Microbiology, Vol. 69, No. 3, 2000, pp. 266–270. Translated from Mikrobiologiya, Vol. 69, No. 3, 2000, pp. 328–333.
Original Russian Text Copyright © 2000 by Krasil'nikova, Zakharchuk.

EXPERIMENTAL ARTICLES

The Activity of the Carbon Metabolism Enzymes in *Chromatium minutissimum* after Long-Term Storage

E. N. Krasil'nikova and L. M. Zakharchuk

Department of Microbiology, Biological Faculty, Moscow State University, Vorob'evy gory, Moscow, 119899 Russia

Received April 27, 1999

Abstract—The activity of the enzymes of the tricarboxylic acid cycle and glyoxylate shunt, as well as of some enzymes involved in carbohydrate metabolism, were determined in the purple sulfur bacterium *Chromatium minutissimum* either maintained by subculturing in liquid medium or stored in the lyophilized state for 36 years. In cultures stored in the lyophilized state, the activities of the key enzymes of the tricarboxylic acid cycle, glyoxylate shunt, and Embden–Meyerhof–Parnas pathway were higher, whereas the activities of glucose-6-phosphate dehydrogenase, pyruvate kinase, and ribulose bisphosphate carboxylase were somewhat lower than in cultures maintained by regular transfers.

Key words: purple sulfur bacteria, enzymes of the tricarboxylic acid cycle, enzymes of carbohydrate metabolism, methods for microorganism storage, lyophilization

To ensure the survival of microorganisms and the stability of their biochemical properties, enzyme activities, and vitamin- and antibiotic-producing abilities, microorganisms are maintained in culture collections by several parallel methods. Regular culture transfers, storage under mineral oil, drying, storage at low and extra low temperatures, lyophilization, and other methods are used [1–3].

Lyophilization is one of most efficient storage methods ensuring the viability of microorganisms and the stability of their enzymatic activities. Tens of microbial cultures of various systematic groups have been stored in the Culture Collection of Moscow State University in the lyophilized state for as long as 35–36 years [4–6]. Most of these cultures have retained their viability.

The aim of the present work was to determine the activity of certain carbon metabolism enzymes in cultures of the purple sulfur bacterium *Chromatium minutissimum* that was stored in the Culture Collection of the Moscow State University in the lyophilized state for 36 years and to compare these activities with those in bacterial cultures maintained by periodical reinoculations in liquid medium.

MATERIALS AND METHODS

The subject of this study was the purple sulfur bacterium *Chr. minutissimum*, strain 1, isolated by researchers from the Department of Microbiology at Moscow State University.

Bacteria were grown anaerobically in the light (2000 lx) in sealed flasks completely filled with Larsen mineral medium containing sulfide and thiosulfate (0.1% each). Cultivation was autotrophic or heterotrophic; in the latter case, the medium was supplemented with 0.1% acetate, malate, or glucose [7].

Bacteria were lyophilized from a 1% gelatin solution containing 10% sucrose [5]. Ampules with lyophilized cultures were stored at 3–6°C. After 36 years of storage, bacteria were inoculated into Larsen medium with sulfide and thiosulfate (0.1% each).

To determine enzyme activities, exponential-phase cultures were used. Cells were sedimented by centrifugation, washed with 0.05 M Tris–HCl buffer (pH 7.8), resuspended in the same buffer, and disrupted ultrasonically at 22 kHz for 2.5–3 min. The supernatant obtained after homogenate centrifugation at 40000 *g* for 30 min was used to determine enzyme activities. Protein in cell extracts was determined by the method of Lowry *et al.* [8].

The activities of the tricarboxylic acid cycle (TCA cycle) enzymes and enzymes of the carbon metabolism were determined spectrophotometrically as described earlier [9, 10]. The proceeding of pyruvate decomposition with the formation of acetyl phosphate (the phosphoclastic reaction involving pyruvate : ferredoxin oxidoreductase, hydrogenase, and phosphotransacetylase) was assayed in a reaction mixture containing methyl viologen and kept under an argon atmosphere [11].

The activities of ribulose bisphosphate carboxylase (RuBPC), phosphoenolpyruvate carboxylase, and phosphoenolpyruvate carboxykinase were determined by the radioisotopic method [12] from the rate of radiocarbon incorporation from [^{14}C]bicarbonate by cellular extracts. To study the effect of NaCN on RuBPC, it was added at a concentration of 1 mM.

0026-2617/00/6903-0266$25.00 © 2000 MAIK "Nauka/Interperiodica"

Table 1. Activities (nmol/(min mg protein)) of the enzymes of the TCA cycle and glyoxylate shunt in *Chr. minutissimum* strain 1 cells grown in media of different compositions

Enzyme	Culture stored by reinoculations			Lyophilically stored culture		
	grown in medium with					
	malate	acetate	sulfide and thiosulfate	malate	acetate	sulfide and thiosulfate
Citrate synthase	17.3	21.8	12.6	15.2	17.8	13.8
Aconitate hydratase	26.4	54.5	62.0	85.0	80.0	61.0
Isocitrate dehydrogenase	46.9	28.6	16.0	51.0	90.0	19.0
2-Oxoglutarate dehydrogenase	0	0	0	0	0	0
Fumarase	42.1	75.7	59.0	46.0	89.0	52.0
Malate dehydrogenase	0	0	0	0	0	0
Malic enzyme	25.0	42.6	19.0	94.0	88.0	24.0
Isocitrate lyase	1.6	10.2	0	3.5	11.6	0.9
Malate synthase	23.5	25.5	27.0	51.0	48.0	39.0

Table 2. Activities (nmol/(min mg protein)) of the carbohydrate metabolism enzymes in *Chr. minutissimum* strain 1 cells grown in media with malate and glucose

Enzyme	Culture stored by reinoculations		Lyophilically stored culture	
	grown in medium with			
	malate	glucose	malate	glucose
Hexokinase	0.6	4.2	0.9	5.9
Glucose-6-phosphate dehydrogenase	3.8	7.5	1.8	4.6
Fructose bisphosphate aldolase	130.0	153.0	230.0	245.0
6-Phosphofructokinase	11.5	16.4	15.8	10.0
Glyceraldehyde-3-phosphate dehydrogenase	39.0	100.0	57.0	119.0
Pyruvate kinase	86.0	163.0	63.4	122.0
Phosphoclastic reaction	12.8	16.3	37.4	38.0

RESULTS

Enzymes of the TCA Cycle and Glyoxylate Shunt

Cultures of *Chr. minutissimum*, strain 1, both those maintained by regular transfers and those lyophilically stored for 36 years, exhibited the activities of all of the main TCA cycle enzymes except 2-oxoglutarate dehydrogenase and malate dehydrogenase (Table 1). The activity of the key TCA cycle enzyme, citrate synthase, was somewhat higher in cells grown on acetate than in cells grown on malate and autotrophically grown cells. The activity of citrate synthase in cells grown on acetate was slightly lower after lyophilic storage of *Chr. minutissimum* than after its maintenance by subculturing.

The level of other TCA enzymes studied was generally higher in lyophilically stored cultures of *Chr. minutissimum* (Table 1); e.g., the activity of aconitate hydratase in such cultures grown on malate or acetate was, respectively, 3 and 1.5 times higher than in cultures maintained by regular transfers.

After autotrophic cultivation, no differences in aconitate dehydrogenase activity were revealed between cultures stored by different methods. The activity of isocitrate dehydrogenase in acetate-grown cells of lyophilically stored cultures was 3 times higher than in acetate-grown cells of cultures maintained by regular transfers (Table 1). After autotrophic growth or heterotrophic growth on malate-containing medium, the activity of isocitrate dehydrogenase was slightly higher in lyophilically stored cultures.

In acetate-grown cells, the activity of fumarase was 20% higher for lyophilically stored cultures; in malate-grown or autotrophically grown cells, the activity of fumarase was independent of the culture storage method (Table 1). The activity of the malic enzyme of the lyophilically stored cultures was higher than that of

Table 3. Activities (nmol/(min mg protein)) of carboxylases in *Chr. minutissimum* strain 1 cells grown in media of different compositions

Enzyme	Culture stored by reinoculations			Lyophilically stored culture		
	grown in medium with					
	malate	acetate	sulfide and thiosulfate	malate	acetate	sulfide and thiosulfate
RuBPC	12.3	14.0	30.2	16.6	8.1	21.2
RuBPC + 1 mM cyanide	–	1.5	2.2	–	1.04	1.64
PEP carboxylase						
Mg^{2+}	0.1	0.1	0.1	0.1	0.1	0.2
Mn^{2+}	0.1	0.2	0.1	0.1	0.1	0.3
PEP carboxykinase						
ADP	0.1	0.1	0.1	0	0	0.1

Note: "–" means that a given experimental variant was not run.

the cultures maintained by regular transfers (fourfold for malate-grown cells and twofold for acetate-grown cells).

The TCA cycle in *Chr. minutissimum* is incomplete, lacking 2-oxoglutarate dehydrogenase and malate dehydrogenase. The absence of these enzymes is compensated for by the presence of the key enzymes of the glyoxylate shunt, isocitrate lyase and malate synthase (Table 1). The activity of malate synthase was higher for lyophilically stored cultures (2-fold in malate- and acetate-grown cells and 1.5-fold in autotrophically grown cells). The activity of citrate lyase was not affected by the method of storage.

Enzymes of Carbohydrate Metabolism

Chr. minutissimum can, at a moderate rate, utilize some sugars as an additional carbon source. Carbohydrate metabolism in this bacterium proceeds via the fructose bisphosphate pathway [10]. This pathway may also be operative during the synthesis of reserve polysaccharides from organic acids, including acetate and malate.

Table 2 shows the activities of hexose catabolism enzymes in *Chr. minutissimum* cells grown in media with malate or glucose. The activities of hexokinase and glyceraldehyde-3-phosphate dehydrogenase in malate- and glucose-grown cells were greater for lyophilically stored cultures. The activities of fructose bisphosphate aldolase (the key enzyme of the Embden–Meyerhof pathway) and of enzymes responsible for the phosphoclastic reaction (pyruvate cleavage with the formation of acetyl phosphate) in malate- and glucose-grown cells were, respectively, 1.6 and 2.5 times higher for lyophilically stored cultures than for cultures maintained by regular transfers (Table 2). On the contrary, the activities of glucose-6-phosphate dehydrogenase and pyruvate kinase in malate- and glucose-grown cells was higher for cultures maintained by regular transfers.

Carboxylases

Table 3 presents data on the activities of some carboxylases in *Chr. minutissimum* cultures as dependent on the storage method. The activity of RuBPC, the key enzyme of the Calvin cycle, was higher after autotrophic cultivation, irrespective of the storage method; the highest value was recorded in autotrophically grown cells of cultures maintained by regular transfers.

In acetate-grown cells, the activity of RuBPC was about two times lower than in autotrophically grown cells, irrespective of the culture storage method (Table 3). In malate-grown cells, the activity of RuBPC was also lower than in autotrophically grown cells: by 25% for the case of lyophilically stored cultures and by 2.5 times for cultures maintained by regular transfers.

Introduction of 10^{-3} M cyanide, an RuBPC inhibitor, into the reaction mixture inhibited the reaction rate by an order of magnitude, irrespective of the storage method and growth substrate (Table 3). Linear kinetics of CO_2 fixation was observed for at least 8–10 min irrespective of the presence of cyanide (Figs. 1, 2), confirming the operation of RuBPC.

It is known that microorganisms possessing the Calvin cycle can also fix CO_2 via other reactions, including the carboxylation of phosphoenolpyruvate and pyruvate. Therefore, in addition to RuBPC activity, we also determined the activities of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase (Table 3). The activity of phosphoenolpyruvate carboxylase proved to be very low, irrespective of the culture storage method and growth substrate. The activity of phosphoenolpyruvate carboxykinase was also very low; in malate- and acetate-grown cells of lyophilically stored cultures, its activity could not even be detected (Table 3).

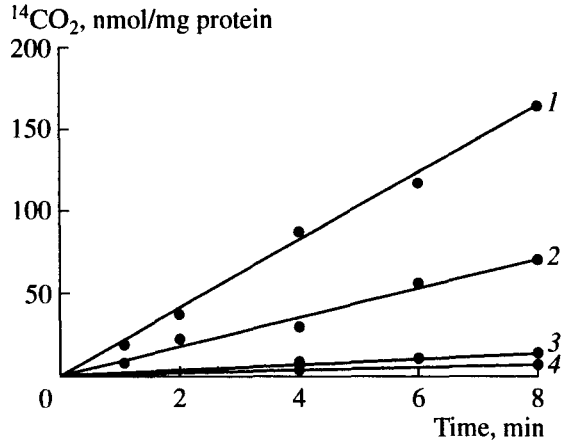

Fig. 1. Activity of RuBPC (nmol/mg protein) in a lyophilically stored culture of *Chr. minutissimum* strain 1: (*1*) cells were grown autotrophically; (*2*) cells were grown in acetate medium; (*3*) cells were grown autotrophically; 1 mM cyanide was added to the reaction mixture; (*4*) cells were grown in acetate medium; 1 mM cyanide was added to the reaction mixture.

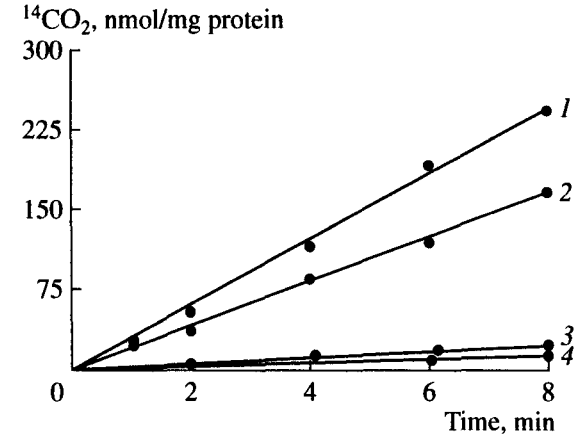

Fig. 2. Activity of RuBPC (nmol/mg protein) in a culture of *Chr. minutissimum* strain 1 maintained by regular reinoculations into liquid medium: (*1*) cells were grown autotrophically; (*2*) cells were grown in acetate medium; (*3*) cells were grown autotrophically; 1 mM cyanide was added to the reaction mixture; (*4*) cells were grown in acetate medium; 1 mM cyanide was added to the reaction mixture.

DISCUSSION

In the course of lyophilization, microorganisms are subject to various stresses, such as freezing, drying, etc.

As follows from the data obtained in the present work, the activity of some enzymes changed in the culture of *Chr. minutissimum* stored for 36 years in a lyophilic state. The activities of aconitate hydratase, isocitrate dehydrogenase, malic enzyme, malate synthase, fructose bisphosphate aldolase, and of the enzyme complex responsible for the phosphoclastic reaction increased (Tables 1, 2), whereas the activities of glucose-6-phosphate dehydrogenase, pyruvate kinase, and ribulose bisphosphate carboxylase somewhat decreased (Tables 2, 3). On the whole, the type of metabolism did not change in the lyophilically stored culture.

The differences in the enzyme activities observed between cultures of *Chr. minutissimum* stored lyophilically and by regular transfers cannot be explained without a special additional investigation. At present, we may assume that these differences were due to mutations arising in the bacterial population as a result of storage in the lyophilic state.

It is known that, during lyophilization, a considerable portion of cells die due to protein, DNA, and RNA denaturation and damage to membranes, cell walls, and other structures. The addition of protectants and the use of sparing lyophilization methods [13–16] increase the cell survival rate but do not allow cell death to be completely avoided.

During the resuscitation of the anabiotic cells that have survived the process of lyophilization and storage in the lyophilized state, damaged DNA is repaired via the postreplicational reparation; errors during the latter process give rise to mutants [17–19]. Lyophilization of bacterial cells may cause multiple DNA breaks [16]; therefore, mutations usually affect entire chromosome segments rather than individual nucleotides.

It was shown that the frequency of mutations depends on the storage temperature of the lyophilized cultures. After the storage of lyophilized *Escherichia coli* cells at temperatures below 5°C, virtually no mutations were detected, whereas at higher temperatures, mutations arose at a frequency of 10^{-7} [16, 18, 19]. A number of chemical compounds can prevent mutations [3, 20]. These are, first of all, adonitol (100 mM), cysteine (3 mM), and thiourea (30 mM). In the Culture Collection of Moscow State University, ampules with lyophilized *Chr. minutissimum* cultures are stored at 3–6°C, and the protecting medium lacks compounds preventing mutations. Therefore, it can be suggested that the changes found in the enzymatic activities of *Chr. minutissimum* cells after 36 years of storage result from mutations. However, relevant experiments are necessary for final conclusions to be made.

Changes in the enzymatic activities of *Chr. minutissimum* cultures maintained by regular transfers and, in particular, the decrease in these activities can also be explained by spontaneous mutations and their selection by cultivation conditions. Surely, in this case, relevant experiments are also necessary for final conclusions. However, culture transfers of photosynthetic bacteria that have been repeated many times are known to sometimes result in culture contamination in spite of all the precautions taken; the consequently needed culture purification may result in the selection of a clone carrying this or that spontaneous mutation. Therefore, culture storage by lyophilization seems a more reliable method; however, the use of storage temperatures no higher than 2–3°C is recommended, and the medium

should be supplemented with mutation-preventing compounds.

REFERENCES

1. Arkad'eva, Z.A., Factors Affecting the Viability and Properties of Microorganisms during Storage by Various Methods, *Biol. Nauki*, 1983, no. 4, pp. 93–105.
2. Malik, K.A., Use of Activated Charcoal for the Preservation of Anaerobic Phototrophic and Other Sensitive Bacteria by Freeze-Drying, *J. Microbiol. Methods*, 1990, vol. 12, pp. 117–124.
3. Kizsop, B.S. and Doyle, A., *Maintenance of Microorganisms and Cultured Cells: A Manual of Laboratory Methods*, London: Academic, 1991, 2nd ed.
4. Kupletskaya, M.B. and Arkad'eva, Z.A., Long-Term Maintenance of Microorganisms in the Culture Collection of the Microbiology Department of Moscow State University, *Mikrobiologiya*, 1997, vol. 66, no. 2, pp. 312–317.
5. Kupletskaya, M.B., On the Lyophilization of Saprophytic Microorganisms, *Mikrobiologiya*, 1961, vol. 30, no. 4, pp. 717–721.
6. Kupletskaya, M.B., The Results of the 25-Year-Long Storage of Lyophilized Microbial Cultures, *Mikrobiologiya*, 1987, vol. 56, no. 3, pp. 488–491.
7. Kondrat'eva, E.N., *Fotosinteziruyushchie bakterii* (Phototrophic Bacteria), Moscow: Akad. Nauk SSSR, 1963.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.I., Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem.*, 1951, vol. 193, no. 1, pp. 265–275.
9. Krasil'nikova, E.N., Pedan, L.V., Firsov, N.N., and Kondrat'eva, E.N., Tricarboxylic Acid Cycle Enzymes in Various Species of Phototrophic Bacteria, *Mikrobiologiya*, 1973, vol. 42, no. 6, pp. 995–1000.
10. Krasil'nikova, E.N., Enzymes of Carbohydrate Metabolism in Phototrophic Bacteria, *Mikrobiologiya*, 1975, vol. 44, no. 1, pp. 5–10.
11. Lovenberg, W., Buchanon, B.B., and Robinowitz, J.C., Studies on the Chemical Nature of Clostridial Ferredoxin, *J. Biol. Chem.*, 1963, vol. 238, no. 10, pp. 3899–3907.
12. Romanova, A.K., *Biokhimicheskie metody izucheniya avtotrofii u mikroorganizmov* (Biochemical Methods for Studying Autotrophy in Microorganisms), Moscow: Nauka, 1980.
13. Ashwood-Smith, M.J. and Grant, E., Mutation Induction in Bacteria by Freeze-Drying, *Cryobiology*, 1976, vol. 13, pp. 206–213.
14. Israeli, E., Shaffer, B.T., and Lighthart, B., Protection of Freeze-Dried *Escherichia coli* by Trehalose upon Exposure to Environmental Conditions, *Cryobiology*, 1993, vol. 30, pp. 519–523.
15. Israeli, E., Shaffer, B.T., Hoyt, J.A., Lighthart, B., and Ganio, L.M., Survival Differences among Freeze-Dried Genetically Engineered and Wild-Type Bacteria, *Appl. Environ. Microbiol.*, 1993, vol. 59, pp. 594–598.
16. Calcott, P.H. and Gargett, A.M., Mutagenicity of Freezing and Thawing, *FEMS Microbiol. Lett.*, 1981, vol. 10, pp. 151–155.
17. Ohnishi, T., Tanaka, Y., Yoh, M., Takeda, Y., and Miwatani, T., Deoxyribonucleic Acid Strand Breaks during Freeze-Drying and Their Repair in *Escherichia coli*, *J. Bacteriol.*, 1977, vol. 130, pp. 1393–1396.
18. Tanaka, Y., Yoh, M., and Miwatani, T., Induction of Mutation in *Escherichia coli* by Freeze-Drying, *Appl. Environ. Microbiol.*, 1979, vol. 37, pp. 369–372.
19. Asada, S., Takano, M., and Shibasaki, I., Mutation Induced by Drying *Escherichia coli* on a Hydrophobic Membrane, *Appl. Environ. Microbiol.*, 1980, vol. 40, pp. 274–281.
20. Sakane, T., Banno, I., and Iijima, T., Compounds Protecting L-Dried Cultures from Mutation, *IFO Res. Commun.*, 1983, vol. 11, pp. 14–26.