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EXPERIMENTAL ARTICLES

Metabolism of the Phase Variants of the Phototrophic Bacterium *Rhodobacter sphaeroides*

E. S. Mil'ko, E. N. Krasil'nikova, O. I. Keppen, N. V. Lebedeva, and R. N. Ivanovsky[1]

Department of Microbiology, Biological Faculty, Lomonosov Moscow State University, Russia

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Abstract—Growth, bacteriochlorophyll *a* content, electron transport chain (ETC), and activities of the tricarboxylic acid (TCA) cycle enzymes were studied in R and M phase variants of *Rhodobacter sphaeroides* cells grown anaerobically in the light and aerobically in the dark. Under all cultivation conditions tested, bacteriochlorophyll *a* content was 2–3 times lower in the cells of the M variant compared to the R variant, which therefore was predominant in the cultures grown in the light. In both variants, activity of all TCA cycle enzymes was higher for the cells grown in the dark under aerobic conditions. When grown aerobically in the dark, the R variant, unlike the M variant, did not contain cytochrome aa_3, acting as cytochrome *c* oxidase, in its ETC. An additional point of coupling the electron transfer to the generation of the proton gradient at the cytochrome aa_3 level provided for more efficient oxidation of organic substrates, resulting in predominance of the M variant in the cultures grown in the dark under aerobic conditions.

Keywords: photosynthetic bacteria, *Rhodobacter sphaeroides*, adaptive variation, R and M variants, tricarboxylic acid cycle, electron transport chain

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Phase variations are defined as reversible variations in bacterial genome. Their frequency (10^{-2} to 10^{-5} per generation) is significantly higher than that of spontaneous mutations (10^{-6}–10^{-8}). The diverse molecular mechanisms of variation in bacterial genome have been described in a number of reviews [1–4]. The biological role of phase variation is to ensure bacterial adaptation to varying environmental conditions. A bacterial population always contains a number of variants, with their ratio depending on the growth phase. Significant changes in environmental conditions result in the substitution of a less adapted variant by another one, which has a higher growth speed under these conditions.

Genetic, physiological, biochemical, and morphological characteristics of phase variants of various bacterial species have been described in the literature. For pathogenic bacteria, which were the main subject of research, variations in the properties of the outer membrane and in capacity for biofilm formation and synthesis of the polysaccharide capsule were studied [5–10]. The data on the biochemical properties of the variants of saprotrophic bacteria, including the data on their carbon metabolism, are scarce, dealing mainly with the differences in utilization of different sugars [11, 12]. No information concerning variations in the central carbon metabolism is available in the literature.

The purple nonsulfur photosynthetic bacterium *Rhodobacter sphaeroides*, which was the subject of our research, has a variable metabolism. It is able to grow aerobically and anaerobically in the light, as well as under aerobic conditions in the dark, using organic substrates as carbon and energy sources [13]. We have previously reported the capacity of this bacterium for phase variation [14]. The R and M variants were isolated from *Rba. sphaeroides* populations. PCR of the 16S rRNA genes revealed their identity with the parent strain. The variants differed in colony morphology, pigmentation, growth rate, and resistance to various physical and chemical factors. The R variant was shown to grow well in the light under both aerobic and anaerobic conditions. Both the growth rate of the M variant in the light and its final yield were lower than in the case of the R variant. Under aerobic conditions in the dark, both the growth rate and final yield of the M variant were higher [14].

The goal of the present work was to investigate the carbon and energy metabolism of the R and M variants of *Rba. sphaeroides* under different growth conditions.

MATERIALS AND METHODS

The purple nonsulfur bacterium *Rba. sphaeroides* strain 2R from the collection of Department of Micro-

[1] Corresponding author; e-mail: mguru@mail ru

Table 1. Biomass yield and bacteriochlorophyll synthesis in *Rba. sphaeroides* phase variants grown anaerobically in the light and aerobically in the dark

Growth conditions	R-variant		M-variant	
	cell number, 10^8 cells/mL	bacteriochlorophyll, μg/mg protein	cell number, 10^8 cells/mL	bacteriochlorophyll, μg/mg protein
Anaerobic, light	35.0	12.7	22.0	3.6
Aerobic, dark	7.0	1.3	18.0	0.4

biology, Moscow State University was the subject of our research. The cultures were grown at 30°C in Ormerod medium [15] with 0.1% malate under anaerobic conditions in the light (2000 lx) in vials filled to capacity or under aerobic conditions in the dark in shake (200 rpm) flasks filled to one-third of capacity. Two- or three-day cultures of the R and M variants, respectively, which were grown in the same medium, were used as inocula (5%, vol/vol). The ratio of the variants in the population and the total number of cells were determined by plating aliquots of the cultures (tenfold dilutions in physiological saline) onto nutrient agar with wart (1 : 1).

Cells from the late exponential growth phase were used for determination of the enzyme activity. The cells were washed with K phosphate buffer (pH 7.8) and sonicated at 22 kHz for 2 min. Undisrupted cells were removed by centrifugation (18000 *g*, 20 min), and the supernatant was used for analyses. Activity of the enzymes of the tricarboxylic acid (TCA) cycle was determined in 50 mM K phosphate buffer or Tris buffer (50 mM, pH 7.8) at room temperature. Citrate synthase (EC 4.1.3.7) activity was determined at 412 nm with 5,5-dithiobis-2-nitrobenzoate [16]. Aconitate hydratase (EC 4.2.1.3) activity was determined by an increase in OD_{240} [17]. Isocitrate dehydrogenase (EC 1.1.1.42) activity was determined by NADP reduction (340 nm) [18]. Succinate dehydrogenase (EC 1.3.99.1) activity was measured at 420 nm by ferricyanide reduction [19]. Fumarate hydratase (EC 4.2.1.2) was determined by an increase in OD_{250} in the presence of malate [20]. Activity of malate dehydrogenase (EC 1.1.1.37) was measured at 340 nm as NADP oxidation in the presence of oxaloacetate [18]. Bacteriochlorophyll was determined at 765 nm after extraction from whole cells with acetone–methanol (7 : 2) [21]. Differential spectra (ferricyanide minus dithionite) in *Rba. sphaeroides* cell-free extracts were determined using a Hitachi 2000 double-beam spectrophotometer. Oxidase activity of cell suspensions was measured as oxygen consumption using a closed platinum Clark electrode. *N*,*N*,*N*,*N*-Tetramethyl-*p*-phenylenediamine (100 μM) with 5 mM Na ascorbate was used as an electron donor. Protein in whole cells and cell extracts was determined by the Lowry method.

RESULTS AND DISCUSSION

Activity of the enzymes of the TCA cycle, which provides keto acids (amino acid precursors), was used as an indicator of the possible differences in metabolism of the R and M variants. TCA cycle is also the major source of energy for photosynthetic bacteria growing in the dark. The concentration of bacteriochlorophyll *a* (Bchl *a*), the main pigment of the *Rba. sphaeroides* photosynthetic apparatus, and the structure of the respiration chain were also studied in the variants grown under different conditions.

Bacteriochlorophyll. Bacteria were grown anaerobically in the light and aerobically in the dark (Table 1). During anaerobic growth in the light, Bchl *a* content in the cells of the M variant was 3.5 times lower than in the R variant. This was probably the reason for lower growth rates of the M variant in the light under anaerobic conditions.

Oxygen suppresses the synthesis of bacteriochlorophyll in *Rba. sphaeroides* both in the light and in the dark. This suppression, however, is never complete. The cells grown under aerobic conditions always contain ~10% of the Bchl *a* concentration in anaerobically grown cells. Importantly, when grown anaerobically in the light, both variants exhibited correlation between their growth rates and bacteriochlorophyll content. This correlation was not, however, observed when the variants were grown aerobically in the dark. Under these conditions, the growth rate of the M variant remained at the initial level, while that of the R variant decreased sixfold.

TCA cycle enzymes. All the TCA cycle enzymes, except for isocitrate lyase, were revealed in the cells of both *Rba. sphaeroides* variants (Table 2). This indicated the absence of the glyoxylate cycle in this organism and confirmed the previous data [22, 23]. In the R variant, 2-oxoglutatrate dehydrogenase exhibited low activity, while in the M variant it was absent. Cultivation of both variants in the dark under aerobic conditions derepressed 2-oxoglutarate dehydrogenase synthesis. This is an indication of the possible functioning of a completely closed TCA cycle under these conditions (Table 2).

The data presented in Table 2 show activity of the TCA cycle enzymes in *Rba. sphaeroides* R variant grown under anaerobic conditions in the light to be comparable to that of the M variant grown under the

Table 2. Activity of the TCA cycle enzymes in *Rba. sphaeroides* variants grown in malate medium under various conditions, nmol/(min mg protein)

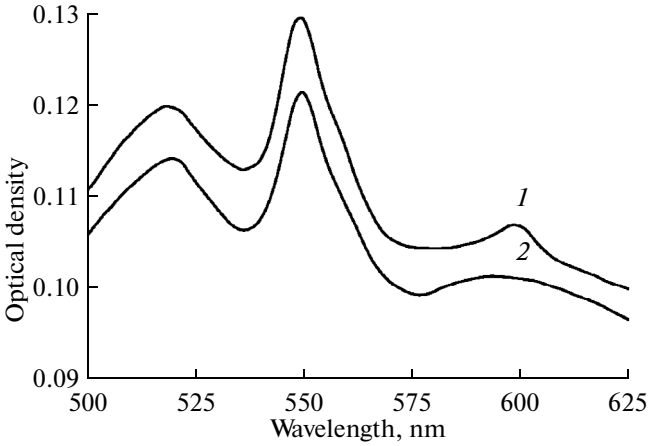
Enzyme	R-variant			M-variant		
	anaerobic, light	aerobic, dark	d/l	anaerobic, light	aerobic, dark	d/l
Citrate synthase	76.4	120.7	1.6	88.1	96.0	1.1
Aconitase	201.2	419.5	2	133.9	100.9	0.7
Isocitrate dehydrogenase	120.7	562.9	4.6	144.4	250.0	1.7
Succinate dehydrogenase	25.3	37.6	1.52	42.1	38.2	0.9
Fumarate hydratase	33.3	40.4	1.2	33.1	42.9	1.3
Malate dehydrogenase	71.6	755.6	10.5	308.3	1006.9	3.2
2-Oxoglutarate dehydrogenase	0.5	10.1	20.2	0.0	7.8	
Isocitrate lyase	0.0	0.0		0.0	0.0	

D/l indicates the ratio of enzyme activity of the cultures grown aerobically in the dark to those grown anaerobically in the light.

same conditions. Activity of the TCA cycle enzymes in the cells of the R and M variants grown under aerobic conditions in the dark suggests a similar conclusion. The considerably higher activity of malate dehydrogenase in the M variant, independent of growth conditions, was an exception in this respect.

Aerobic cultivation of the R variant in the dark resulted in a significant increase in the activity of all the TCA cycle enzymes, compared to the values in the cells grown in the light under anaerobic conditions. This was an indication of additional synthesis of the TCA cycle enzymes in the R variant switching from the phototrophic to organotrophic metabolism, with oxidation of organic substrates via the TCA cycle becoming the main energy source.

Activity of the TCA cycle enzymes in the cells of the M variant grown under aerobic conditions in the dark also increased, although to a lesser degree than in the cells of the R variant. These data indicate that the M variant of *Rba. sphaeroides* is better adapted to aerobic growth in the dark than the R variant. Unlike the R variant, in the M variant switching from anaerobic phototrophic to aerobic heterotrophic metabolism does not require additional synthesis of the TCA cycle enzymes.



Differential absorption spectrum (ferricyanide–dithionite) of cell-free extracts of *Rba. sphaeroides* variants grown in the dark under aerobic conditions: M variant (*1*) and R variant (*2*).

Electron transport chain. In the case of *Rba. sphaeroides* grown aerobically in the dark, the R variant, unlike the M variant, did not contain cytochrome aa_3, acting as a cytochrome *c* oxidase (figure).

An additional site for coupling electron transfer with generation of the proton gradient at the cytochrome aa_3 level provides for more efficient oxidation of organic substrates by the M variant cells. This corresponded with the lower cytochrome *c* oxidase activity of the R variant, compared to the M variant (99.8 and 117.8, respectively).

A similar result was previously obtained for *Azospirillum lipoferum* grown under aerobic and microaerobic conditions [24]. During aerobic growth, the laccase-negative variant $4V_I$ with cytochrome aa_3 as a cytochrome *c* oxidase predominated in *A. lipoferum* populations. Growth under microaerobic conditions resulted in predominance of the laccase-positive variant $4V_{II}$, which lost the ability to synthesize cytochrome aa_3 [25].

Thus, *Rba. sphaeroides* grown in the dark under aerobic conditions resulted in substitution of the R variant, which is adapted to photoheterotrophic growth, by the M variant, better adapted to aerobic organotrophic growth. The respiratory chain of the M variant contains cytochrome aa_3, which acts as cytochrome *c* oxidase. The presence of an additional site for coupling electron transfer with the generation of the proton gradient at the cytochrome aa_3 level provides for the energetically more efficient oxidation of

organic substrates and may be considered an adaptation of *Rba. sphaeroides* cells to aerobic metabolism

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REFERENCES

1. Ryall, B., Eydallin, G., and Ferenci, T., Culture history and population heterogeneity as determinants of bacterial adaptation: the adaptomics of a single environmental transition, *Microbiol. Mol. Biol. Rev.*, 2012, vol. 76, no. 3, pp. 597–625.
2. van der Woude, M.W., Phase variation: how to create and coordinate population diversity, *Cur. Opin. Microbiol.*, 2011, vol. 14, no. 2, pp. 205–211.
3. van der Woude, M.W. and Bäumler, A.J., Phase and antigenic variation in bacteria, *Clin. Microbiol. Rev.*, 2004, vol. 17, no. 3, pp. 581–611.
4. Vial, L., Lavire, C., Mavingui, P., Blaha, D., Haurat, J., Moenne-Loccoz, Y., Bally, R., and Wisniewski-Dye, F., Phase variation and genomic architecture changes in *Azospirillum, J. Bacteriol.*, 2006, vol. 188, no. 15, pp. 5364–5373.
5. Kirisits, M., Prost, L., Starkey, M., and Parsek, M.R., Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms, *Appl. Environ. Microbiol.,* 2005, vol. 71, no. 8, pp. 4809–4821.
6. Golod, N.A., Loiko, N.G., Mulyukin, A.L., Neiymatov, A.L., Vorobjeva, L.E., Suzina, N.E., Shanenko, E.F., Gal'chenko, V.F., and El-Registan, G.I., Adaptation of lactic acid bacteria to unfavorable growth conditions, *Microbiology* (Moscow), 2009, vol. 78, no. 3, pp. 280–289.
7. Doroshenko, E.V., Loiko, N.G., Il'inskaya, O.N., Kolpakov, A.I., Gornova, I.B., Klimova, E.V., and El'Registan, G.I., Characterization of *Bacillus cereus* dissociants, *Microbiology* (Moscow), 2001, vol. 70, no. 6, pp. 698–705.
8. Mil'ko, E.S. and Egorov, N.S., *Geterogennost' populyatsii bakterii i protsess dissotsiatsii* (Heterogeneity of Bacterial Populations and the Process of Dissociation), Moscow: Mos. Gos. Univ., 1991.
9. Fursova, P.V., Mil'ko, E.S., and Levich, A.P., *Limitiruyushchie resursy i sostav soobshchestva bakterii: eksperimenty i model'nyi analiz* (Limiting Resources and Composition of Bacterial Communities: Experiments and Model Analysis), Moscow: GEOS, 2008.
10. Mulyukin, A.L., Kozlova, A.N., and El-Registan, G.I., Properties of the phenotypic variants of *Pseudomonas aurantiaca* and *P. fluorescens, Microbiology* (Moscow), 2008, vol. 77, no. 6, pp. 681–690.
11. Mil'ko, E.S. and Il'inykh, I.A., The effect of major nutrient elements on the growth and population homogeneity of the R, S, and M dissociants of *Pseudomonas aeruginosa* and the glucose oxidation and fermentation pathways, *Microbiology* (Moscow), 2004, vol. 73, no. 1, pp. 30–36.
12. Mil'ko, E.S. and Krasilnikova, E.N., Peculiarities of carbohydrate metabolism of R-, S- and M-dissociants of *Pseudomonas aeruginosa, Microbiology* (Moscow), 1999, vol. 68, no. 2, pp. 175–178.
13. Kondrat'eva, E.N., *Fotosinteziruyushchie bakterii* (Photosynthetic Bacteria), Moscow: Izdatel'stvo Akademii nauk SSSR, 1963.
14. Ivanovsky, R.N., Mil'ko, E.S., and Mil'ko D.M., Isolation of the phase variants of the purple photosynthetic bacterium *Rhodobacter sphaeroides* and investigation of their molecular, physiological, biochemical, and morphological characteristics, *Microbiology* (Moscow), 2013, vol. 82, no. 2, pp. 169–185.
15. Ormerod, J.G., Ormerod, K.S., and Gest, H., Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism, *Arch. Biochem. Biophys.*, 1961, vol. 94, pp. 449–463.
16. Srere, P.A., Citrate synthase, in *Methods in Enzymology*, Lowenstein, J.M., Ed., New York: Academic, 1969, vol. 13, pp. 3–11.
17. Anfinsen, C.B., Aconitase from pig heart muscle, in *Methods in Enzymology*, Colowick, S.P. and Kaplan, N.O., Eds., New York: Academic, 1955, vol. 1, pp. 695–698.
18. Holo, H. and Sirevag, R., Autotrophic growth and CO_2 fixation of *Chloroflexus aurantiacus, Arch. Microbiol.*, 1986, vol. 145, pp. 173–180.
19. Veeger, C., Der Vartanian, D.V., and Zeylemaker, W.P., Succinate degydrogenase, in *Methods in Enzymology*, Lowenstein, J.M., Ed., New York: Academic, 1969, vol. 13, pp. 51–61.
20. Bergmeyer, H.U., Gawehn, K., and Grasse, M., Fumarate hydratase, in *Methods of Enzymatic Analysis*, New York: Academic, 1974, vol. 1, pp. 425–522.
21. Cohen-Bazire, G., Sistrom, W.R., and Stanier, R.Y., Kinetic studies of pigment synthesis by non-sulfur purple bacteria, *J. Cell Physiol.*, 1957, vol. 49, no. 1, pp. 25–32.
22. Filatova, L.V., Berg, I.A., Krasil'nikova, E.N., and Ivanovsky, R.N., A study of the mechanism of acetate assimilation in purple nonsulfur bacteria lacking the glyoxylate shunt: enzymes of the citramalate cycle in *Rhodobacter sphaeroides, Microbiology* (Moscow), 2005, vol. 74, no. 3, pp. 270–278.
23. Filatova, L.V., Berg, I.A., Krasil'nikova, E.N., Tsygankov, A.A., Laurinavichene T.V., and Ivanovsky, R.N., A study of the mechanism of acetate assimilation in purple nonsulfur bacteria lacking the glyoxylate: acetate assimilation in *Rhodobacter sphaeroides, Microbiology* (Moscow), 2005, vol. 74, no. 3, pp. 265–269.
24. Alexandre, G. and Bally, R., Emergence of a laccase-positive variant of *Azospirillum lipoferum* occurs via a two-step phenotypic switching process, *FEMS Microbiol. Lett.*, 1999, vol. 172, no. 2, pp. 371–378.
25. Alexandre, G., Bally, R., Taylor, B.L., and Zhulin, I.B., Loss of cytochrome *c* oxidase activity and acquisition of resistance to quinone analogs in a laccase-positive variant of *Azospirillum lipoferum, J. Bacteriol.*, 1999, vol. 181, no. 21, pp. 6730–6738.

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