Regulation of Cyclic AMP Levels in *Arthrobacter crystallopoietes* and a Morphogenetic Mutant

R. W. HAMILTON† and P. E. KOLENBRANDER‡ *

*Department of Microbiology and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802*

Received for publication 16 December 1977

The extracellular levels of cyclic AMP (cAMP), cAMP phosphodiesterase activity, and adenylyl cyclase activity were measured at various intervals during growth and morphogenesis of *Arthrobacter crystallopoietes*. There was a significant rise in the extracellular cAMP level at the onset of stationary phase, and this rise coincided with a decrease in intracellular cAMP. The phosphodiesterase activity measured in vitro increased in the early exponential phase of growth as intracellular cAMP decreased, and, conversely, prior to the onset of stationary phase the phosphodiesterase activity decreased as the intracellular cAMP levels increased. Adenylyl cyclase activity was greater in cell extracts prepared from cells grown in a medium where morphogenesis was observed. Pyruvate stimulated adenylyl cyclase activity in vitro. A morphogenetic mutant, able to grow only as spheres in all media tested, was shown to have altered adenylyl cyclase activity, whereas no significant difference compared to the parent strain was detectable in either the phosphodiesterase activity or the levels of extracellular cAMP. The roles of the two enzymes, adenylyl cyclase and phosphodiesterase, and excretion of cAMP are discussed with regard to regulation of intracellular cAMP levels and morphogenesis.

A distinguishing characteristic of members of the genus *Arthrobacter* is their ability to undergo a unique morphogenetic cycle. Stationary-phase cells are spherical, but upon inoculation into a rich organic medium, the spheres elongate into rod-shaped cells and continue to grow and divide as rods throughout exponential growth. At the onset of the stationary phase, the rod-shaped cells return to a spherical form by regressive cell division or fragmentation of the rods.

*Arthrobacter crystallopoietes* offers an additional advantage for study in that the cellular morphology can be nutritionally controlled (1). Cells growing in a glucose-based minimal medium grow only as spheres and fail to undergo the normal morphogenetic cycle. However, addition of one of a number of specific amino acids or organic acids such as asparagine, succinate, or butyrate results in an increased growth rate and induction of sphere-to-rod morphogenesis. The inducer compound is the preferred substrate for growth, and its depletion from the medium results in resumption of the slower growth rate and a return to spherical-shaped cells.

In a previously published report (3) we demonstrated that cyclic AMP (cAMP) was involved in morphogenesis of *A. crystallopoietes*. In this report it was found that the intracellular concentration of cAMP remained relatively constant during growth of *A. crystallopoietes* as spheres in glucose-based medium (see Fig. 1A). However, after inoculation of glucose-grown cells into succinate-based medium, there was an immediate 30-fold rise in intracellular levels of cAMP, which then rapidly fell to a rather stable level throughout exponential growth (see Fig. 1B). This level was four- to fivefold higher than that found in glucose-grown cells. This initial peak of intracellular cAMP just preceded the morphological change of spherical- to rod-shaped cells. At the onset of the stationary phase, there was a second peak of intracellular cAMP, which rapidly dropped to a stable low concentration in stationary-phase cells. Again, this peak of intracellular cAMP just preceded a point of morphological change, i.e., the change from rod- to spherical-shaped cells.

An investigation of *A. crystallopoietes*, Mph-3, a morphogenetic mutant of *A. crystallopoietes* which is unable to undergo morphogenesis and grows solely as spheres in both succinate- and glucose-based media, revealed the absence of the two peaks of intracellular cAMP (3). Moreover, the intracellular cAMP level in spherical...
cells growing in succinate-based medium was nearly identical to the parent strain growing as spheres in glucose-based medium (see Fig. 1C).

This report is concerned with the mechanisms involved in the control of cAMP levels during growth and morphogenesis of *A. crystallopoietes*. These mechanisms include the extraction of cAMP, hydrolysis of cAMP by phosphodiesterase, and formation of cAMP by adenylyl cyclase. The regulation of adenylyl cyclase activity and the relationship of cAMP to catabolite repression in *A. crystallopoietes* compared to that reported in other procaryotic systems are discussed.

**MATERIALS AND METHODS**

**Strains and culture conditions.** A culture of *A. crystallopoietes* (ATCC 15481) was obtained from J. C. Ensign, Department of Bacteriology, University of Wisconsin. The morphological mutant of *A. crystallopoietes*, Mph-3, was obtained as previously described (3). Cultures were grown in a mineral salts phosphate medium as described previously (3). Carbon sources, glucose and sodium succinate, were filter sterilized separately and added after autoclaving to the mineral salts-phosphate growth medium.

For measurement of extracellular cAMP, cultures were grown at 30°C with shaking aeration in 1,500-ml capacity Erlenmeyer flasks containing 200 ml of medium. Bacterial growth was monitored by use of a Klett-Summerson colorimeter with a red filter (660 nm).

Cells used to prepare cell extracts for assay of phosphodiesterase and adenylyl cyclase were grown in a 20-liter fermenter (Microferm Fermenter model MF-1285, New Brunswick Scientific Co., New Brunswick, N.J.). Incubation was at 30°C, with aeration at 40 liters/min and agitation set at 350 rpm.

**Assay of cAMP.** The protein-binding assay of Gilman (2) was used to measure cAMP levels as previously described (3). Concentrations of cAMP in bacterial extracts were determined from a standard curve, which was linear in the range between 1 and 20 pmol of cAMP.

Extracellular cAMP was measured by filtering 10 ml of culture fluid through a membrane filter (Millipore Corp., 0.45-μm porosity, 25 mm in diameter) into a screw-capped test tube which was then capped and heated for 10 min at 95°C. Samples of the filtered culture medium were assayed in duplicate for cAMP by the protein-binding assay by use of test kits supplied by Boehringer Mannheim Corp.

**Preparation of cell extract for enzyme assays.** Samples of 500 to 2,000 ml were removed from the fermentor and immediately cooled to 4°C with ice. The bacterial cells were pelleted by centrifugation at 15,000 × g for 20 min at 4°C; the supernatant was removed and discarded. Cell pellets were suspended in 5 to 10 ml of 10 mM tris(hydroxymethyl)amino- methane-hydrochloride (pH 8.0), and the bacterial cells were disrupted by three passages through an Amino French pressure cell at 20,000 lb/in². The resulting crude extract was clarified by centrifugation at 15,000 × g for 15 min. The clarified supernatant containing the enzyme activities was removed and stored at −55°C until assayed. Protein concentration of the clarified crude extracts was determined by the method of Lowry et al. (9) using bovine serum albumin as the standard.

**Phosphodiesterase assay.** The activity of cAMP phosphodiesterase was assayed with slight modification of the procedure described by Thompson and Appleman (24). The complete reaction mixture contained (final concentration in 250 μl): 32 mM tris(hydroxymethyl)aminomethane-maleate-hydrochloride (pH 7.0), 2 mM dithiothreitol, 6.25 mM MgCl₂, 0.5 mM cAMP, and enough [³H]cAMP (20 Ci/mmol) to give approximately 200,000 cpm per assay tube. The reaction was started by the addition of 100 μl of crude extract (containing 0.2 mg of protein). The reaction was incubated for 30 min at 30°C and then terminated by heating for 2.5 min at 95°C. After cooling to 30°C, 50 μg (in 50 μl of distilled water) of snake venom (*Crotalus atrox*) was added, and the mixture was incubated for 15 min. The reaction vessels (10- by 75-mm borosilicate test tubes) were placed in an ice bath and cooled to 4°C, and 1 ml of 1:3 (wt/vol) 16% ethanolic slurry of Bio-Rad resin (AG2-X8, 200 to 400 mesh) was added, blended in a Vortex mixer, and allowed to equilibrate for 15 min. The tubes were then blended in a Vortex mixer and centrifuged for 5 min at maximum speed in a swinging-bucket rotor in a clinical centrifuge. The resin formed a pellet at the bottom of the test tube, and 0.5 ml of the resulting supernatant was drawn off and added to 10 ml of Hydromix (Yorktown Research, Hackensack, N.J.) for scintillation counting. Crude extract added to the reaction mixture and immediately boiled was used as a control. Under these conditions, the formation of product in the phosphodiesterase reaction mixture was linear with respect to time for 60 min.

**Adenylyl cyclase assay.** The adenylyl cyclase assay followed the two-column method of Salomon et al. (18) with minor modification. The reaction mixture contained 25 mM Tris-hydrochloride (pH 7.9), 5.0 mM MgCl₂, 20 mM creatine phosphate, 100 U of creatine phosphokinase per ml, 0.75 mM cAMP, 5 mM ATP, 1,500,000 cpm of a-[³²P]ATP (11 Ci/mmol), 1 mg of bovine serum albumin per ml, 2.5 mM dithiothreitol, and, if indicated, 75 mM sodium pyruvate. The reaction was started by the addition of 20 μl of crude extract (40 μg of protein), bringing the total incubation mixture to 50 μl. The reaction mixture was incubated at 30°C for 45 min, and the reaction was terminated by heating for 2 min at 95°C. [³H]cAMP (approximately 30,000 cpm) in 50 μl of distilled water was added to monitor cAMP recovery in the following chromatographic procedures.

After addition of 0.8 ml of water to each reaction tube, the tube contents were mixed and decanted into a column (2.5-ml Sarstedt pipette tips, Walter Sarstedt Inc., Princeton, N.J.) containing 1 ml of Bio-Rad cat- ion-exchange resin (AG-50W-X8, 200-400 mesh, hydrogen form). The eluants from this and two successive 1-ml glass-distilled water washes were discarded. A 3-ml volume of glass-distilled water was then added to the column; the eluant passed directly into a column containing 0.6 g of neutral alumina (which had been previously washed with 8.0 ml of 0.1 M imidazole), and
the subsequent eluant was discarded. A 4-ml volume of 0.1 M imidazole was then added to the column, and the eluant was collected directly into a scintillation vial. A 10-ml volume of Hydromix (Yorktown Research) was added for scintillation counting of the aqueous sample.

The columns can be recycled for further use by addition of 5 ml of 0.1 N HCl to the Bio-Rad AG 50WX8 cation exchange columns, followed by a 10-ml, glass-distilled water wash. The neutral alumina columns can be recycled by washing with 10 ml of 0.1 M imidazole.

In A. crystallopoietes, the rate of reaction of adenylate cyclase was maximal at pH 7.9 using tria-(hydroxymethyl)aminomethane-hydrochloride buffer. A divalent cation was essential for the reaction, and addition of 5.0 mM Mg\(^{2+}\) resulted in optimal activity. It was found that Mn\(^{2+}\) at 5.0 mM concentration could replace Mg\(^{2+}\), and was equally effective as Mg\(^{2+}\). Often, cell-free preparations of adenylate cyclase are unstable, and although this is more of a problem in particulate preparations, e.g., plasma membranes, it can also be a problem in soluble preparations (20). It was necessary to stabilize the adenylate cyclase of A. crystallopoietes with 1 mg of bovine serum albumin per ml to achieve proportional product formation over an extended period of time.

Many crude preparations used for adenylate cyclase assays contain high levels of ATP-degrading enzymes. Therefore, the ATP-regenerating system was added to the reaction mixture. Creatine phosphate and creatine phosphokinase were used for this purpose as the phosphoenolpyruvate and pyruvate kinase-regenerating system has been demonstrated to be capable of activating or inhibiting adenylate cyclase (30). The 0.75 mM cAMP which was added to the reaction mixture served a dual purpose; it reduced interference due to phosphodiesterase activity and it could prevent an accelerated formation of product during boiling, which was used to terminate the reaction (20).

The amount of cyclic nucleotide (product) degradation in the assay was examined using A. crystallopoietes extract from succinate-grown cells containing 8 U of phosphodiesterase activity (the most active preparation tested) as measured in the phosphodiesterase assay. This phosphodiesterase preparation is catalytically capable of degrading at least 50% of the cAMP present in the adenylate cyclase reaction mixture. However, under the conditions employed in the adenylate cyclase assay, the phosphodiesterase activity was much lower and capable of degrading only 2.5% of the cAMP (Table 1). This low value of activity probably resulted because the higher pH that was required for the adenylate cyclase activity (pH 7.9) was well above the optimal pH for phosphodiesterase activity (pH 7.0). The product of the adenylate cyclase assay was identified as cAMP (Table 2) by using thin-layer chromatography as outlined below. The formation of cAMP during the course of the adenylate cyclase reaction was linear for at least 45 min assayed in the presence or absence of pyruvate.

**Thin-layer chromatography.** The thin-layer chromatographic analysis employed was a modified procedure of Tao (22). Using a capillary tubing, the radioactive sample was applied to the cellulose thin-

<table>
<thead>
<tr>
<th>Table 1. Phosphodiesterase activity detectable in the reaction mixture used to measure adenylate cyclase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (cpm) recovered from:</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Expta</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
</tbody>
</table>

* a The reaction was identical to the standard reaction of adenylate cyclase, except for the addition to the reaction mixture of 2,000,000 cpm of [\(^3\)H]cAMP (20 Ci/mmol) to monitor phosphodiesterase activity. After 45 min of incubation at 30°C, the reaction was terminated by heating, and the reaction tubes were centrifuged in a clinical centrifuge to pellet denatured protein. A 40-ml volume of the 50-ml reaction mixture was spotted and developed on the cellulose chromatogram.

<table>
<thead>
<tr>
<th>Table 2. Identification of product of adenylate cyclase assay as cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expta</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
</tbody>
</table>

* a The reaction was the standard reaction for adenylate cyclase as described in Materials and Methods. After terminating the reaction by heating, two samples were processed by the two-column method, and two samples were centrifuged in a clinical centrifuge to pellet denatured protein. A 5-ml volume of each supernatant was spotted onto a cellulose thin-layer sheet, which was developed as described in Materials and Methods.

Percent identified as cAMP = [(counts per minute of cAMP from columns)/counts per minute from cAMP spot] × 100.

layer sheet (Eastman Chromogram Sheet 6064, without fluorescent indicator) and dried under a stream of cool air, and 5 μl of a 5-mg/ml nonradioactive cAMP solution was added as carrier. The sheet was placed in an Eastman Chromogram developing apparatus (model 6071) and developed at room temperature with a solvent system composed of 1.0 M ammonium acetate–95% ethanol (30:75, vol/vol). After the solvent front had migrated to the top of the thin-layer sheet, the chromatogram was removed and allowed to dry. The nucleotides were visualized under short-wave UV light (254 nm). Each of the sample lanes was cut into 8-mm strips, and each strip was placed in 7 ml of scintillation fluid consisting of 6.4 g of 2,5-diphenyloxazole dissolved in 800 ml of toluene and 300 ml of 2-methoxyethanol. Radioactivity was measured in a Beckman model LS 3155T scintillation counter.

The cAMP, with an Rf of 0.44, was well separated from ATP, ADP, and AMP, which have Rf values of
0.02, 0.04, and 0.11, respectively, and from adenosine and adenine, which have respective $R_f$ values of 0.63 and 0.58 (22).

Materials. [8-$\beta$H]cAMP (specific activity, 20 Ci/mmol) was purchased from Schwarz/Mann (Orangeburg, N.Y.), $\alpha$-[32P]ATP (11 Ci/mmol) was supplied by Amersham/Searle (Arlington Heights, Ill.). Cyclic nucleotides, their dibutyryl derivatives, phosphorylated nucleic acid bases, dithiothreitol, imidazole, creatine phosphate, creatine phosphokinase, snake venom, and neutral alumina (WN-3) were purchased from Sigma Chemical Co. (St. Louis, Mo.). 2-Methoxyethanol was supplied by Eastman Kodak Co. (Washington, D.C.). All other chemicals were obtained commercially and were of the highest purity available.

RESULTS

Extracellular concentration of cAMP. The concentration of cAMP found in the growth medium for *A. crassa* was growing in glucose-based medium is illustrated in Fig. 1A. No cAMP was immediately detectable, but a small amount (0.5 $\times$ 10$^{-9}$ M) was found at mid-exponential growth and continued to increase at a slow rate until the onset of the stationary phase. At this point there was a significant rise in the external cAMP concentration (Fig. 1A, dotted line). After this rise, the level of cAMP in the medium remained relatively constant throughout the stationary phase at $20 \times 10^{-9}$ M.

In succinate-based medium the extracellular cAMP could be detected early in the exponential growth phase (Fig. 1B). The level rose relatively slowly throughout this growth phase despite the elevated level of internal cAMP during exponential growth. It was demonstrated previously that during growth in succinate there is a sudden increase and ensuing decrease in internal cAMP at the onset of stationary phase, coincident with the change from rod- to spherical-shaped cells (3). It should be noted that, just prior to the highest point in this peak of internal cAMP, the external cAMP level increased and continued to increase as the internal level decreased. It is possible that the internal level after the second peak was regulated by excretion of the cyclic nucleotide. During stationary phase the external level continued to increase but at a markedly slower rate and reached levels of approximately $90 \times 10^{-9}$ M.

The external cAMP levels in the morphogenetically altered mutant, Mph-3, growing on succinate nearly paralleled that of the parent strain, but reached slightly lower levels (about $75 \times 10^{-9}$ M) during stationary phase (Fig. 1C). Once again the rise in external cAMP concentration occurred at the point of decrease in internal cAMP. However, the change in the internal level was slight when compared to the parent strain, whereas the external levels of cAMP were nearly identical in the two strains. It is possible that in Mph-3 any cAMP synthesized during the onset of stationary phase is immediately excreted into the medium and not allowed to accumulate in the cell.

The pattern of cAMP excretion (as well as internal cAMP levels) by Mph-3 growing in glucose-based medium was nearly identical to the parent strain growing in this medium (Fig. 1A). However, the levels of external cAMP only reached $15 \times 10^{-9}$ M during stationary phase (data not shown).

Phosphodiesterase activity during growth. The phosphodiesterase activity measured using cells of the parent strain grown in glucose-based medium is shown in Fig. 2A. Mid-exponential glucose-grown cells were transferred to fresh glucose medium, and cells were harvested and assayed for phosphodiesterase activity at various intervals during growth. The level of phosphodiesterase activity remained relatively constant at 2.0 U throughout exponential growth. In early stationary phase there was an increase in activity which soon leveled off at 4.0 U of activity. The pattern of phosphodiesterase activity in the mutant strain, Mph-3, grown in glucose-based medium was similar to the parent as demonstrated in Fig. 2D, although the exponential values of activity were slightly higher at 3.0 U, and the decrease in activity during exponential phase began earlier in the mutant than in the parent strain.

An examination of the parent strain growing in succinate-based medium gave a different profile of activity (Fig. 2B). The levels of phosphodiesterase activity during exponential growth rose to a much higher value than found in glucose-grown cells. The activity of phosphodiesterase continued to rise, from 3.5 to 8.0 U, as the internal cAMP level decreased from its initial peak value (cf. Fig. 1B). This suggests that the internal concentration of cAMP during exponential growth may be controlled by the phosphodiesterase activity since no rise in external cAMP was found at this point, and the level of internal cAMP falls much too rapidly to be due to dilution of cyclic AMP by cell growth. In late exponential phase there was a drop in phosphodiesterase activity, and this drop just precedes the second rise in the internal cAMP level (Fig. 2B, dotted line). During the stationary phase the phosphodiesterase activity remained relatively constant at 4 U, which is similar to the level observed in glucose-grown cells. Mph-3 growing in succinate-based medium showed a pattern of phosphodiesterase activity which was nearly identical to wild-type cells on that medium, except for a slight increase in activity of phospho-
Adenylate cyclase activity during growth. A comparison of the internal levels of cAMP in Mph-3 and parent strain, each growing on succinate, indicated an alteration in the regulation of cAMP metabolism in the mutant. However, an investigation of the external cAMP levels and phosphodiesterase activities of the mutant showed they were nearly identical to those observed in wild-type cells. The absence of significant differences in the mutant and parent strains regarding the external cAMP levels and phosphodiesterase activity led to an investigation of adenylate cyclase, the enzyme responsible for the formation of cAMP.

The activity of adenylate cyclase in the parent
strain during growth in glucose-based medium is shown in Fig. 3A. As expected from the internal cAMP data, the adenylate cyclase activity remained low throughout exponential growth (less than 3 U). There was a slight increase in activity prior to the onset of stationary phase which coincided with the excretion of cAMP into the cytoplasm.
medium. The activity continued to increase until late stationary phase, where the activity reached 15 U.

When glucose-grown cells (100 Klett units, adenylyl cyclase activity, 0.7 U) were transferred to succinate-based medium, there was an immediate increase of adenylyl cyclase activity to approximately 15 U, or a 20-fold rise in activity (Fig. 3B). The pattern of adenylyl cyclase activity fluctuated during exponential growth with an increase to 27 U of activity early in growth and decreasing to 8 U of activity at mid-logarithmic growth. There was a small increase in activity to 15 U before stationary phase, and a rise to 50 U of activity in stationary-phase cells.

The mutant, Mph-3, showed an altered pattern of activity when grown in succinate-based medium (Fig. 3C). There appeared to be little change in the adenylyl cyclase activity, which remained fairly constant around 2 to 4 U throughout exponential growth. In general the activity appeared to be four- to fivefold less than the parent strain, which is consistent with the decreased levels of intracellular cAMP found in Mph-3.

The pattern of adenylyl cyclase activity in Mph-3 grown in glucose-based medium was also altered from that of the wild type (Fig. 3D). However, in this medium the activity was greater than that of the parent strain. The significance of this finding is not certain, since the internal levels of cAMP in Mph-3 parallel the levels of the wild-type strain on glucose. When compared to the parent strain, the difference in adenylyl cyclase activity in the mutant during growth in either succinate or glucose medium is evident and suggests that the adenylyl cyclase is altered in Mph-3.

Adenylyl cyclase activity in the presence of pyruvate. In many prokaryotic systems the activity of adenylyl cyclase is affected by small molecules (5, 6, 12, 23, 26). Pyruvate and other keto-group compounds have been shown to stimulate adenylyl cyclase activity of *Brevibacterium liquefaciens* both in vivo and in vitro (4, 10). Pyruvate was also found to stimulate adenylyl cyclase activity in *A. crystallopoietes*.

An examination of the adenylyl cyclase activity of glucose-grown *A. crystallopoietes* in the presence of 75 mM pyruvate is shown in Fig. 4A. The activity appeared to remain relatively constant throughout growth at approximately 55 U of activity, except for a slight decrease to 45 U at the onset of stationary phase. This activity is more than 20 times the activity observed with no pyruvate. When mid-exponential glucose-grown cells (activity 60 U) were transferred to succinate, there was an immediate decrease in adenylyl cyclase activity to about 20 U of activity (Fig. 4B). This value rose to 35 U in 3 h.

![Fig. 4](http://jb.asm.org/) Adenylyl cyclase activity measured in the presence of pyruvate and cell growth of *A. crystallopoietes* in glucose (A) or succinate (B); and *A. crystallopoietes*, Mph-3, growing in succinate (C) or glucose (D). Adenylyl cyclase activity is plotted as units of activity and 1 U equals 1 nmol of cAMP formed from ATP per min per mg of protein. Dotted line is a reference line for comparison with other figures. Symbols: O, bacterial growth; □, units of adenylyl cyclase activity.
activity then dropped until the cells reached about 80 Klett units of turbidity. Just prior to the second peak of internal cAMP (Fig. 4B, dotted line), there was a peak in the adenylate cyclase activity (50 U), and the activity remained relatively high throughout stationary phase. Note that the adenylate cyclase of glucose-grown cells was capable of great stimulation by pyruvate, whereas the succinate-grown cells exhibited a decreased stimulation. Hence, in the presence of pyruvate the activity of adenylate cyclase in glucose-grown cells was actually greater than succinate-grown cells, which is just opposite of the results obtained by assaying adenylate cyclase activity without pyruvate in the reaction mixture. It may be significant that the peak of adenylate cyclase activity in succinate-grown wild-type cells just preceded, but paralleled, the second peak of internal cAMP.

The pattern of adenylate cyclase activity was altered in the mutant with respect to the parent strain. In succinate-based medium the activity peaked initially at 15 U, but then remained low at approximately 5 U throughout the exponential phase of growth (Fig. 4C). This activity is three- to fourfold lower than in the wild-type strain. The decreased activity of adenylate cyclase in the mutant growing in succinate, with respect to the parent, correlates well with the lack of elevated internal cAMP in the mutant, and suggests that the failure to produce cAMP in normal amounts is due to an alteration in adenylate cyclase. In glucose-based medium the initial activity was slightly higher than the parent strain (Fig. 4D), but the activity continued to decrease throughout exponential phase. The activity remained constant during stationary phase at 25.0 U. These data suggest that small molecules, e.g., pyruvate, may be involved in the regulation of adenylate cyclase activity, in vivo, in A. crystallopoietes.

DISCUSSION

Saier et al. (17) showed that in Escherichia coli and Salmonella typhimurium extraction of cAMP was an important mechanism in the regulation of internal cAMP levels. Since the dry weight of A. crystallopoietes growing in succinate, at the point where the external level of cAMP began to rise (dotted line, Fig. 1B), was equal to 0.77 mg (dry weight) per ml of culture, and the peak of internal cAMP was equal to 100 pmol/mg (dry weight), it can be calculated that the intracellular concentration of cAMP was about \(3 \times 10^{-5}\) M. To determine if all of the intracellular cAMP was excreted into the culture medium or if additional cAMP was synthesized at this time and immediately excreted, the intracellular and extracellular levels of cAMP were compared by expressing both as picomoles of cAMP per milliliter of culture. When expressed in this way, the intracellular concentration at the point in growth described above (Fig. 1B, dotted line) was 77 pmol/ml of culture and fell to 5.0 pmol/ml of culture as the cells entered the stationary phase of growth. Note that this intracellular change of 72 pmol/ml of culture is nearly identical to the rise in extracellular cAMP of 68 pmol/ml of culture. Similar calculations for glucose-grown cells give a cellular concentration change of 8.7 pmol/ml of culture, whereas the extracellular concentration changes by 17 pmol/ml of culture. Thus, it appears that cAMP is excreted into the medium, resulting in a lowering of internal cAMP level at the onset of the stationary phase in A. crystallopoietes. When Mph-3 was grown in succinate-based medium, a change in external cAMP level of 65 pmol/ml of culture was observed at the onset of the stationary phase. This value was nearly identical to that found in the parent strain. However, the intracellular level was calculated to change only 3 pmol/ml of culture. A possible explanation for this phenomenon is that, at the onset of the stationary phase, Mph-3 synthesizes nearly an equivalent amount of cAMP to that in the parent strain, but that all cAMP made is immediately excreted, which prevents an intracellular accumulation of cAMP.

The profile of phosphodiesterase activity in the parent strain, growing in succinate-based medium, suggests that phosphodiesterase activity is involved in the regulation of internal cAMP levels during exponential growth. At the beginning of exponential growth, the phosphodiesterase activity continues to rise as the intracellular cAMP level decreases from its initial peak value. An opposite pattern is found before the second peak of cAMP (Fig. 2B, dotted line), i.e., the phosphodiesterase activity progressively decreases prior to the second increase in internal cAMP. The fact that little extracellular cAMP can be detected during the decrease of the initial peak of intracellular cAMP (cf. Fig. 1B) further substantiates the role of the phosphodiesterase enzyme in the regulation of internal cAMP concentration during exponential growth. The pattern of phosphodiesterase activity in Mph-3 during growth in succinate- or glucose-based medium does not appear appreciably altered from that of the parent strain, which suggests that an altered phosphodiesterase activity is not responsible for the lower intracellular cAMP level in Mph-3 growing in succinate-based medium.

Opposite to the findings during exponential growth, the excretion of cAMP appears to be involved in the regulation of internal cAMP levels during the stationary phase of growth.
Only during the stationary phase do both succinate- and glucose-grown cells have equivalent phosphodiesterase activities and nearly equivalent levels of intracellular cAMP. However, there is an increase in adenylate cyclase activity during the stationary phase of growth (Fig. 3). Therefore, it is likely that regulation of internal cAMP concentration during the stationary phase involves the concerted action of adenylate cyclase, phosphodiesterase, and excretion of cAMP.

The activity of adenylate cyclase in *A. crystallopoietes* measured in the absence of pyruvate reflects the activity expected from the intracellular cAMP data. There is little adenylate cyclase activity measurable in glucose-grown cells, but upon inoculation of mid-exponential glucose-grown cells into succinate-based medium, there is an immediate 20-fold increase in adenylate cyclase specific activity, which correlates well with the finding of an initial peak of intracellular cAMP. Also, as predicted by the external cAMP and phosphodiesterase activity data, the activity of adenylate cyclase in Mph-3 is much lower than in the parent strain, remaining at least four- to fivefold lower throughout exponential growth.

Similar to *B. liquefaciens* (10), the activity of adenylate cyclase in *A. crystallopoietes* is capable of stimulation by pyruvate. The finding of greater adenylate cyclase activity in glucose-grown cells, compared to succinate-grown cells, in the presence of pyruvate is not consistent with the low internal cAMP level found in cells grown in this medium. Perhaps the adenylate cyclase activity in the presence of pyruvate is a measure of the maximum possible rate of cAMP synthesis, and is not a true measure of the in vivo activity. Alternatively, the activity of succinate-grown cells may already be stimulated by small molecules which were present in vivo, which prevent further stimulation by pyruvate in vitro. The observation of a peak in adenylate cyclase activity in succinate-grown wild-type cells just preceding, but paralleling, the second peak of intracellular cAMP may indicate that small molecules are regulatory during this later stage of growth, but are not effective at earlier stages. This is further substantiated by the absence of a pyruvate-stimulated peak of adenylate cyclase activity paralleling the first peak of intracellular cAMP. Although the interpretation of these findings is not clear, the observation of increased adenylate cyclase activity by pyruvate does suggest that metabolic intermediates may play a role in vivo in the regulation of adenylate cyclase activity in *A. crystallopoietes*.

The activity of pyruvate-stimulated adenylate cyclase in Mph-3 growing in succinate-based medium is four- to fivefold less than that of the parent strain grown in the same medium. Moreover, a similar reduction in activity in the mutant compared to the parent strain was observed when adenylate cyclase was assayed in the absence of pyruvate. These findings suggest that an alteration in the adenylate cyclase activity in Mph-3 may be a major cause of the reduced intracellular level of cAMP which is involved in the sphere-to-rod-to-sphere morphogenesis in the parent strain.

The mechanism of regulation of the cAMP levels in *E. coli* has recently been examined and involves interaction of the adenylate cyclase enzyme with the phosphoenolpyruvate-dependent phosphotransferase system (13). The model includes the concept that adenylate cyclase is normally complexed with enzyme I of the phosphotransferase system. Adenylate cyclase can express a high level of activity, when enzyme I exists in the phosphorylated form, but its activity is low when enzyme I is dephosphorylated. The high-activity state is favored by the presence of phosphoenolpyruvate and the absence of glucose, whereas the opposite conditions favor a low-activity state. Hence, the observation of low levels of cAMP during growth of *E. coli* in glucose-based medium (14) is due to the dephosphorylation of enzyme I and the subsequent inactivation of adenylate cyclase.

However, there is no evidence that cAMP is regulated in *A. crystallopoietes* in the same manner as in *E. coli*, or that cAMP is involved in catabolite repression. *A. crystallopoietes* is an obligate aerobe with an oxidative physiology, in contrast to the facultative anaerobes (e.g., *E. coli*), and *A. crystallopoietes* utilizes organic acids preferentially to glucose and other carbohydrates. Krulwich and Ensign (7) studied the diauxic growth of *A. crystallopoietes*. Unlike *E. coli*, glucose transport was found to involve active transport with a glucose-specific permease. This system was inducible by glucose, but in the presence of succinate the synthesis of the permease was repressed, and the activity was inhibited. This led them to the conclusion that glucose utilization was directly inhibited by the organic acid (i.e., succinate). Schechter et al. (19) investigated enzyme induction and repression in *A. crystallopoietes*. Histidase induction by histidine was reduced by incubation of cells with either glucose or succinate. Each required an extended period of time for maximum effect (greater than 100 min). Also, succinate or glucose inhibited the transport of histidine. Induction of L-serine dehydratase by glycine was severely and permanently repressed by glucose, but unaffected by succinate. Isocitrate lyase was severely repressed by succinate or fumarate, but
glucose had no effect. In each case, exogenously added cAMP had no effect on enzyme production or repression. Schechter et al. (19) suggested that, in _A. crystallopoietes_, the extent to which a compound is involved in repression has nothing to do with its ready utilization as a growth substrate, but may be related to the pathway by which it is utilized. Thus, it appears unlikely that, in _A. crystallopoietes_, cAMP plays a significant role in catabolite repression. Regulation of adenylate cyclase in _A. crystallopoietes_ must also be different from _E. coli_, since no phosphotransferase system for glucose or other sugars (T. A. Kruwich, personal communication) has been found to exist in _A. crystallopoietes_.

This conclusion is supported by recent findings in _Pseudomonas aeruginosa_. _P. aeruginosa_ is also an obligate aerobe which has no apparent phosphotransferase system, which utilizes organic acids preferentially to glucose, and in which synthesis of inducible enzymes for carbohydrate utilization is strongly repressed by succinate (8, 11, 15, 16, 25). Siegel et al. (21) investigated the role of cAMP in catabolite repression in _P. aeruginosa_. The intracellular cAMP remained at a constant value regardless of the carbon source, and exogenous cAMP failed to reverse catabolite repression. They concluded that cAMP is unlikely to have a role in catabolite repression in _P. aeruginosa_. It is possible that both _P. aeruginosa_ and _A. crystallopoietes_, which exhibit oxidative metabolism and preferential growth on organic acids, fail to utilize cAMP as a controlling element in catabolite repression.

**ACKNOWLEDGMENTS**

This study was supported by a Public Health Service Biomedical Support grant. R. W. Hamilton was supported by a Public Health Service training grant in microbiology, GM00512-15, from the National Institute of General Medical Sciences.

**LITERATURE CITED**


Downloaded from http://jb.asm.org/ on July 10, 2017 by guest