Adenylate and Guanylate Energy Charges in a Subsurface *Pseudomonas* sp.

Sharon R. Ford, Marliese S. Hall, Valerie R. Vaden[1] ,
JoAnn J. Webster[2], and Franklin R. Leach*

Department of Biochemistry and Molecular Biology,
246B Noble Research Center, Oklahoma State University, Stillwater, OK 74078

Received: 1993 Dec 15; Revised: 1994 Apr 15

Growth and energy relationships of a *Pseudomonas* sp., strain Lula V, that was isolated from a well-characterized pristine shallow aquifer site at Lula, Oklahoma, have been studied. The Lula, Oklahoma site has had extensive study of its ground water and the influence of the subsurface material on ground water. Lula V is a subsurface organism that can influence the transport and fate of pollutants in ground water. When grown with a doubling time of 3.5 to 4 h in a peptone/yeast extract/glucose medium, strain Lula V had normal adenylate (AEC = 0.87) and guanylate (GEC = 0.75) energy charges. Strain Lula V maintained a higher than normal adenylate energy charge when in the stationary phase and this value did not decrease during a 40 h-starvation period. The adenylate energy charges of *Escherichia coli*, *Bacillus subtilis*, and *B. stearothermophilus* cells were used for comparison. The maintenance of a high AEC value of Lula V may reflect a nutritional adaptation of this subsurface soil organism to allow faster responses to changes in the availability of substrates.

INTRODUCTION

The adenylate energy charge (AEC) concept was introduced by Atkinson and Walton (*1*) to account for the regulation of enzymatic activity by ATP, ADP, and/or AMP. Atkinson (*2*) expanded this discussion to integrate biochemical mechanisms with physiological responses such as growth. Wiebe and Bancroft (*3*) proposed that AEC measurements could indicate the metabolic status of natural microbial populations. They demonstrated that AEC changes reflected several biological phenomena (such as diauxie) and concluded that when high AEC values are present there are metabolically active microbial populations. Methodology for measurement of AEC values for environmental samples was established by Karl and Holm-Hansen (*4*) who concluded that AEC can be a useful indicator of mean metabolic activity and potential for cell growth of environmental samples. Given the division of function of various nucleotides, Karl (*5*) has suggested that guanylate energy charge (GEC) would reflect the ability to synthesize protein. This paper reports the determination of several energy-related parameters such as AEC and GEC during growth and starvation of a subsurface soil microbe.

Pseudomonas sp., strain Lula V, was isolated by Balkwill and Ghiorse (*6*) from a subsurface soil sample collected at a depth of 3 m from the interface between the water-saturated and unsaturated zones at Lula, Oklahoma in November 1983. The Lula site is located in the margin of the flood plain of a small creek with an in situ temperature of 17 °C and has been used for a number of environmental studies emphasizing ground water [some 18 papers have been based on study of this site; for example see Beloin et al., (*7*); Webster, et al., (*8*); and Wilson, et al., (*9*)]. The depth to the permanent water table at this site was ca. 3 m; the depth to bedrock was 7 m. Sample 3 from which Lula V was isolated had a moisture content of 17% and was fawn colored sand with a minor clay content. The bacteria were first grown aerobically on aquifer sediment extract agar and then on peptone/yeast extract/glucose (PYG) agar. This isolate could also grow on nutrient agar, an indication of greater nutrient flexibility as compared to other isolates (*10*); many such isolates were

[1]Present address: Department of Biochemistry/Biophysics/Genetics, University of Colorado Health Sciences Center, Denver, CO 80262.
[2]Present address: Symex Corp., 2720 N. Hemlock Ct., Broken Arrow, OK 74012.
*Corresponding author.
*Review and processing of this manuscript was controlled by Associate Editor Otis C. Dermer.
Proc. Okla. Acad. Sci. 74: 31-36 (1994)

unable to grow on such a rich medium. Lula V can store large amounts of poly-β-hydroxybutyrate, glycogen, and polyphosphate under the proper nutrient conditions (*11*). This organism is a short, nonfluorescent Gram-negative rod with polar flagella. Lula V is of interest because of its remarkable ability to store two different carbon reserves and polyphosphate, and because it is from a well-characterized site that is the subject of several environmental studies. Since Lula V can store large carbon and energy reserves, it is interesting to ask questions concerning its nucleotide content and utilization during growth and starvation. This paper reports on studies of the adenylate and guanylate energy charges during laboratory growth of Lula V, an organism that influences the transport and fate of pollutants as they impinge on ground water.

EXPERIMENTAL PROCEDURES

Lula V was a gift from Dr. William C. Ghiorse of Cornell University. It was isolated from a subsurface sample collected by a method developed at the Robert S. Kerr Environmental Research Laboratory (*11*). *Escherichia coli* strain W3100 was from Dr. Richard C. Essenberg of this department and was grown on M-9 medium (*12*) with 0.2% glucose. Firefly luciferase was purchased from Analytical Luminescence Laboratory. Nucleotides, other enzymes, and reagents were procured from Sigma Chemicals.

Growth of Lula V. Lula V was grown on PYG medium: Bacto peptone, 0.25 g; Bacto yeast extract, 0.25 g; glucose, 0.25 g; $MgSO_4 \bullet 7H_2O$, 0.6 g; $CaC1_2 \bullet 2H_2O$, 0.07 g; and water to one liter (*10* and Ghiorse, personal communication) [the peptone component supplies phosphorus]. To eliminate interference by endogenous AMP in adenylate energy charge determinations, this medium was treated with acid-washed charcoal [2.5 g per liter (*13*)] before use. This allowed direct energy charge determinations to be made. Lula V was transferred three times in liquid cultures to obtain a rapidly growing culture before determination of the growth curve. The cells were grown at 25 °C with shaking at 150 rpm in a New Brunswick Controlled Environmental Shaker Model G25. The cells started growing immediately with a doubling time of 3.5-4 h. Growth was followed by A_{600} measurement in a 1-cm cell on either a Gilford Model 2000 or a Zeiss PM-6 spectrophotometer. Appropriate samples were taken, diluted in 0.1% peptone, and plated on PYG containing 1.5% agar. The colonies were counted after at least 48 h incubation at room temperature.

Growth of *E. coli*. *E. coli* W3000 was grown in liquid M-9 medium with 0.2% glucose (*12*) at 37 °C and plated on the same medium containing 1.5% agar. Otherwise they were treated exactly as were the Lula V cells. Colony plate counts were made after 24 h incubation at 37 °C. This strain of *E. coli* grew very poorly in PYG medium.

Extraction of Nucleotides. Samples of 0.5 ml were taken with a Pipetman and were injected into 4.5 ml of 20 mM Tricine buffer, pH 7.8, that had been heated for 5 min in a boiling-water bath. The mixtures were heated for 3 min in a boiling-water bath and then rapidly cooled in an ice bath (at least 10 min). The samples were frozen in a Dry Ice-acetone bath and stored overnight frozen at -20 °C. The samples were thawed and warmed to room temperature and then the amounts of the adenine and guanine nucleotides were determined. Medium blanks were extracted and used for controls.

Nucleotide Measurement. ATP was measured in a 500-μl reaction mixture that contained 50 μl of sample; 100 μl of firefly luciferase (Firelight™, dissolved in 50 mM Tricine, 10 mM $MgSO_4$, and 1 mM EDTA, 1 mM dithiothreitol); 50 μl of buffer, pH 7.8, containing 250 mM Tricine, 50 mM $MgSO_4$, 10 mM EDTA, and 10 mM dithiothreitol; and water to volume in a Lumac/3M Model 2010A Biocounter for 10 s (*14*). The AEC was determined according to Webster et al. (*15*). Guanylates were determined by methods modified from Karl (*16*). There were four steps in the determinations of the guanylates. Three separate incubation tubes were used to determine the indicated guanylates: A for GTP, B for GTP + GDP, and C for GTP + GDP + GMP. Samples of 200 μl were added to the appropriate reaction mixture. The first step in the reaction series converted GDP and GMP into GTP. The reaction mixture (50 μl) contained in tube

A: 75 mM Tricine buffer, pH 7.5; 0.5 mM $MgC1_2$; 0.0125 mM KCl; tube B contained in addition 0.5 mM phosphoenolpyruvate and 4 μg pyruvate kinase. For tube C 100 ng ATP and 0.02 units of guanylate kinase (Sigma G-7570 reconstituted in 20 mM Tricine, pH 7.5, containing 0.1% bovine serum albumin; selection of a lot of guanylate kinase with a very low adenylate kinase activity was required) were added to the components of tube B. The reaction mixtures were incubated at 30 °C in a New Brunswick Metabolyte Water Bath Shaker (150 rpm, 90 min); the reaction was stopped by placing the tubes in a boiling-water bath for 3 min. The tubes were cooled in ice for at least 10 min.

In step 2, ATP and UTP were destroyed. To each of the previous reaction mixtures was added 50 μl of a mix that contained 75 mM Tricine buffer, pH 7.5; 0.5 mM $MgC1_2$; 0.012 mM KCl; 0.5 mM D-glucose (Sigma G-5000); 0.5 mM D-glucose 1-phosphate (Sigma G-6875); 0.5 mM $NADP^+$ (Sigma N-0505); 0.5 unit of hexokinase and 0.2 unit of glucose 6-phosphate dehydrogenase (Sigma H-8629, contains both enzymes); and 0.025 units of uridine diphosphate glucose pyrophosphorylase (Sigma U-8501). The reaction mixtures were incubated for 30 min at 30 °C and treated as above.

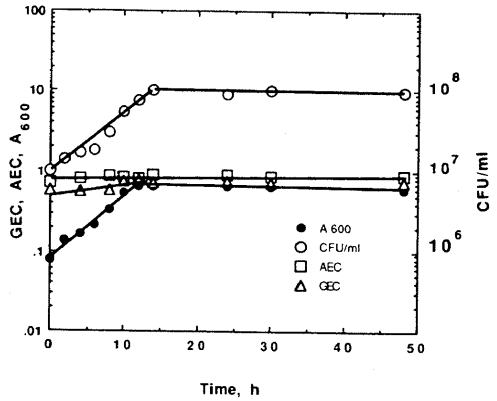

Figure 1. AEC and GEC during Lula V Growth. The adenylate and guanylate energy charges were determined during growth. Each value is the average of triplicate assays with a standard deviation of ≤5%. The entire experiment was repeated three times. Known samples with varying AEC and GEC values were used as internal controls to ascertain proper functioning of all assay components. The experimental AEC and GEC control values were within 2.5% of the theoretical values.

Step 3 converted GTP into ATP and step 4 assayed ATP. The reaction for step 3 was done in a Lumacuvette™ with 50 μl of sample from step 2, and 350 μl of a mix containing 25 mM Tricine, 5 mM $MgSO_4$, 1 mM EDTA, 1 mM dithiothreitol, 100 μunits of nucleoside diphosphate kinase (Sigma N-3380), and 1.5 μM ADP. The samples were incubated 5 min at room temperature. For step 4, 60 light units of Firelight™ in 100 μl were injected. Light production was measured in a Lumac/3M Model 2010A Biocounter for 10 s. Standards of guanylates with energy charges ranging from 0 to 1.0 were also done as controls.

RESULTS and DISCUSSION

Growth of Lula V. Figure 1 shows a growth curve for Lula V grown in charcoal-treated PYG medium. The measurements of growth by A_{600} determination and by plate counts are parallel, with a doubling time of 3.5 to 4 h. Since the cells used for inoculation were taken from a rapidly growing liquid culture and diluted into fresh medium, there was no lag period. When the inoculum was taken from a stationary culture or from a slant or plate, there was a lag period of about 6 h. The number of cells increased logarithmically for about 12 h and then became stationary under these conditions; the titer of viable cells did not change during the next 36 h. Thus there is no death in these experiments. Figure 1 also shows the AEC and GEC values during growth of Lula V in the charcoal-treated PYG medium. The AEC value remained fairly constant between values of 0.7 and 0.9 over the 48-h period. The GEC value (analogous to AEC, but for the guanine nucleotides) was less than the AEC value, starting at 0.56, rising to 0.74 at the end of the growth period, and then remaining constant. After 24-h growth the pH of the medium was 7.18 (the starting value was pH 6.82) and more than 99.8% of the glucose had been utilized. The glucose was the growth-limiting factor. Under the low nutrient conditions of these experiments, the glucose was completely used.

Comparison of Energy Charges. This laboratory has determined AEC and GEC values of *E. coli* strain W3100, and AEC

TABLE 1. Comparison of Adenylate and Guanylate Energy Charges

Organism	AEC Value				GEC Value			
	Log	Stationary			Log	Stationary		
		Early	Mid	Late		Early	Mid	Late
Pseudomonas Lula V	0.88	0.85	0.94	0.94	0.66	0.72	0.71	0.62
Escherichia coli W3100	0.76	0.65	0.48	0.14	0.73	0.65	0.77	**
Bacillus subtilis	0.85	0.76						
Bacillus stearothermophilus	0.76	0.52						

The *Pseudomonas* and *E. coli* values are the averages of two experiments with the values taken at 7–8 and 2 h for mid log, respectively ; and at 10–12, 12–14, and 24 and 5–6, 9–12, and 24 h for early, mid, and late stationary phase, respectively. The *Bacillus* data are from Webster, et al. (*15*); work done in this laboratory used the same procedures.
**Anomalously low results with tube C occurred in both experiments. The GTP amount was about one tenth the amount in the mid-stationary phase.

values for *Bacillus subtilis* and *B. stearothermophilus* during growth (*15*) (a positive control of the methods). Table 1 compares these values to those for Lula V. Both AEC and GEC values are maintained at a high level in strain Lula V well after the cells have entered the stationary phase (measured up to 48 h). The adenylate energy charge in *E. coli* decreases to a low level after 24 h of incubation. Under these growth conditions the pH changed from 6.93 to 6.69 in 24 h and the glucose was 99.9% used. The glucose was again the growth-limiting factor. This strain of *E. coli* will not grow in PYG medium and Lula V will not grow on the M9 medium. In the case of *B. subtilis* the AEC values were higher and remained higher than those of the thermophile *B. stearothermophilus*. Since both organisms are spore formers, they probably have mechanisms to maintain the energy required for sporulation.

Balkwill and Ghiorse (*6*) found that aerobic, metabolically versatile microorganisms predominated in the Lula subsurface environment. The organisms could subsist on low concentrations of organic compounds without forming specialized resting cells. Most organisms from these subsurface samples grew better on diluted medium than on rich medium, supporting speculations that most subsurface organisms grow and survive under low-nutrient conditions (*11*). Bone and Balkwill (*17*) found that the subsurface microorganisms are nutrient-stressed and specially adapted for growth under starvation conditions. The current paper discloses that Lula V cells, even under laboratory culture conditions, may have made special adaptations in their nucleotide metabolism consistent with the environmental pressure to survive and grow under low-nutrient (oligotrophic) and fluctuating conditions. Under limited growth conditions marine bacteria produce smaller cells and effectively utilize reserve materials and cellular constituents to allow survival (*18*). Similar processes also occurred with Lula V; both the ratio (surface area/volume) and the production of poly-β-hydroxybutyrate increased under limiting growth conditions (see Magill and Ghiorse, ref. 10). The high adenylate energy charge during the stationary phase could make possible storage of nutrients for survival of Lula V, as has been suggested by Atkinson (*2*) in his analysis of the interrelationships of biosynthesis, growth, and storage. Jones and Rhodes-Roberts (*19*) demonstrated that a marine *Pseudomonas* sp. maintained a high energy charge at high cell densities during a 35-d starvation.

The high adenylate energy charge value of Lula V in stationary cultures suggests that it has developed metabolic means to sustain high energy reserves while waiting for addition of substrate and while preparing for low-nutrient conditions. This observation is consistent with the metabolic flexibility discussed by Balkwill and Ghiorse (*6*) and with the synthesis of large amounts of poly-β-hydroxybutyrate (up to 35% of the dry weight, see Magill and Ghiorse, ref. 10) and polyphosphate [up to 0.9% of the cell's dry weight (*10*)] during this stationary period. Polyglucose is stored only when the original growth medium contains 14 mM glucose (*10*). Polyphosphates are "metabolic traps" that aid in bacterial survival and in resuming metabolic activities immediately under favorable conditions (*22*); the functioning of polyphosphates as an energy source in Lula V has not been examined. Poly-β-hydroxybutyrate storage is a mechanism for survival during periods of low nutrient availability (nutritional stress) (*11, 20*).

Dawes (*21*) also believes that maintenance of a high AEC under starvation conditions is correlated to survival in prokaryotes.

During logarithmic growth, nucleotide metabolism in Lula V is like that occurring in surface microorganisms; differences occur during the stationary phase. With Gram-positive spore-forming *Bacillus subtilis* and *B. stearothermophilus*, we observed [(*15*) and unpublished data] that the AEC values decrease as the log growth phase ends. This is the normal pattern with AEC, but the situation in Lula V with continued high AEC would allow the cells to prepare for long nutritional deprivation that the organism might encounter. Table 1 shows that in the case of *E. coli* (another Gram-negative organism) the AEC value is markedly reduced after 24-h incubation. In data not shown there is also a decrease in the ATP concentration.

Wiebe and Bancroft (*3*) state that, in general, actively growing and dividing bacterial cells have AEC values of 0.8-0.95, cells in stationary growth maintain an AEC of 0.6, and the AEC in senescent cells is below 0.5. The specific changes occurring in AEC values upon starvation vary from organism to organism and with conditions. Adenylate kinase is the essential factor in controlling the AEC of *E. coli* (*22*). In the marine bacterium *Vibrio fluvialis* substantial concentrations of ATP and GTP are maintained after 48-h starvation, but there was no measurement of AMP alone so no calculation of AEC can be made. The ATP level changes from 1.2 to 0.68 nmol per mg of bacterial dry weight during the 48-h starvation (*23*). Oliver and Stringer (*24*) observed a 59% decrease in the levels of ATP during a 21-day starvation of a marine *Vibrio* sp. Cells of *Arthrobacter crystallopoietes* survive total starvation of at least 4 weeks (*25*). When growing on succinate, the cells entered the stationary phase at approximately 13 h (*25*); after a slight decrease in the AEC value during 24 h, the cells then maintained their AEC values for the next 144 h. Both the spherical and rod-shaped cells remained more than 90% viable during this time.

The subsurface organism, *Pseudomonas* sp., strain Lula V, isolated from a pristine shallow aquifer demonstrates the nutritional flexibility required of subsurface organisms. The high AEC values are consistent with utilization by the organism of its energy for synthesis of reserve compounds for long-term survival. Maintenance of high energy charge permits the cells to use immediately any suitable nutrient that come their way.

ACKNOWLEDGMENTS

A portion of this work was supported by the National Center for Ground Water Research through CR 812808 administered by the Robert S. Kerr Environmental Research Laboratory, Ada, Oklahoma. This is manuscript J-5579 of the Oklahoma Agricultural Experiment Station and was supported in part by Project 1806. It is published with the approval of the Director of the Oklahoma Agricultural Experiment Station. Drs. William Ghiorse, E.C. Nelson, James B. Blair, and Otis Dermer made helpful suggestions.

Disclaimer:

Although the research in this article has been funded wholly or in part by the United States Environmental Protection Agency, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency and no official endorsement should be inferred.

REFERENCES

1. Atkinson, D.E., and Walton, G.M., Adenosine Triphosphate Conservation in Metabolic Regulation: Rat Liver Citrate Cleavage Enzyme, *J. Biol. Chem.* **242**, 3239-3241 (1967).
2. Atkinson, D.E., *Cellular Energy Metabolism and Its Regulation,* Academic Press, New York (1977) 293 pp.
3. Wiebe, W.J., and Bancroft, K., Use of the Adenylate Energy Charge Ratio to Measure Growth State of Natural Microbial Communities, *Proc. Nat. Acad. Sci. USA.* **72**, 2112-2115 (1975).
4. Karl, D.M., and Holm-Hansen, O., Methodology and Measurement of Adenylate Energy Charge Ratios in Environmental Samples, *Mar. Biol.* **48**, 185-197 (1978).
5. Karl, D.M., Cellular Nucleotide Measurements and Applications in Microbial Ecology, *Microbiol. Rev.* **44**, 739-796 (1980).
6. Balkwill, D.L., and Ghiorse, W.C.,

Characterization of Subsurface Bacteria Associated with Two Shallow Aquifers in Oklahoma, *Appl. Environ. Microbiol.* **50**, 580-588 (1985).

7. Beloin, R.M., Sinclair, J.L., and Ghiorse, W.C., Distribution and Activity of Microorganisms in Subsurface Sediments of a Pristine Study Site in Oklahoma, *Microb. Ecol*. **16**, 85-97 (1988) and other papers contained in this special issue on ground-water organisms.
8. Webster, J.J., Hampton, G.J., Wilson, J.T., Ghiorse, W.C., and Leach, F.R., Determination of Microbial Cell Numbers in Subsurface Samples, *Ground Water* **23**, 17-25 (1985).
9. Wilson, J.T., Miller, G.D., Ghiorse, W.C., and Leach, F.R., Relationship Between the ATP Content of Subsurface Material and the Rate of Biodegradation of Alkylbenzenes and Chlorobenzene, *J. Contam. Hydrol.* **1**, 163-170 (1986).
10. Magill, N.G., and Ghiorse, W.C., Morphological Changes and Poly-β-Hydroxybutyrate Utilization by a Subsurface *Pseudomonas* sp. During Starvation, *Abst. Ann. Mtg. Am. Soc. Microbiol.* **87**, 176 (1987). For additional details see Magill, N.G., Starvation-survival of Subsurface Bacteria, Dissertation, Cornell University, Ithaca, NY, (1988) 187 pp., available from University Microfilms International, Order Number DA 8900839.
11. Wilson, J.T., McNabb, J.F., Balkwill, D.L., and Ghiorse, W.C., Enumeration and Characterization of Bacteria Indigenous to a Shallow Water-table Aquifer, *Ground Water* **21**, 134-142 (1983).
12. Anderson, E.H., Growth Requirements of Virus-resistant Mutants of *Escherichia coli* Strain "B", *Proc. Nat. Acad. Sci. USA.* **32**, 120-128 (1946).
13. Kornberg, A., and Stadtman, E.R., Preparation of Coenzyme A, *Meth. Enzymol.* **3**, 907-913 (1957).
14. Webster, J.J., and Leach, F.R., Optimization of the Firefly Luciferase Assay for ATP, *J. Appl. Biochem.* **2**, 469-479 (1980).
15. Webster, J.J., Walker, B.G., and Leach, F.R., ATP Content and Adenylate Energy Charge of *Bacillus stearothermophilus* During Growth, *Curr. Microbiol.* **16**, 271-275 (1988).
16. Karl, D.M., Determination of GTP, GDP, and GMP in Cell and Tissue Extracts, *Met. Enzymol.* **57**, 85-94 (1978).
17. Bone, T.L., and Balkwill, D.L., Morphological and Cultural Comparison of Microorganisms in Surface Soil and Subsurface Sediments at a Pristine Study Site in Oklahoma, *Microb. Ecol.* **16**, 49-64 (1988).
18. Novitsky, J.A., and Morita, R.Y., Possible Strategy for the Survival of Marine Bacteria under Starvation Conditions, *Mar. Biol.* **48**, 289-295 (1978).
19. Jones, K.L., and Rhodes-Roberts, M.E., The Survival of Marine Bacteria under Starvation Conditions, *J. Appl. Bacteriol.* **50**, 247-258 (1981).
20. Smith, G.A., Nickels, J.S., Kerger, B.D., Davis, J.D., Collins, S.P., Wilson, J. T., McNabb, J.F., and White, D.C., Quantitative Characterization of Microbial Biomass and Community Structure in Subsurface Material: a Prokaryotic Consortium Responsive to Organic Contamination, *Can. J. Microbiol.* **32**, 104-111 (1986).
21. Dawes, E.A., Starvation, Survival, and Energy Reserves, in *Bacteria in Their Natural Environment* (Fletcher, M., and Floodgate, G.D., Eds.) Publication **16** of the Soc. Gen. Microbiol., Academic Press, London, (1985) pp. 43-79.
22. Glembotski, C.C., Chapman, A.G., and Atkinson, D.E., Adenylate Energy Charge in *Escherichia coli* CR341T28 and Properties of Heat-sensitive Adenylate Kinase, *J. Bacteriol.* **145**, 1374-1385 (1981).
23. Smigielski, A.J., Wallace, B.J., and Marshall, K.C., Changes in Membrane Functions During Short-term Starvation of *Vibrio fluvialis* Strain NCTC 11328, *Arch. Microbiol.* **151**, 336-341 (1989).
24. Oliver, J.D., and Stringer, W.F., Composition of a Psychrophilic Marine *Vibrio* sp. During Starvation-induced Morphogenesis, *Appl. Environ. Microbiol.* **47**, 461-466 (1984).
25. Leps, W.T., and Ensign, J.C., Adenylate Nucleotide Levels and Energy Charge in *Arthrobacter crystallopoietes* During Growth and Starvation, *Arch. Microbiol.* **122**, 69-76 (1979).