefore, we harvested cells at early log, midlog, and early stationary phases and incubated the cells sepa-

rately in the presence or absence of pyruvate. Figure 4 shows that pyruvate accentuated the accumulation of intracellular [³H]cAMP in all these cells, indicating that adenylate cyclase can be stimulated by pyruvate throughout the various phases of cell growth.

Although the percentage of conversion of [³H]adenosine to [³H]cAMP in the young cells was less than that in the older cells, the extent of stimulation by pyruvate was comparable. The lower [³H]cAMP in the young cells was not due to a diminished cellular uptake of [³H]adenosine, which was comparable in these cells (data not shown). The intracellular level of cAMP represents the balance of the rate of synthesis and that of degradation. The lower [³H]cAMP level in the younger cells may be a

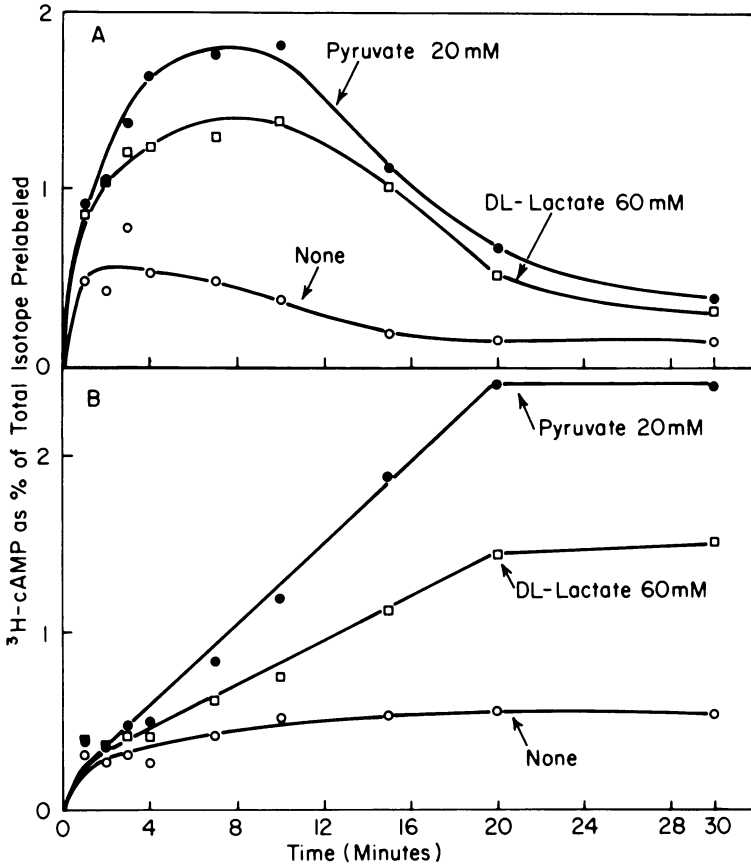


FIG. 3. Effect of pyruvate and DL-lactate on the intracellular (A) and extracellular (B) accumulation of ^3H cAMP in *B. liquefaciens*. Fifteen milliliters of a washed cell suspension was prelabelled with $600 \mu\text{Ci}$ of ^3H adenosine prior to their exposure to 20 mM pyruvate or 60 mM DL-lactate. At the times indicated, 1-ml samples were withdrawn and the ^3H cAMP was isolated from the medium and the trichloroacetic acid extract was determined.

result of lower adenylate cyclase activity or a smaller ratio of the activities of the synthetic to degradative enzymes. The activities of the two enzymes in the homogenate from cells harvested at different times after inoculation were therefore examined. As shown in Table 1, both enzymic activities were higher in younger cells; however, the ratio of the synthetic activity, relative to the degradative activity, was lower in these cells. This smaller ratio of the two enzymic activities could account for the lower percentage of intracellular ^3H cAMP in the young cells in Fig. 4. On the other hand, a smaller intracellular conversion of ^3H adenosine to ^3H ATP could also give the same results.

Functional state of adenylate cyclase in cells of different growth phases. Because the amount of ^3H cAMP synthesis in *B. liquefaciens* was a function of pyruvate concentration in

the medium (see Fig. 2), it was important to determine the minimum level of pyruvate which stimulates adenylate cyclase in vivo. Table 2 shows that 2 mM pyruvate definitely stimulated ^3H cAMP synthesis. This concentration of pyruvate was present in the medium in early log phase of cell growth (see Fig. 1). Thus, even before the microorganism reached log phase, adenylate cyclase existed in a stimulated state; conceivably stimulation of adenylate cyclase by pyruvate became more pronounced at stationary phase, during which time the level of pyruvate reached 20 mM (see Fig. 1).

The growth rate of *B. liquefaciens* in a medium containing arginine or $(\text{NH}_4)_2\text{SO}_4$ as nitrogen source was comparable to that in a medium containing DL-alanine. However, as shown by Ide et al. (5), the concentration of medium cAMP was much lower when the cells were grown in arginine or in ammonium chlor-

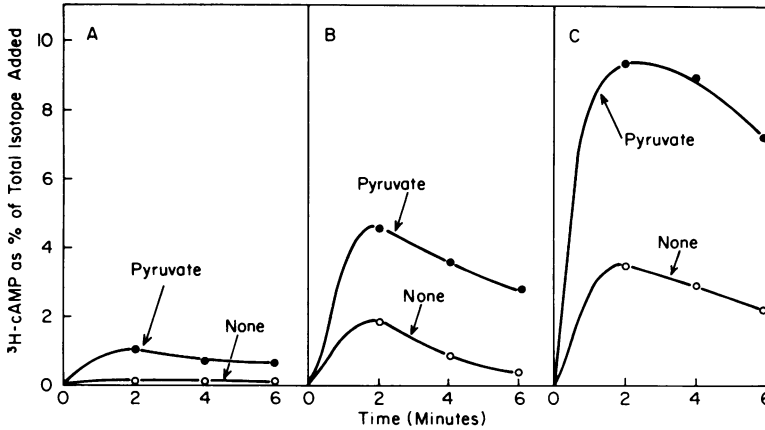


FIG. 4. Effect of pyruvate on the extracellular accumulation of [³H]cAMP in *B. liquefaciens* of different cell ages. Cells harvested at different phases of growth were resuspended in medium A to a comparable cell density as determined by turbidity reading at 650 nm. Three milliliters of a washed cell suspension was added to a 25-ml Erlenmeyer flask containing 3 ml of medium A, 100 μCi of [³H]adenosine, and 0 or 200 mM pyruvate. At the times indicated, 1-ml samples were withdrawn and the [³H]cAMP was isolated from the medium and trichloroacetic acid extract was determined. (A) Early log cells; (B) midlog cells; and (C) early stationary cells.

TABLE 1. Adenylate cyclase and phosphodiesterase activities in a crude homogenate of *B. liquefaciens* harvested at various times after inoculation^a

Cell age (A ₆₅₀)	Adenylate cyclase (pmol/mg of protein/min)	Phosphodiesterase (pmol/mg of protein/min)	Adenylate cyclase/phosphodiesterase
1.7	318	29	11
2.7	393	84	5
7.1	139	9	15
9.6	131	7	19
11.0	184	9	20
14.0	186	4	47

^a A cell suspension (40 ml) was removed at different times and centrifuged at 12,000 × g for 10 min. The pellet was washed in cold growth medium and then sonicated for 30 s. The homogenate was assayed separately for adenylate cyclase and cAMP phosphodiesterase activities as described in Materials and Methods. A₆₅₀, absorbancy at 650 nm.

ide. Because the pyruvate level in the absence of DL-alanine was low (10 μM or less), it appears that the functional activity of adenylate cyclase is not critical for cell growth under this condition. Perlman and Pastan (13) reported that a mutant of *Escherichia coli* that lacked adenylate cyclase activity also grew relatively well.

Association of adenylate cyclase with cell membranes. Cell membranes prepared from *B. liquefaciens* according to the procedure of Kaback (6) contained adenylate cyclase, which could be solubilized readily by sonication. The membrane enzyme was also stimulated by pyr-

uvate and DL-lactate. It is probable that most, if not all, of the adenylate cyclase in this microorganism is associated with the cell membrane and that the soluble enzymes studied earlier by Hirata and Hayaishi (3) and by Chiang and Cheung (1) may have been detached from the bacterial cell membrane by sonication, an initial step in the purification procedure.

In conclusion, our experiments indicate that pyruvate stimulates adenylate cyclase in vivo and that the allosteric site for pyruvate is present in the enzyme throughout the various phases of cell growth.

TABLE 2. Effect of pyruvate concentration on the extracellular accumulation of [³H]cAMP *B. liquefaciens*^a

Medium pyruvate (mM)	Relative concn of extracellular [³ H]cAMP
0	100
0.2	103
2	147
5	194
10	225
20	242

^a Two milliliters of a washed cell suspension was added to a 25-ml Erlenmeyer flask containing 2 ml of medium A, 50 μCi of [³H]adenosine, and various concentrations of pyruvate. At the end of 8 min, 1-ml samples were withdrawn and the [³H]cAMP isolated from the medium was determined as described in Materials and Methods.

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William L. Maguire isolated and assayed cell membranes from *B. liquefaciens*, and Edwin Thomas assisted us greatly in the isolation of these membranes.

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ADDENDUM

The earlier report that the soluble adenylate cyclase of *Brevibacterium liquefaciens* was detached from the bacterial cell membrane was based on the observation that the enzyme in vitro was inactivated by a preliminary incubation with neuraminidase, phospholipase A, or phospholipase C (1). We subsequently failed to reproduce these earlier experiments. Nevertheless, the present finding, that membranes prepared from *B. liquefaciens* contained an active adenylate cyclase, supports the hypothesis that the soluble adenylate cyclase was detached from the cell membrane.

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