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Adenylate Energy Charge in *Escherichia coli* CR341T28 and Properties of Heat-Sensitive Adenylate Kinase

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Escherichia coli strain CR341T28 will not grow at temperatures above 34°C in liquid medium, and the adenylate kinase of this strain is heat sensitive. When a culture was shifted from a permissive (30°C) to a nonpermissive (36°C) temperature, the adenylate energy charge fell from 0.9 to 0.2, with a concurrent decrease in the number of viable cells and in the specific activity of adenylate kinase. When cultures of the temperature-sensitive strain were grown at temperatures above 30°C, the adenylate energy charge, the specific activity of adenylate kinase, and the growth rate were lower than the corresponding parameters for the parental strain. By isotopic labeling of the adenine nucleotides in vivo, it was determined that increasing growth temperatures between 30 and 34°C for the heat-sensitive strain resulted in a decrease in the adenosine triphosphate-to-adenosine monophosphate and adenosine triphosphate-to-adenosine diphosphate ratios. Between 26 and 30°C the adenosine triphosphate-to-adenosine diphosphate ratio was essentially normal in the temperature-sensitive strain, but the adenosine triphosphate-to-adenosine diphosphate ratio was decreased. The adenylate ratios in the parental strain did not change between 30 and 34°C. The adenylate kinase mass action ratio for each strain was essentially constant under all growth conditions. When assayed at 30°C, the affinities of the enzyme from the mutant strain were somewhat lower than those of the parent adenylate kinase. The mutant enzyme also did not exhibit the substrate inhibition that was observed at high adenosine monophosphate concentrations with the parental enzyme. An increase in the assay temperature from 30° to 40°C had little or no effect on the K_m values determined for the parental adenylate kinase, but caused the K_m values determined for the mutant adenylate kinase to increase by a factor of two or more.

Adenylate kinase catalyzes interconversion of the adenine nucleotides: AMP + ATP $\rightleftharpoons$ 2 ADP. This reaction provides the main route for reconversion of metabolically generated AMP to ADP (and subsequently to ATP). Adenylate kinase thus is an essential enzyme, and is present at high levels in energetically active tissues (20). Its activity is probably high enough in most cases to hold the reaction components near equilibrium. If so, the three-component AMP/ADP/ATP system is reduced to one degree of freedom with respect to mole fractions, relative concentrations, or concentration ratios, and the specification of the energy charge [(ATP + 0.5 ADP)/(ATP + ADP + AMP)] or of any two-component ratio fixes all mole fractions and concentration ratios. A severe decrease in the activity of adenylate kinase would restore a second degree of freedom and partially uncouple the two-component ratios from each other or from the energy charge.

The activities of major regulatory enzymes in vitro as a function of energy charge have been reviewed (1, 2). Regulatory enzymes in anabolic, or ATP-utilizing (U-type), sequences show an increase in activity as the energy charge increases from 0 to 1. Regulatory enzymes in catabolic, or ATP-regenerating (R-type), sequences show a decrease in activity as the energy charge increases. The midpoints and the steepest regions of the response curves of these enzymes to changes in energy charge in vitro occur at energy charge values above 0.80. These sensitive and oppositely directed responses appear to play a major role in the kinetic partitioning of metabolites between catabolic and anabolic sequences. They should also hold the value of the energy charge within a narrow range. This expectation has been borne out by analyses showing that in intact, growing, and actively metabolizing cells of many types the energy charge is stabilized at

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a value of about 0.90 (3–5), and protein synthesis appears to be depressed when the charge falls below this value (25, 26).

Escherichia coli CR341T28 is a heat-sensitive strain with a thermolabile adenylate kinase (6–8, 13) and a thermolabile glycerol-3-phosphate acyltransferase (10); both labilities apparently result from a single mutation (9, 10, 13). The adenylate kinase defect is the more thoroughly studied of the two. Adenylate kinase of this strain is inactivated at elevated temperatures (above 36 to 37°C) both in vivo and in vitro (6, 8, 13). The inactivation in vivo is associated with a rapid decline in the cellular ATP concentration and concomitant cell death (7, 13).

An objective of this study was to exploit the temperature sensitivity of adenylate kinase in *Escherichia coli* CR341T28 in an attempt to determine the consequences of limiting activity of this enzyme on growth and regulation. We hoped that growth of this strain at temperatures at which the activity of adenylate kinase was growth limiting would allow for some degree of uncoupling of the ATP/AMP and ATP/ADP ratios from each other and from the energy charge. It might then be possible to evaluate the relative impacts of these ratios, which might be considered in a sense to be component factors of the energy charge, on the growth rate. The simplest prediction was that the ADP/AMP and ATP/AMP ratios would decrease with increasing temperature. Changes, if any, in the ATP/ADP ratio and the energy charge could not be predicted.

We also wished to establish whether the reduced adenylate kinase activity in the mutant strain might be at least in part due to altered kinetic properties of the mutant enzyme, rather than merely to inactivation. Since the activity of this enzyme in the mutant cells appears to decrease with a rise in culture temperature between 30 and 34°C, it was also of interest to establish whether the kinetic properties of the mutant enzyme are affected by changes in temperature. We therefore purified adenylate kinase from the temperature-sensitive and parental strains and compared their kinetic properties over the relevant temperature range. The enzyme has been partially purified from *E. coli* by Holmes and Singer (16) and by Cousin et al. (8).

(Portions of these results have been presented previously [A. G. Chapman, C. C. Glembotski, and D. E. Atkinson, Abstr. 11th FEBS Meet. 1977, C-9, p. 816].)

MATERIALS AND METHODS

Organism. *Escherichia coli* strains CR341T28 f⁻ *thr leu B1 thy lacY met* and CR34 (19) were derived from *E. coli* K-12 (6, 8, 18). Strain CR341T28 is a temperature-sensitive mutant containing a thermolabile adenylate kinase (EC 2.7.4.3). This strain also has a thermolabile mutant form of glycerol-3-phosphate acyltransferase (EC 2.3.1.15) (13), the first enzyme in the phospholipid biosynthetic pathway, apparently as a consequence of the same mutation that causes adenylate kinase to be temperature sensitive (9). Strain CR34 (called the parent in this paper) is isogenic to strain CR341T28 except for the mutation in adenylate kinase and for the absence of a methionine requirement. Strain CR341 was not available for use during this study. Strain CR34 has been used in the past for comparison to strain CR341T28 (19).

Growth and sampling. Cells were grown aerobically at 30°C unless otherwise stated in medium containing 5 g of KH_2PO_4, 17 g of $K_2HPO_4 \cdot 3H_2O$, 2 g of $(NH_4)_2SO_4$, 0.2 g of $MgCl_2$, 5 g of glucose, 10 mg of thiamine-Cl, 200 mg each of thymidine, L-methionine, L-isoleucine, L-leucine, L-threonine, and L-valine, and 1.0 g of Casamino Acids (Difco Laboratories, Detroit, Mich.) per liter. Guanine (12.8 mg/liter) was added when [^{3}H]adenosine was to be incorporated into the adenine nucleotides. Starter cultures (50 ml) were grown at 28°C for 12 to 14 h in Erlenmeyer flasks with rotary shaking. Experimental cultures were incubated in gas dispersion flasks in a circulating water bath, with moist air forced through fritted-glass disks at a rate of 150 ml/min. Cell growth was followed by measuring the optical absorbance at 540 nm (A_{540}). Growth was followed by measuring the number of particles per ml with an Electrozone Celloscope (Particle Data, Inc., Elmhurst, Ill.) and by plating to determine the number of viable cells. A 0.1- to 0.5-ml sample of a $1{:}10^6$ dilution of the culture was mixed with 10 ml of a solution containing 0.5% agar and 1% nutrient broth, and the resulting colonies were counted. For enzyme purification, a 15-liter culture of *E. coli* CR341T28 was grown in a glass carboy with moist air forced through a fritted-glass tube at a rate of 150 ml/min; *E. coli* CR34 (parent) was grown in six 2,000-ml Erlenmeyer flasks each containing 1 liter of medium and aerated by rotary shaking. Cells were harvested by centrifugation in late exponential phase (A_{540} = 1.0 for strain CR341T28 and A_{540} = 2.0 for strain CR34), and washed twice with 50 mM Tris-chloride buffer, pH 7.4. For experiments involving a shift-up of the culture temperature, the mutant cells were grown in a water-jacketed gas dispersion flask equipped with a magnetic stirrer and aerated with moist air at the rate of 150 ml/min.

Perchloric acid extraction of adenine nucleotides. For the determination of adenine nucleotides by the luciferase method, a 1.0-ml sample of the bacterial culture was removed through a sampling port in the incubation flask and pipetted into 0.2 ml of cold 17% $HClO_4$. After 30 min at 4°C, the sample was centrifuged at 7,000 × *g* for 15 min. A 0.8-ml portion of the supernatant was removed and neutralized with 0.14 ml of 0.58 M $KHCO_3$ in 2.6 M KOH. After 15 min the sample was centrifuged at 7,000 × *g* for 15 min. The supernatant was then removed and stored at −70°C (5, 26). For the determination of adenine nucleotides by the radioisotope method, the same extraction procedure was used but the volumes were scaled down by a factor of ten.

Luciferase assay. ATP was assayed by the luciferase reaction (24) with a Luminescence Biometer (Du Pont Co., Wilmington, Del.). ADP was determined by difference after converting it to ATP with pyruvate kinase and assaying for ATP. AMP was determined by difference after conversion to ADP with adenylate kinase and conversion of ADP to ATP with pyruvate kinase. These procedures have been previously described (26).

Determination of intracellular adenine nucleotides. The intracellular nucleotide levels were determined by subtracting the values for adenine nucleotides in the medium after the cells had been removed by filtration from values for the total culture. This correction is important in the CR341T28 cultures because up to 15% of the total adenine nucleotides are in the medium. The filtering apparatus was a 5-ml syringe to which a 25-mm (biometer samples) or 13-mm (radioisotope samples) filter adapter (Millipore Corp., Bedford, Mass.) had been attached. Millipore filters with a pore size of 0.22 μm were used to remove cells.

Incorporation of [^{3}H]adenosine into adenine nucleotides. Cultures were grown to a density of about 7×10^8 cells per ml either in an Erlenmeyer flask on a shaker or in a sparger flask. A 0.27-ml sample was then placed in a small tube that contained 30 μCi (35 to 50 Ci/mmol) of [2,8-^{3}H]adenosine in 0.03 ml of water at the same temperature as the culture flask. This procedure is similar to one described previously (13, 15). The incorporation was allowed to proceed for 90 s, at which time the adenine nucleotide pool was considered to be uniformly labeled. After the pulse-labeling, 0.20 ml of culture was removed and filtered through a 0.22-μm pore Millipore filter 13 mm in diameter. A 0.1-ml aliquot of the filtrate was extracted with 0.02 ml of 17% $HClO_4$, as was 0.1 ml of the culture that had not been filtered. The samples were then treated as previously described (see extraction of the adenine nucleotides above.).

PEI-cellulose TLC separation of adenine nucleotides. A 5-μl volume of each sample was spotted 1.8 cm from the short side of a poly(ethylene)imine cellulose thin-layer chromatography plate (10 cm by 20 cm) that was impregnated with a fluor to facilitate viewing UV-absorbing compounds. Unlabeled ATP, ADP, and AMP were spotted as markers. The plate was soaked in anhydrous methanol for 10 min and thoroughly dried under a stream of cool air. The plate was then soaked in deionized water for 10 min and again dried. The methanol and water washes removed salts that otherwise interfere with the chromatography. The plate was developed in deionized water until the solvent front reached the top. This step required about 60 min. After thorough drying, the plate was placed in aqueous 0.5 M LiCl and developed until the solvent front was 12 cm above the origin (22). This step required 30 min. The plate was again dried.

Elution and counting of adenine nucleotides. The spots corresponding to the marker nucleotides were located with the aid of a UV lamp, cut out, and placed in scintillation vials. A 0.7-ml volume of 4 M HCl was added to elute the nucleotides. After 30 min, 15 ml of scintillation fluid {57% toluene, 43% anhydrous ethanol containing 0.46 g of PPO (2,5-diphenyloxazole) and 0.6 g of POPOP [bis(2-[5-phenyloxazolyl])benzene) per liter} was placed in the vials, which were counted in a Tri-Carb Scintillation Spectrometer (model 2002; Packard Instrument Co., Inc., Rockville, Md.).

Enzyme assays. For estimation of the specific activity of adenylate kinase in vivo, cell samples were taken directly from the liquid culture and frozen at −70°C. The samples were later thawed, and the cells were broken by sonication. After centrifugation at $27,000 \times g$ for 50 min, the supernatant was dialyzed against 4 liters of 50 mM Tris-chloride (pH 7.4) at 4°C for 15 to 20 h.

Adenylate kinase assays were performed, at 30°C unless otherwise indicated, in 1.0-ml volumes by using a temperature-controlled recording spectrophotometer. For estimations of activity in vivo and during enzyme purification, the reverse reaction (production of ATP and AMP from ADP) was routinely used. The reaction was coupled to the hexokinase and glucose-6-phosphate dehydrogenase reactions, and the production of NADPH was observed as a function of time (21). Each assay contained 100 mM Tris-Cl (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 10 mM glucose, 1 mM $NADP^+$, 4 mM ADP unless otherwise stated, and 50 μg of dialyzed hexokinase-glucose-6-PO_4 dehydrogenase (1:1 by weight). All components except the adenylate kinase were incubated together in a cuvette for 5 min at 30°C before the reaction was initiated by addition of adenylate kinase. To monitor the production of ADP from ATP and AMP (forward reaction), the adenylate kinase reaction was coupled to the pyruvate kinase and lactate dehydrogenase reactions, and the oxidation of NADH was followed spectrophotometrically. Each assay contained 50 mM Tris-chloride (pH 7.4), 5 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate, 0.26 mM NaDH, the indicated amounts of AMP and ATP, and 30 μg of dialyzed lactate dehydrogenase-pyruvate kinase mixture. Again, a stable background rate was obtained before the reaction was initiated by the addition of adenylate kinase. The specific activity of adenylate kinase was expressed as micromoles of product formed per minute per milligram of protein. Affinity constants and Hill coefficients were calculated from Hill plots $\{\log[v/(V_{max} - v)]$ versus $\log S\}$ after the maximum velocity had been estimated from Lineweaver-Burk plots.

Protein determination. The total protein was determined by the amido black method (23). Cells were collected by centrifuging a 0.6-ml sample at $12,000 \times g$ for 5 min at 4°C, and the supernatant was discarded. The pellet was suspended in 0.81 ml of water, and 0.09 ml of Tris-chloride (pH 7.5) with 0.1% sodium dodecyl sulfate was added; then 0.18 ml of 60% trichloroacetic acid was added and a portion of the resulting precipitate was spotted on a sheet of type GS Millipore filter paper and assayed as published (23).

Materials. Poly(ethylene)imine cellulose thin-layer chromatography plates on plastic sheets were obtained from E. Merck, AG, Darmstadt, Germany. The nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo. Hexokinase, glucose-6-phosphate dehydrogenase, myokinase, and pyruvate kinase were obtained from Boehringer Mannheim Corp., New York, N.Y., and [2,8-^{3}H]adenosine was obtained from New England Nuclear Corp., Boston, Mass.

Purification of adenylate kinase. Table 1 summarizes the purification of adenylate kinase from *E. coli* CR341T28. The enzyme from the parental strain was purified according to the same procedure, except that only 5 g of packed cells was used, and all volumes were scaled down by a factor of seven. All steps of the purification were carried out at 0 to 4°C. Packed *E. coli* CR341T28 cells (35 g) were suspended in 350 ml of 50 mM Tris-chloride (pH 7.4), and sonicated in a Branson Sonifier, model LS75, at an intensity of 7 and a power of 7 mA for 45 s (three pulses of 15 s each). Further sonication led to partial inactivation of the temperature-sensitive adenylate kinase. Cell debris was removed by centrifugation at 20,000 × *g* for 15 min. The supernatant solution was dialyzed against the extraction buffer (Tris-chloride, pH 7.4) for 12 to 14 h. Streptomycin-SO_4 was added slowly at the rate of 1 μl of 25% streptomycin sulfate per ml of cell-free extract per *A* unit at 260 nm. The suspension was stirred for an additional 60 min, and the resulting precipitate was removed by centrifugation for 30 min at 20,000 × *g*. Solid ammonium sulfate (291 mg/ml) was added to the supernatant solution slowly with rapid stirring to give a 50% saturated solution. After an additional 30 min of stirring, the precipitate was removed by centrifugation at 20,000 × *g* for 30 min. To the supernatant fraction was added 194 mg of solid ammonium sulfate per ml to give an 80% saturated solution. The suspension was centrifuged, and the precipitate was dissolved in 35 ml of 50 mM Tris-chloride (pH 7.4). The 50 to 80% ammonium sulfate fraction was applied directly to a Sephadex G-75 column (2.6 by 92 cm) equilibrated with 50 mM Tris-chloride (pH 7.4). The column was eluted with the same buffer at a flow rate of 0.7 ml/min. The adenylate kinase activity was collected between 110 and 160 ml of eluent volume.

Blue dextran 2000 was covalently bound to Sepharose 4B by the method of Thompson et al. (27). The pooled G-75 fractions were added to a column of blue dextran-Sepharose (1.6 by 38 cm) previously equilibrated with 50 mM Tris-chloride until the A_{280} of the eluent was zero. The enzyme was eluted with a 0 to 0.5 mM linear gradient of adenylates (0.25 mM AMP plus 0.25 mM ATP). The peak of the adenylate kinase activity eluted at approximately the 0.4 mM adenylate concentration. The peak adenylate kinase fractions (12 ml) were pooled and added to a column (1.3 by 15 cm) of DEAE-cellulose (Whatman DE 52) equilibrated with 50 mM Tris-chloride (pH 7.4). The column was eluted with a 0 to 0.2 M linear KCl gradient. Adenylate kinase activity appeared in the eluent at approximately 0.15 M KCl. Fractions containing the highest activity were pooled and stored in aliquots at −20°C in the presence of 1 mg of bovine serum albumin per ml. The protein concentration before the bovine serum albumin addition was 20 μg/ml.

Enzyme purity was estimated with the use of disc electrophoresis in 7% polyacrylamide gels by the procedure of Gabriel (11). Gels were stained with 0.5% amido black in 7% acetic acid for 60 to 120 min at room temperature and later destained with 7% acetic acid for 24 to 48 h. For purity estimations, 120 μg of protein was layered on each gel. Parallel gels were stained for enzyme activity with nitroblue tetrazolium and phenazine methosulfate as terminal electron acceptor dyes (12). For comparison, a standard of commercially available muscle adenylate kinase was run on an identical gel.

RESULTS

If adenylate kinase were partially inactivated, the conversion of AMP to ADP would presumably be impaired. Changes in the relative concentrations of the adenine nucleotides might adversely affect regulation of the energy metabolism of the cell. Further, a decrease in the concentration of ADP might become a limiting factor in oxidative phosphorylation and, hence, in growth. Growth of CR341T28 cells at partially permissive temperatures that cause partial inactivation of adenylate kinase thus seemed to be a promising approach to study of the correlation between alterations in energy charge values or adenylate ratios and functional capacities of the cell such as growth and viability.

Among a population of *E. coli* CR341T28 cells, over 90% form colonies on nutrient agar at 34°C, but only 10% do so at 38°C (Fig. 1). There is no obvious explanation for this range of temperature sensitivities, since the heat lability of the adenylate kinase is apparently caused by a point mutation. The distribution may be due to microheterogeneity in culture caused by physiological individuality among the cells. Alternatively, the probability that any given cell will form a colony when plated may decrease with temper-

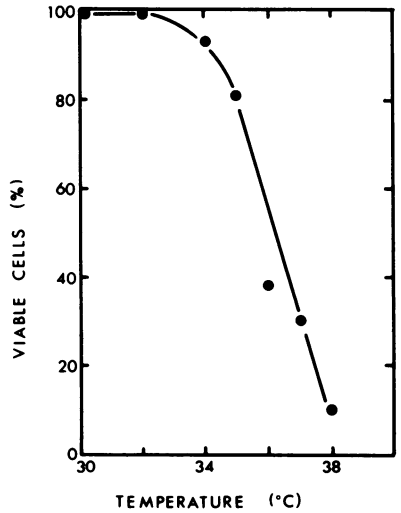

FIG. 1. *Colony-forming ability of E. coli CR341T28 incubated in pour plates at various temperatures. For each temperature, cells were grown in liquid culture at 30°C to a density of 5 × 10^8 cells per ml as determined by particle counts. Then a sample was serially diluted, plated in nutrient agar, and incubated at the indicated temperature for 2 days.*

ature even though the cells may be essentially identical. In liquid medium, the cut-off temperature was slightly lower and appeared to be sharper, occurring at about 34°C.

When the mutant strain was shifted from a permissive (30°C) to a nonpermissive (36°C) temperature, the viable-cell count declined in agreement with previous observations (6, 8). The change was coordinant with the decrease in adenylate energy charge (Fig. 2). The cells could not be revived by shifting back to 30°C after 1.5 h at 36°C. The specific activity of adenylate kinase also decreased rapidly following the shift in temperature.

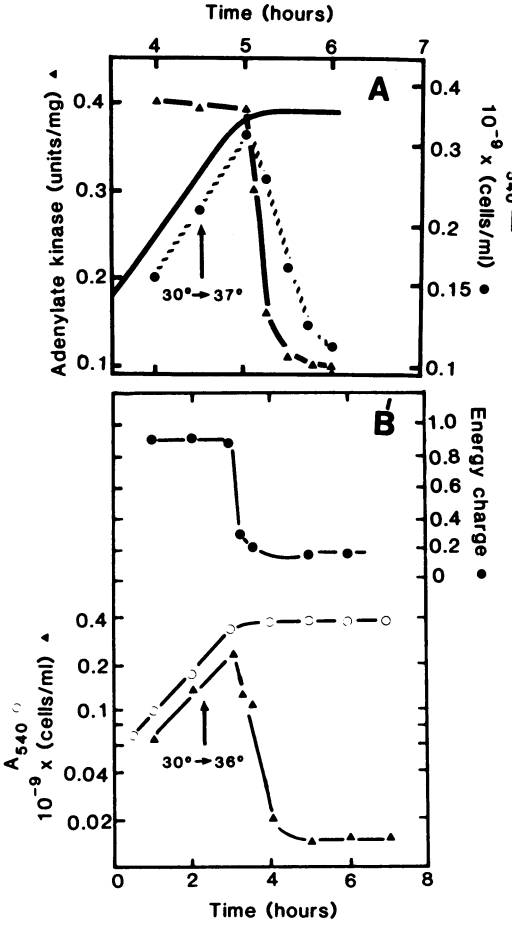

FIG. 2. *Effect on the adenylate energy charge, adenylate kinase activity, and viability of E. coli CR341T28 of a shift from a permissive to a nonpermissive temperature. (A) Cells were grown in liquid culture at 30°C and then shifted to 37°C. The specific activity of adenylate kinase was determined in dialyzed cell-free extracts according to the procedure given in the text for the reverse reaction. (B) Cells were grown in liquid culture for 2 h at 30°C and then shifted to 36°C. The viability was estimated by ability to form colonies at 30°C and the energy charge by the luciferase method.*

Exponential growth of *E. coli* CR341T28 was observed at temperatures up to 34°C. At 34°C and below, the viable colony counts and particle counts correlated very well (e.g., Fig. 1). At temperatures below 30°C, the growth rate of the mutant strain was the same as that of the nearly isogenic strain CR34 (Fig. 3). This indicated that at these temperatures, the adenylate kinase activity in strain CR341T28 was probably not growth-rate limiting. Above 30°C, strain CR341T28 grew exponentially but at rates substantially lower than those of CR34 cells (Fig. 3). In this temperature range the ratio of the growth rate constants of the mutant and wild-type strains decreased with temperature in an essentially linear manner (Fig. 4). At 34.5°C or higher, colony counts and particle counts correlated poorly for strain CR341T28, as shown in Fig. 1.

The relative specific activities of adenylate kinase in the two strains provided further apparent correlation between the lowered growth rates of strain CR341T28 at temperatures above 30°C and the adenylate kinase lesion (Fig. 4). The specific activities for the mutant and parent strains differed by a factor of about two, even at permissive temperatures (Fig. 5). This difference in specific activities was reported by Cousin et

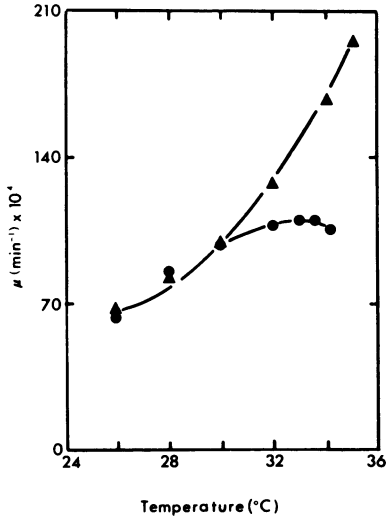

FIG. 3. *Growth rates of the parental (CR34) (▲) and the temperature-sensitive (CR341T28) (●) strains of E. coli at various temperatures. The growth constant is defined as $0.69/t_{gen}$, where t_{gen} is the time required to double the number of cells. The percentage of cells able to form colonies was monitored by comparing particle counts with colony counts and was always at or near 100%.*

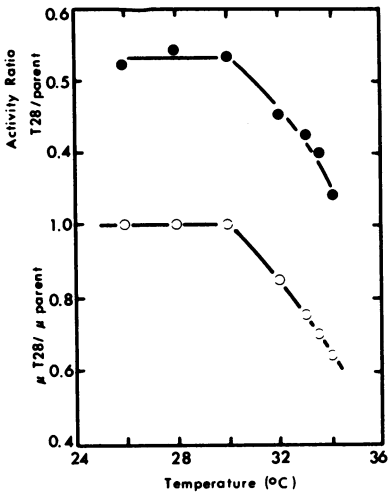

FIG. 4. *The ratios of the growth constant μ (○) and of adenylate kinse specific activity (●) for E. coli CR341T28 to the corresponding parameters for E. coli CR34 as a function of temperature.*

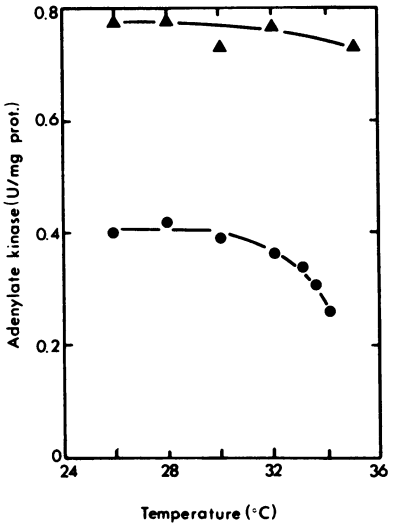

FIG. 5. *The specific activities of adenylate kinase in E. coli CR341T28 (●) and E. coli CR34 (▲) grown at various temperatures. Samples were taken from a liquid culture at the indicated temperature at a density of about 9 × 10⁸ cells per ml. The samples were sonicated and assayed as described in the text.*

al. (8). The K_m of adenylate kinase for ADP is 0.14 mM for strain CR34 and 0.56 mM for strain CR341T28 (see below). The experiments that led to Fig. 5 were performed at an ADP concentration of 4 mM; thus, the wild-type enzyme was about 90% saturated, and the mutant enzyme, about 75%. This difference thus accounts for about 20% of the observed twofold difference in rate. The difference in specific activities at permissive temperatures may be due to a lower turnover rate of catalysis (V_{max}) for the enzyme from the mutant strain, a lower molar level of enzyme in the cell, or differential loss of activity during purification. In an attempt to test the last possibility, we found that, for each strain, the extracts obtained by sonication and by use of the French pressure cell had equal adenylate kinase specific activities.

The adenylate energy charge was initially determined by the luciferase method for both strains at permissive and partially permissive temperatures. In the luciferase assay, errors in ADP and especially AMP values were large since these compounds must be estimated by difference. Energy charge can be determined with comparatively little error because this parameter is relatively insensitive to changes in the concentration of AMP, but ratios between the adenylates are subject to large error. The energy charge at 30°C and below was about 0.90 in both strains and remained at this value at higher temperatures in the CR34 cells, but declined in strain CR341T28 to a value of 0.82 at 34°C. The close correlation in the mutant strain between the decreases at temperatures above 30°C in specific activity of adenylate kinase, in the energy charge, and in the growth rate further supports the suggestion that the lowered enzyme activity limits the growth rate at these temperatures.

The [^{3}H]adenosine incorporation technique (see above) permitted determination of adenine nucleotide levels, and therefore ratios and energy charge values, with substantially less error than in the luciferase method. Since the turnover time of an ATP molecule in *E. coli* is about 0.1 s (17), it was considered that after 90 s the adenylate pool was uniformly labeled. The energy charge values obtained with this method were slightly higher than those obtained by the luciferase technique. The energy charge declined in strain CR341T28 with increasing temperature from 0.90 at 30°C to 0.88 at 34°C (Fig. 6A). Energy charge values near 0.9 have been observed consistently in actively metabolizing cells (4, 5). The lowered growth rate observed when the energy charge fell below 0.90 in the mutant strain suggests that regulatory interactions involving the adenine nucleotides may be limiting the growth rate. The energy charge in the parental strain remained relatively high, 0.93, throughout the entire temperature range.

The ATP/ADP and ATP/AMP ratios, as determined by the isotope method (Fig. 6B and 6C), increased as temperature decreased below 30°C in both strains. This has also been observed in *E. coli* B (C.C.G., unpublished data). The reason for this increase is not clear. The ATP/

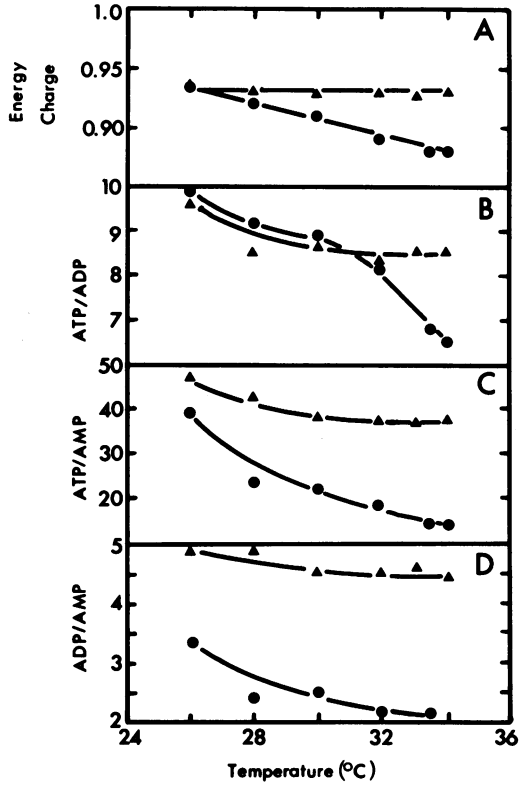

FIG. 6. *Adenine nucleotide concentration ratios as determined by the incorporation of [^{3}H]adenosine. Adenylate energy charge (A), ATP/ADP ratio (B), ATP/AMP ratio (C), and ADP/AMP ratio (D) of the temperature-sensitive (●) and parental (▲) strains were determined at various growth temperatures. Cultures were pulse-labeled with [^{3}H]adenosine and extracted, and the adenine nucleotides were separated by poly(ethylene)imine-cellulose thin-layer chromatography.*

ADP ratio was constant in strain CR34 above 30°C, but declined noticeably in the mutant strain above this temperature and was about 25% to 30% lower at 34°C than at 30°C. The ADP/AMP ratio in the mutant strain was about 50% of that in the parental strain at nearly all growth temperatures (Fig. 6D). The ATP/AMP ratio was also lower at all temperatures in strain CR341T28 than in strain CR34 (Fig. 6C). At about 30°C, the ATP/AMP ratio in strain CR34 tended to become constant with respect to temperature. The ATP/AMP ratio in strain CR341T28 declined as the temperature increased from 26 to 34°C. At 34°C, the ATP/AMP ratio in the mutant strain was about 60% of the value observed at 30°C.

Thermal inactivation of adenylate kinase. In a culture of *E. coli* CR341T28 at 30°C, the specific activity of adenylate kinase determined in the cell-free extract remained constant, at approximately 0.4 μmol/min per mg of protein when assayed at 30°C, during the exponential growth phase (Fig. 2). When the growth temperature of the culture was raised to 37°C, the cells stopped growing after 30 min. Coincident with the cessation of growth, the specific activity of adenylate kinase decreased rapidly. A shift-up in temperature for this mutant strain of *E. coli* has previously been shown to result in a rapid decline in the intracellular ATP concentration (7, 13) in accordance with the adenylate kinase inactivation.

Adenylate kinase from the temperature-sensitive mutant grown at 30°C is thermolabile in cell-free extracts (8, 13). The temperature-sensitive adenylate kinase was 95% inactivated when a crude extract (3.2 mg/ml of protein) was incubated at 37°C for 30 min. This observation agrees with previous results of Cousin et al. (8), whereas Glaser et al. (13) showed little or no adenylate kinase inactivation in the *E. coli* CR341T28 extract under similar conditions. The purified temperature-sensitive enzyme, even in a much more dilute solution (51 μg/ml of protein), was more resistant to thermal inactivation, retaining 31% of initial activity after 30 min at 37°C. Adenylate kinase from the parent strain was fully active after the same treatment.

Purification of adenylate kinase. The method used to purify adenylate kinase was a modification of that used by Holmes and Singer (16).

Blue dextran-Sepharose chromatography (27) was employed because it is a rapid procedure that results in considerable purification with relatively little inactivation of the unstable mutant adenylate kinase. Since adenylate kinase requires both ATP and AMP as substrates, it was hoped that the use of low concentrations of both adenine nucleotides would be effective in eluting this enzyme, rather than others that bind either ATP or AMP.

Table 1 summarizes the purification, which resulted in a 278-fold purification of the mutant enzyme and a final specific activity of 50 μmol/min per mg of protein at 30°C. The details of the purification are given above. Because the enzyme was needed only for kinetic comparisons, extensive efforts to maximize yield did not seem necessary. The same procedure, applied to adenylate kinase from the parent strain, resulted in a 475-fold purification and a final specific activity of 976 μmol/min per mg of protein at 30°C. The difference between the specific activities of the mutant and the parent enzymes increased during the course of the purification. The similar degree of apparent purity observed in polyacrylamide gel electrophoresis of the two

TABLE 1. *Purification of adenylate kinase from a temperature-sensitive mutant, E. coli CR341T28*

Fraction	Total activity	Yield (%)	Sp. Act (μmol of ATP/min per mg of protein at 30°)	Purification factor
Streptomycin-SO_4 ppt[a]	130	100	0.18	1
$(NH_4)_2SO_4$ ppt	145	112	0.42	2
Sephadex G-75	48	40	1.68	9
Blue dextran-Sepharose	15	12	27.3	152
DEAE-cellulose	7	5	50.0	278

[a] ppt, Precipitate.

purified enzymes suggests that the mutant enzyme was partially inactivated during the purification procedure, and that the major band may have contained both active and inactive mutant adenylate kinase protein.

Polyacrylamide gel electrophoresis. Before bovine serum albumin was added for stabilization, 100 μg (5 ml) of the purified adenylate kinase from each strain was concentrated by ultrafiltration and subjected to electrophoresis on a 7% acrylamide gel. Duplicate gels were run; one was stained for protein and the other for enzyme activity. A similar pattern of one major and two minor protein bands was observed with both enzymes. The mobility of the major band (estimated as approximately 90% of total protein) corresponded to that of the adenylate kinase activity in the enzyme-stained gel. The mobility of the major protein band also matched that of commercial muscle adenylate kinase when run as a standard in the same gel system and stained for either protein or enzyme activity.

Kinetic properties of mutant and parent adenylate kinase at 30°C. Table 2 summarizes the kinetic parameters observed for the adenylate kinase reaction when using dialyzed, partially purified enzymes from the mutant and parent strains. The assay temperature (30°C) corresponded to a permissive growth temperature for the mutant strain, and the highest temperature at which maximum adenylate kinase activity was observed in the mutant cells (Fig. 5). At this temperature, the parent adenylate kinase had slightly higher affinities than the mutant enzyme for all three substrates, with affinity ratios ranging from 1.5 for ATP to 4 for ADP. The Hill coefficients, approximately 1 for ATP and AMP and 2 for ADP, were the same for both enzymes. When adenylate kinase was isolated from the mutant strain grown at 34°C, where the enzyme level has been shown to be reduced by 40%, the specific activity in extracts was reduced, as expected, but the K_m values for

TABLE 2. *Kinetic parameters of adenylate kinase from E. coli CR34 and from the temperature-sensitive mutant E. coli CR341T28 assayed at 30°C*

Strain	ATP[a]		AMP[a]		ADP	
	K_m	n	K_m	n	K_m	n
CR34	0.17	0.09	0.08	1.0	0.14	1.8
CR341T28	0.25	0.9	0.17	1.0	0.56	1.8

[a] At saturating concentration of cosubstrate: 1 mM for the parental enzyme, and 2 mM for the temperature-sensitive enzyme.

all substrates were the same as those for the enzyme isolated from the mutant strain grown at 30°C. Variation of the concentration of one of the cosubstrates, AMP or ATP, over a 10-fold range (0.05 mM to 0.5 mM) produced a less than twofold variation in the affinity of either enzyme for the remaining substrate.

The substrate inhibition observed at high AMP concentrations for the parent adenylate kinase was not observed for the mutant enzyme even at much higher AMP concentrations (Fig. 7).

Kinetic properties of adenylate kinase as a function of temperature. Cultures of *E. coli* CR341T28 show a gradually decreasing level of adenylate kinase activity with an increase in the growth temperature above 30°C, reaching complete inactivation (and concomitant cell death) around 37°C. The affinities of the partially purified mutant and parent adenylate kinases for the three substrates were determined as a function of temperature over the same range (Fig. 8). The reaction mixtures were incubated in cuvettes at the indicated temperatures for 10 min, and the reactions were initiated by the addition of adenylate kinase. Control experiments showed that in the presence of substrate the temperature-sensitive adenylate kinase was stable for the duration of the assay even at 40°C. The affinities of adenylate kinase from the parent strain for all three nucleotides were essentially unchanged over the temperature range 30 to 40°C.

In contrast, the affinities of the mutant adenylate kinase for all three substrates decreased substantially as the assay temperature was raised from 30°C to 40°C. The change was greatest in the K_m value for ATP, which increased from 0.25 mM at 30°C to 0.70 mM at 40°C. The K_m values for AMP and ADP increased about twofold over the same temperature range. A control experiment showed that the temperature-sensitive adenylate kinase, when incubated at 40°C for 10 min (in the presence of 1 mM ADP to reduce inactivation) and subsequently assayed at 30°C, exhibited the same affinity for

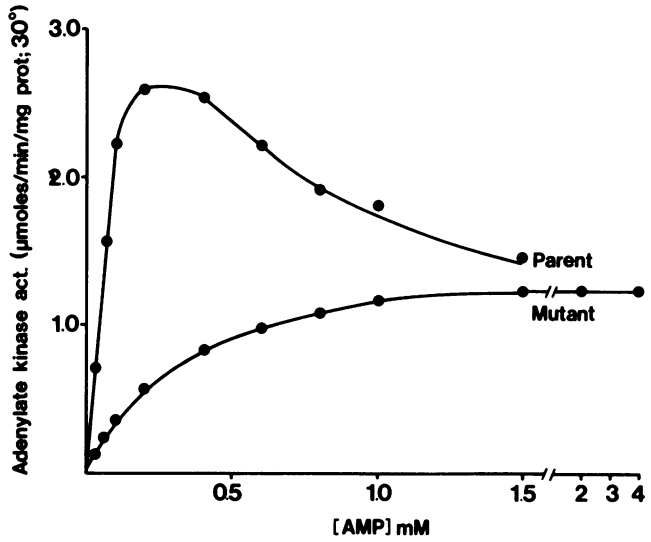

FIG. 7. *Reaction rate as a function of AMP concentration for partially purified adenylate kinase from E. coli CR34 (parent) and E. coli CR341T28 (mutant) at 30°C. The concentrations of ATP were 4 mM and 1 mM for the mutant and the parent enzymes respectively.*

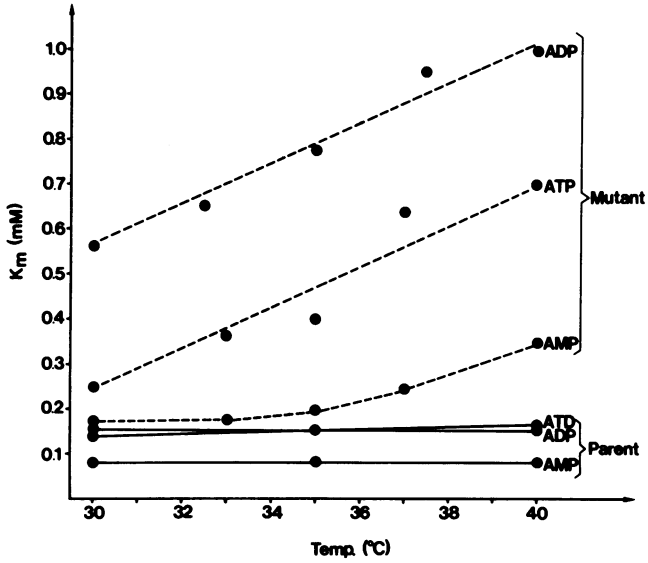

FIG. 8. *Michaelis constants for adenylate kinase from E. coli CR34 (parent, bottom 3 lines) and E. coli CR341T28 (mutant, top 3 lines) as functions of temperature. Cosubstrate concentration (AMP when ATP was varied; ATP when AMP was varied) was constant at 2 mM. Partially purified adenylate kinases from the two strains grown at 30°C were assayed according to the procedures described in the text.*

ADP as did the enzyme assayed at 30°C without any prior incubation at elevated temperature.

The Hill coefficients for the three substrates were the same at 30°C and at 40°C (1.8 for ADP, 1.0 for AMP, and 0.9 for ATP) for the mutant enzyme. However, when the log of the maximum velocity of the reaction was plotted against the inverse of the temperature, the resulting Arrhenius plot for the parent enzyme showed the expected straight line relationship, whereas there was a deviation from linearity for the mutant enzyme at elevated (37.5 to 42.5°C) temperatures (Fig. 9).

DISCUSSION

At temperatures below 30°C, the adenylate ratios were altered in the mutant strain in a way that is consistent with a less active adenylate

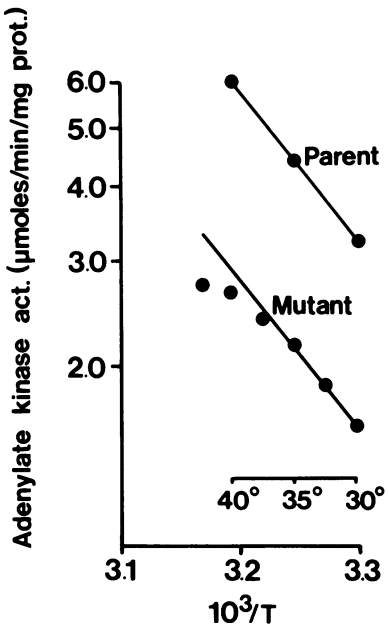

FIG. 9. *Arrhenius plots of the maximum velocity of the adenylate kinase reaction for the enzymes partially purified from E. coli CR34 (parent) and E. coli CR341T28 (mutant). Activation energies of 11.9 and 11.3 kcal/mol were calculated from the slope of the curve for the parental enzyme and from the linear part of the curve for the mutant enzyme, respectively.*

kinase. The value of $(ADP)^2/[(ATP)(AMP)]$ averaged 0.54 in the parent strain and 0.31 in the mutant, and did not vary with temperature in either strain over the range 26°C to 34°C. The ATP/AMP and ADP/AMP ratios were also lower in the mutant in this permissive temperature range (Fig. 6C and 6D). However, at 26°C to 30°C, growth of the mutant strain was essentially identical to that of the parent. It may be significant that the ATP/ADP ratio in the mutant was equal to that of the parental strain at these temperatures. Although no definitive conclusions in this regard are possible, these results suggest that the ATP/ADP ratio may, under some conditions, be more important than the ATP/AMP ratio in relation to growth.

The pattern of nucleotide concentrations in the mutant strain at temperatures above 30 or 32°C is puzzling. Although the specific activity of adenylate kinase in extracts of the mutant was lower than in the same strain at lower temperatures, the mass action ratio was as high as at lower temperatures; as expected, the ADP/AMP ratio was lower and ATP/AMP ratio much lower than in the 26°C to 30°C range. However, the ATP/ADP ratio was also lower. This effect balanced the decrease in ADP/AMP, with the result that the mass action ratio, which may be expressed as the product of ADP/AMP and ADP/ATP, remained essentially constant.

The reason for the decrease in the ATP/ADP ratio in the mutant at higher temperatures is not clear. It may perhaps not be related directly to the heat lability of adenylate kinase. The glycerol-3-phosphate acyltransferase activity of this strain is also heat labile (8), apparently as a consequence of the same mutation that affects adenylate kinase (9). It is possible that this defect in phospholipid biosynthesis may affect membrane function in such a way as to limit electron transport phosphorylation. Thus, the analytical results are consistent with the suggestion of simultaneous decreases in vivo at higher temperatures in the activity of adenylate kinase (hence, the decreases in the ATP/AMP and ADP/AMP ratios) and in the capacity for oxidative phosphorylation (hence, the decrease in the ATP/ADP ratio).

The close correlation between growth rate and specific activity of adenylate kinase shown in Fig. 4 is also puzzling in view of the fact that the mass action ratio, expected to be the most sensitive indicator of enzyme function in vivo, did not decrease in the temperature range where the activity of the enzyme, as measured in extracts, fell sharply with increasing temperature. The results indicate that a value of the ATP/AMP ratio nearly 50% below its normal range is compatible with normal growth (mutant strain at 30°C; Fig. 4). This observation is surprising, since several enzymes have been shown to respond to the ATP/AMP ratio. At temperatures above 30°C, decreases in the adenylate energy charge and in the ATP/ADP ratio accompanied a further decrease in the ATP/AMP ratio, so that it is not possible to attribute the sharp decline in growth rate relative to the parental strain with confidence to any one factor.

The altered primary structure of the mutant enzyme might lead to lower specific activity of adenylate kinase in mutant cells growing at permissive temperatures either by causing a reduction in the turnover number for the enzyme or by increasing the susceptibility of the mutant enzyme to hydrolysis by intracellular proteases. Previous studies (8, 13) have ruled out an increased level of general proteolytic activity in the temperature-sensitive strain, but it is known that defective enzymes may be more susceptible than normal enzymes to proteolysis (14). The susceptibility might become more pronounced at higher temperatures, and might thus contribute to the gradual decrease in adenylate kinase activity in the mutant as a function of growth temperature above 30°C. The inactivation of the mutant adenylate kinase in vivo at 36°C to 37°C might also reflect thermal denaturation of the

mutant enzyme in addition to or instead of proteolytic cleavage. Substrates and ammonium sulfate offer protection against thermal inactivation (8); however, this could equally affect either mechanism of inactivation. The degree of inactivation that occurs during incubations of the mutant enzyme, at different stages of purification, at different temperatures and other incubation conditions, varies somewhat both in our own experiments and according to previous reports (8, 13). In general, however, the mutant enzyme appears to be less sensitive to temperature increase in vitro than in vivo. Furthermore, the enzyme appears to become more heat stable during the purification procedure. This would argue that thermal inactivation may be at least in part proteolytic.

At the permissive temperature (30°C), the kinetic properties of the temperature-sensitive adenylate kinase are quite similar to those of the parent adenylate kinase or to those previously reported for adenylate kinase from wild-type *E. coli* (16) except that affinities for AMP, ADP, and ATP are somewhat reduced.

The kinetic properties of the parent and the mutant adenylate kinases are affected differently by changes in the assay temperature. The maximal velocity of the reaction catalyzed by the parent enzyme yielded a linear Arrhenius plot over the temperature range studied (30 to 40°C). The K_m values for the three substrates remained nearly constant over the same temperature range. In contrast, the maximum velocity of the reaction catalyzed by the mutant adenylate kinase gave a linear Arrhenius plot only below approximately 37°C. Above this temperature, the experimental points diverged downward from linearity, suggesting structural alterations. Although nominally suggesting a decrease in activation energy, this curve probably really reflects inactivation of the enzyme. There is a progressive twofold or larger decrease in the affinity of the mutant enzyme for all three substrates as the assay temperature is increased between 30 and 40°C, again suggesting conformational alterations in the mutant enzyme in this critical temperature range. The reaction remained second order with respect to ADP (Hill slope = 1.8) between 36 and 40°C, but this presumably merely reflects the participation of two molecules of ADP in the reaction, rather than any regulatory interactions between sites.

In summary, both a reduction in the level of adenylate kinase and a reduction in the affinity of the enzyme for its substrates probably contribute to the decreased adenylate kinase activity and the decreased rate of cell growth observed between 30 and 34°C in *E. coli* CR341T28.

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