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EXPERIMENTAL ARTICLES

Metabolism of the Thermophilic Bacterium *Oceanithermus profundus*

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Abstract—The metabolism of the novel facultatively anaerobic thermophilic bacterium *Oceanithermus profundus* was studied during growth on maltose, acetate, pyruvate, and hydrogen. The utilization of carbohydrates was shown to proceed via the glycolytic pathway. Under microaerobic growth conditions, the metabolism of *O. profundus* grown on maltose depended on the substrate concentration. At an initial maltose concentration of 1.4 mM, *O. profundus* carried out oxygen respiration, and in the presence of 3.5 mM maltose, facilitated fermentation occurred, with the formation of acetate and ethanol and limited involvement of oxygen. The use of pyruvate and acetate occurred via the TCA cycle. In cells grown on acetate, the activity of glyoxylate pathway enzymes was revealed. Depending on the energy-yielding process providing for growth (oxygen respiration or nitrate reduction), cells contained cytochromes *a* and *c* or *b*, respectively. The results obtained demonstrate the plasticity of the metabolism of *O. profundus*, which thus appears to be well-adjusted to the rapidly changing conditions in deep-sea hydrothermal vents.

Key words: Oceanithermus profundus, oxygen respiration, nitrate reduction, the TCA cycle.

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The family *Thermaceae* is among the best studied groups of thermophilic prokaryotes [1]. This is a phylogenetically well-defined group of bacteria that are mostly obligately respiring chemoorganotrophs, aerobes or facultative anaerobes capable of dissimilatory reduction of nitrate to nitrite and Fe(III) to Fe(II). The organisms of the family *Thermaceae* mainly inhabit continental hydrothermal vents of various geographical locations [1]. *Oceanithermus profundus*, a representative of a novel genus and species [2] of the family *Thermaceae*, has recently been isolated from a deep-sea hydrothermal vent. Phenotypically, the new organism is in many respects close to other representatives of the family *Thermaceae*; however, it has a number of features likely to be connected with its habitat, deep-sea hydrothermal vents, where volcanic fluid rapidly mixes with oceanic water. Like other members of the family *Thermaceae, O. profundus* uses a wide range of organic substrates; however, it is the first *Thermaceae* representative capable of lithoheterotrophic growth with molecular hydrogen. *O. profundus* is also the first microaerophile in the family *Thermaceae*. Like many other representatives of *Thermaceae, O. profundus* can grow anaerobically at the expense of nitrate reduction to nitrite.

The aim of this work was to study the metabolism of *O. profundus* during growth on various energy substrates under microaerobic conditions and conditions of nitrate reduction.

MATERIALS AND METHODS

The subject of study. The facultatively anaerobic thermophilic bacterium *Oceanithermus profundus* DSM 14977 was the subject of this study.

Cultivation. *O. profundus* cells were grown on medium of the following composition (g l^{-1}): NH_4Cl, 0.33; KCl, 0.33; KH_2PO_4, 0.33; $CaCl_2 \cdot 2H_2O$, 0.33; $MgCl_2 \cdot 6H_2O$, 0.33; NaCl, 25; yeast extract, 0.1; $NaHCO_3$, 0.5; trace element solution [3], 1 ml l^{-1}; and vitamins [4], 1 ml l^{-1}. When *O. profundus* was grown anaerobically with nitrate, the medium contained 2 g l^{-1} $NaNO_3$. Depending on the aim of the experiment, maltose at varied concentrations (1.4–3.5 mM), acetate, or sodium pyruvate(2 g l^{-1}) was added to the medium as an energy substrate. After sterilization, the medium pH was 7.0–7.2. The cultivation was carried out at 60°C in 15-ml Hungate tubes with 10 ml of medium or in

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500-ml bottles with 300 ml of medium closed with rubber stoppers. The free space was filled with an oxygen-free N_2/CO_2 (8 : 2) mixture into which 2% oxygen was introduced in the case of microaerobic growth. If hydrogen was the substrate, the medium volume was reduced to 5 ml (Hungate tubes) or 100 ml (serum bottles) and the free volume was filled with 100% hydrogen.

Obtaining cell extracts. Cell suspensions were obtained by culture centrifugation at 4500 *g* and 4°C for 1 h. The precipitate was washed several times with 20 mM Tris–HCl or MES buffer, pH 7.0. In order to obtain cell homogenate, the cells were disrupted in a UZDN-2T ultrasonic disintegrator for 3 min with pauses for cooling. The supernatant obtained after homogenate centrifugation at 15000 *g* and 4°C for 30 min (cell extract) was used immediately or stored at –18°C.

Determination of metabolic products. Nitrate was determined with sulfanilic acid [5]; other metabolic products were determined gas chromatographically on a Kristall 5000.1 chromatograph (ZAO Khromatek, Russia) equipped with a flame ionization detector and a katharometer; acetate and ethanol were determined using a 10 m × 0.5 mm × 1.2 μm Superox capillary column (Alltech, United States) at 60–160°C (10°C/min) with helium as the carrier gas; hydrogen was determined using a 3 m × 2 mm column with 5 Å molecular sieve at 40°C with argon as the carrier gas. The maltose concentration was determined in the reaction with phenol spectrophotometrically at 488 nm [6].

Protein measurement. Protein was analyzed by the method of Lowry et al. [7] in cell extracts with a protein concentration of 0.02–0.08 mg ml^{-1}.

Determination of the activity of enzymes. All measurements of the enzyme activity were carried out spectrophotometrically on Specord UV-VIS at 60°C. The activities of nitrate reductase (EC 1.7.99.4), hydrogenase (EC 1.12.1.2), aconitate hydratase (EC 4.2.1.3), succinate dehydrogenase (SDH) (EC 1.3.99.1), NAD-dependent malate dehydrogenase (MDH) (EC 1.1.1.37), acetate kinase (EC 2.7.2.1), phosphoacetyltransferase (EC 2.3.1.8), NADP-dependent glutamate dehydrogenase (GDH) (EC 1.4.1.3), isocitrate lyase (EC 4.1.3.1), malate synthase (EC 4.1.3.2), NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42), citrate synthase (EC 4.1.3.7), fumarate hydratase (EC 4.2.1.2), NAD-dependent 2-oxoglutarate dehydrogenase (EC 1.2.4.2), lactate dehydrogenase (LDH) (EC 1.1.1.27), alanine aminotransferase (EC 2.6.1.2), aspartate aminotransferase (EC 2.6.1.1), catalase (EC 1.11.1.6), and superoxide dismutase were determined by the methods indicated in our previous publication [8]. The activities of NAD-dependent decarboxylating malate dehydrogenase (MDH_{CO_2}) (EC 1.1.1.38) [9], pyruvate carboxylase (EC 6.4.1.1), PEP-carboxylase (EC 4.1.1.31) [10], pyruvate kinase (EC 2.7.1.40) [11], pyruvate dehydrogenase [12], NADH-oxidase and NADH-peroxidase (EC 1.11.1.1) [13], as well as the activities of the glycolysis enzymes hexokinase (EC 2.7.1.1), glucose-6-phosphate isomerase (EC 5.3.1.9), fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and alcohol dehydrogenase (EC 1.1.1.1) [14], were determined by the methods described in the corresponding publications. Succinate dehydrogenase activity was determined in cell homogenate, while the activity of the rest of the enzymes was studied in cell extract. In order to study the cytochromes, the disintegrated cells were centrifuged for 1 min at 15000 *g*; 3 ml of the extract obtained was oxidized with atmospheric oxygen, and the same volume was reduced with sodium dithionite [15]. The cytochrome composition was determined using an SF-56 spectrophotometer (Russia).

In inhibition experiments, the succinate dehydrogenase and isocitrate lyase inhibitors thenoyltrifluoroacetone (TTFA) and itaconate were used at a concentration of 5 mM [16]. The inhibitors were introduced into the medium for cultivation of the bacteria.

The specific growth rate (h^{-1}) and the cell yield coefficient (cells/mmol substrate) were calculated using the standard formulas.

All the experiments were performed in three or four replicates; the analytical determinations for each sample were performed in three replicates. The statistically significant differences ($p < 0.05$) are discussed.

RESULTS

Carbohydrate metabolism in *O. profundus*. *O. profundus* shows good growth on medium with maltose, both under microaerobic conditions and anaerobically with nitrate, attaining a final cell concentration of 3.0–9.0 × 10^7 cells/ml. In the absence of nitrate, no growth under anaerobic conditions with maltose was observed. The activities of the main enzymes of carbohydrate metabolism were determined in the extract of cells grown on medium with 3.5 mM (1.26 g l^{-1}) maltose under microaerobic conditions (Table 1). We revealed the activity of hexokinase, an enzyme carrying out glucose phosphorylation, and the activity of the key glycolysis enzymes, namely, glucose-6-phosphate isomerase, whose activity results in the formation of fructose-6-phosphate, fructose-1,6-bisphosphate aldolase, splitting fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, and pyruvate kinase, converting phosphoenolpyruvate to pyruvate. Thus, the utilization of carbohydrates by *O. profundus* follows the glycolytic pathway. No activity of glucose-6-phosphate dehydrogenase, the key enzyme of the pentosephosphate and Entner–Doudoroff pathways that converts glucose-6-phosphate to 6-phosphogluconolactone, was recorded.

The influence of maltose concentration on the metabolism of *O. profundus*. To study the influence of the substrate concentration on the growth of *O. profundus*, we used media with two maltose concentrations,

1.4 and 3.5 mM. The growth of *O. profundus* on maltose was suppressed by high substrate concentrations both under microaerobic conditions and anaerobic conditions in the presence of nitrate (Fig. 1). Both the specific growth rate and the ultimate cell yield decreased, as well as the cell yield coefficient (Figs. 2, 3; Table 2). Other physiological and biochemical differences in the *O. profundus* culture and cells determined by the maltose concentration were also revealed (Table 2). Thus, with an increase in the maltose concentration under microaerobic growth conditions, the products of its incomplete oxidation, acetate and ethanol, were revealed in the medium. The same products, 1.5 mM acetate and the trace amounts of ethanol, were also revealed in the culture liquid of *O. profundus* grown anaerobically on medium with nitrate. No hydrogen was detected in the gas phase in either of the cases. In the difference spectra of *O. profundus* cells grown on medium with 1.4 mM maltose under microaerobic conditions, cytochromes *c* (525- and 552-nm peaks) and *a* (603-nm peak) were detected. In cells grown microaerobically on medium with 3.5 mM maltose, cytochromes were absent. Under these conditions, a high NADH-oxidase (1 μmol min^{-1} mg^{-1} protein) and NADH-peroxidase activity (0.6 μmol min^{-1} mg^{-1} protein) was revealed. Finally, the inhibitors of the TCA and glyoxylate cycle enzymes, succinate dehydrogenase (TTFA) and isocitrate lyase (itaconate), inhibited the growth of *O. profundus* on medium with 1.4 mM maltose under microaerobic conditions but did not influence growth in the presence of 3.5 mM maltose. In the latter case, despite the fact that we did reveal the activity of certain TCA cycle enzymes in the cells (aconitate hydratase, malate dehydrogenase, succinate dehydrogenase, and fumarate hydratase) (Table 1), it was 2–10 times lower than the activity in cells grown on acetate (Table 3).

Thus, we observed inhibition of *O. profundus* growth by high maltose concentrations both under microaerobic conditions and under anaerobic conditions in the presence of nitrate. Inhibitory analysis and analysis of cytochromes allow the assumption that, at high maltose concentrations, the organism grew at the expense of the substrate phosphorylation and that regeneration of electron carriers occurred via idle discharge of electrons to the available acceptor.

Pyruvate metabolism in *O. profundus*. In the extract of cells grown under microaerobic conditions on medium with maltose (3.5 mM), we revealed activity of the pyruvate dehydrogenase complex, which consisted of NAD-dependent pyruvate dehydrogenase, converting pyruvate formed in the process of glycolysis to acetyl-CoA, and phosphoacetyltransferase, producing acetyl phosphate from acetyl-CoA (Table 1). Acetate and one ATP molecule are formed from acetyl phosphate under the action of acetate kinase, whose activity was also detected.

Table 1. Enzyme activity in the extract of *O. profundus* cells grown on medium with 3.5 mM maltose under microaerobic conditions

Enzyme	Activity, μmol min^{-1} mg^{-1} protein
Hexokinase	0.12–3.0
Glucose-6-phosphate dehydrogenase	0.0
Glucose-6-phosphate isomerase	0.25–5.9
Fructose-1,6-bisphosphate aldolase	0.5
Pyruvate kinase	0.7
Pyruvate dehydrogenase	0.13–0.24
Lactate dehydrogenase	0.0
Phosphoacetyltransferase	0.5
Acetate kinase	0.9
Alcohol dehydrogenase (direct)	0.11
Alcohol dehydrogenase (reverse)	0.03–0.13
NADH oxidase	1.0
NADH peroxidase	0.6
Aconitate hydratase	0.2
Malate dehydrogenase	0.9
Succinate dehydrogenase	0.5
Fumarate hydratase	0.09
Catalase	11.7–17.5
Superoxide dismutase	4.3

Pyruvate is one of the energy substrates used by *O. profundus* in the process of growth. In the extract of cells grown on medium with pyruvate and nitrate, the activity of TCA and glyoxylate cycle enzymes was

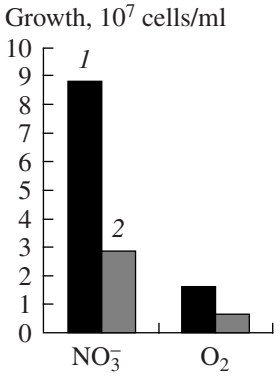

Fig. 1. Relationship between the *O. profundus* cell yield and the initial maltose concentration in the medium (*1*, 0.5 mM; *2*, 3.5 mM) during anaerobic growth with nitrate and microaerobic growth.

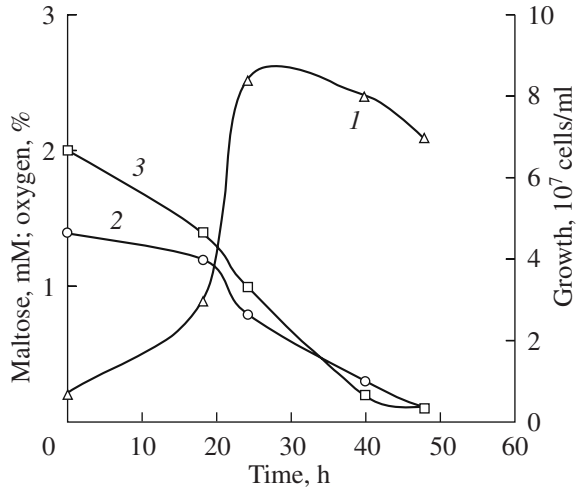

Fig. 2. Cell yield (*1*) and utilization of maltose (*2*) and oxygen (*3*) by *O. profundus* on medium with 1.4 mM maltose.

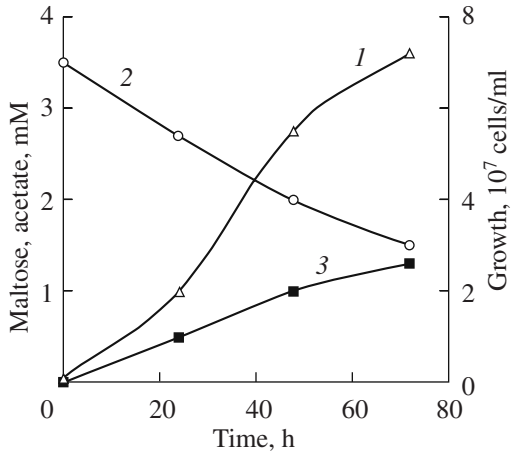

Fig. 3. Cell yield (*1*), maltose utilization (*2*), and acetate production (*3*) by *O. profundus* on medium with 3.5 mM maltose.

revealed (Table 3). Pyruvate may be involved in the TCA cycle not only through acetyl-CoA but also through oxaloacetate. In the former case, the process is catalyzed by NAD-dependent pyruvate dehydrogenase as shown above for *O. profundus* growth under microaerobic conditions on medium with maltose; in the latter case, pyruvate is converted to phosphoenolpyruvate by pyruvate kinase and, then, to oxaloacetate by phosphoenolpyruvate carboxylase. Since we revealed the activity of pyruvate kinase and phosphoenolpyruvate carboxylase (Table 3), the involvement of pyruvate in the TCA cycle via oxaloacetate is quite possible.

Table 2. Physiological and biochemical characteristics of microaerobically grown *O. profundus* cultures as dependent on the initial maltose concentration in the medium

Parameter	Maltose concentration, mM	
	1.4	3.5
Specific growth rate, h^{-1}	0.17	0.04
Cell yield coefficient over 24 h of growth, 10^8 cells/mmol	1.2	0.21
Fermentation products	Not found	Acetate (1.3 mM), ethanol (0.5 mM)
Cytochromes in cells	Cytochromes *a* and *c*	Not found
Effect of the TCA cycle enzyme inhibitors	Growth is inhibited by itaconate and TTFA	Itaconate and TTFA do not influence growth

Acetate metabolism in *O. profundus*. When *O. profundus* grew on medium with acetate as an energy substrate and nitrate as an electron acceptor, the acetate concentration in the medium decreased from 20 to 7 mM over 96 h (Fig. 4). Simultaneously, nitrite, the nitrate reduction product, was formed (13 mM). No other nitrate reduction products were revealed. Under these cultivation conditions, we studied *O. profundus* acetate metabolism (Table 3). The involvement of acetate in the metabolism occurred through acetyl-CoA, formed under the action of acetate kinase and phosphoacetyltransferase. We revealed the activity of all of the TCA and glyoxylate cycle enzymes, as well as the activity of Mg-NAD-dependent MDH_{CO_2}, Mn-NAD-dependent MDH_{CO_2}, and pyruvate carboxylase, which are capable of bypassing the TCA cycle at the malate–oxaloacetate section (Table 3).

In the extracts of *O. profundus* cells grown on medium with acetate and nitrate, cytochrome *b* with absorption maximums at 430 and 560 nm was revealed.

Nitrate reductase activity of *O. profundus*. In *O. profundus* cells grown on medium with acetate and nitrate, nitrate reductase activity was revealed. The enzyme was active in the range of temperatures between 45 and 85°C with the optimum at 70°C and in the pH range of 6.0–9.6 with the optimum at pH 7.4. Under optimal conditions, the nitrate reductase activity was 1 μmol min^{-1} mg^{-1} protein.

Hydrogenase activity of *O. profundus*. In *O. profundus* cells grown on medium with nitrate in the presence of hydrogen, hydrogenase activity (7.5 μmol min^{-1} mg^{-1} protein) was revealed.

Antioxidant system enzymes. In *O. profundus* cells grown under microaerobic conditions on maltose, we revealed the activity of catalase and superoxide dismutase (SOD), the enzymes involved in the removal of toxic oxygen reduction products (O_2^-, H_2O_2) (Table 1).

In cells grown at the expense of nitrate respiration on acetate, we only succeeded in revealing SOD activity (Table 3).

DISCUSSION

The results of this investigation agree with the original phenotypic description of *Oceanithermus profundus* [2], a thermophilic bacterium carrying out various types of metabolism: microaerobic respiration on fermentable and nonfermentable substrates, nitrate reduction, and lithoorganotrophic growth on molecular hydrogen. The study of the metabolism of *O. profundus* grown on maltose showed carbohydrates to be involved in metabolism via the glycolytic pathway. This, as well as the functioning of the TCA cycle and the glyoxylate bypass, is also characteristic of other members of the family *Thermaceae*, for which the presence of these metabolic pathways was established by both biochemical and genomic studies [17, 18]. Our investigations also demonstrated that, when *O. profundus* grew on acetate, it had a bypass functioning at the malate–oxaloacetate site, which involves Mg-NAD-dependent MDH_{CO_2}, Mn-NAD-dependent MDH_{CO_2}, and pyruvate carboxylase. Pyruvate is involved in the metabolism both via acetyl-CoA, formed by pyruvate dehydrogenase (NAD), and via oxaloacetate through reactions catalyzed by pyruvate kinase and phosphoenolpyruvate carboxylase.

Unexpected were the results that we obtained when studying the growth of *O. profundus* at different maltose concentrations. It follows from these results that, at a maltose concentration of 3.5 mM, *O. profundus* grows at the expense of substrate phosphorylation and, in fact, carries out fermentation (Fig. 5, Table 2). Such a conclusion is supported by the formation of acetate and ethanol, absence of cytochromes from cells grown in medium with 3.5 mM maltose, and lack of effect of TCA cycle inhibitors on growth. However, fermentation in *O. profundus* appeared to be only possible in the presence of electron acceptors (oxygen or nitrate). The NADH oxidase activity revealed by us testifies to the fact that oxygen might be involved in the metabolic processes occurring during fermentation: apparently, oxygen and its derivatives induce the performance of NADH-oxidase and NADH-peroxidase, which compensate for the low alcohol dehydrogenase activity, thus ensuring the regeneration of NAD necessary for glycolysis. This is the first evidence of growth at the expense of fermentation in *Thermaceae* representatives, which have so far been considered to be obligately respiring organisms [1]. It may be supposed that similar mechanisms are quite widespread. So far, a few examples of such metabolism have been revealed in several representatives of different phylogenetic groups of prokaryotes. Thus, O_2 involvement in fermentation process appeared to be inherent in several spirochetes from the symbiotic "Thiodendron" association [13], in the facultatively anaerobic strain Ep01 [19], and in *Leuconostoc* [20]; it results in glucose fermentation along a shortened pathway with the formation of more oxidized end products. Under microaerobic conditions, these microorganisms exhibit more efficient glucose utilization and an increased cell yield as compared to strictly anaerobic conditions. However, as distinct from the above bacteria, *O. profundus* is incapable of regeneration of electron carriers in the absence of electron acceptors; hence, it is incapable of fermentation in the classical sense. The cause may be a low alcohol dehydrogenase activity and a lack of hydrogen-producing

Table 3. Enzyme activity in the extracts of *O. profundus* cells grown on medium with acetate or pyruvate and nitrate

Enzyme	Activity, μmol min^{-1} mg^{-1} protein during growth on	
	pyruvate	acetate
Pyruvate kinase	0.3	ND
Acetate kinase	ND	0.15
Phosphoacetyltransferase	ND	0.7
Citrate synthase	0.19	0.33
Aconitate hydratase	1.0	2.4
Isocitrate dehydrogenase	0.7	0.3
2-Oxoglutarate dehydrogenase	ND	0.25
Succinate dehydrogenase	1.8	1.5
Fumarate hydratase	0.11	0.19
Malate dehydrogenase	1.3	3.6
Isocitrate lyase	1.3	0.16
Malate synthase	0.04	0.05
MDH_{CO_2}, Mg-NAD-dependent	0.0	0.1
MDH_{CO_2}, Mn-NAD-dependent	0.0	0.1
Pyruvate carboxylase	ND	1.1
Phosphoenolpyruvate carboxylase	0.4	ND
Nitrate reductase	1.0	1.0
Glutamate dehydrogenase	ND	0.6
Alanine aminotransferase	ND	0.06
Aspartate aminotransferase	ND	0.03
Catalase	0.0	0.0
Superoxide dismutase	0.0	4.4

Note: Pyruvate, acetate, and nitrate were introduced at a concentration of 2 g l^{-1}. ND stands for "not determined."

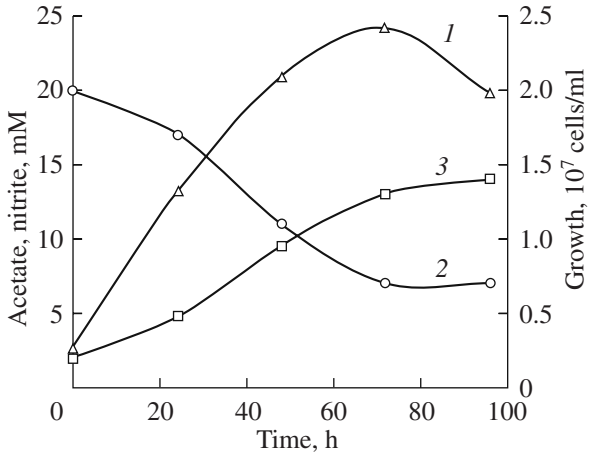

Fig. 4. Cell yield (*1*) and acetate utilization (*2*) and production (*3*) by *O. profundus* on medium with acetate and nitrate.

hydrogenase, as evidenced by the absence of hydrogen production.

At a maltose concentration of 1.4 mM, *O. profundus* carries out, depending on the conditions, aerobic respiration or nitrate respiration. In the case of microaerobic growth, this is confirmed, in particular, by the high cell yield coefficient, absence of incomplete maltose oxidation products, and inhibition of growth by TCA cycle enzyme inhibitors. The corresponding cytochromes were revealed in cells grown microaerobically and anaerobically with nitrate. The cytochrome *b* revealed in cells grown with nitrate is part of the nitrate reductase complex and mediates electron transfer to nitrate with nitrite formation.

In *O. profundus* cells grown under microaerobic conditions, we revealed enzymes removing toxic oxygen species (catalase and SOD). The absence of catalase activity in nitrate-respiring cells seems to be linked to chemical detoxification of hydrogen peroxide by nitrite. Thus, the microaerophilic nature of *O. profundus* is probably related to the inhibitory effect exerted by oxygen on a number of TCA cycle enzymes, primarily, aconitate hydratase and fumarate hydratase [21], rather than to the absence of oxygen detoxification mechanisms.

Thus, our study showed that, although *O. profundus* metabolism has many features in common with the metabolism of other *Thermaceae* representatives, it also exhibits some novel features, in particular, fermentation capacity, which is realized only in the presence of external electron acceptors (oxygen or nitrate). Substrate concentration appeared to play a regulatory role, directing carbohydrate utilization along one or another pathway with an accordingly higher or lower energy yield. Further studies will show whether the regularities

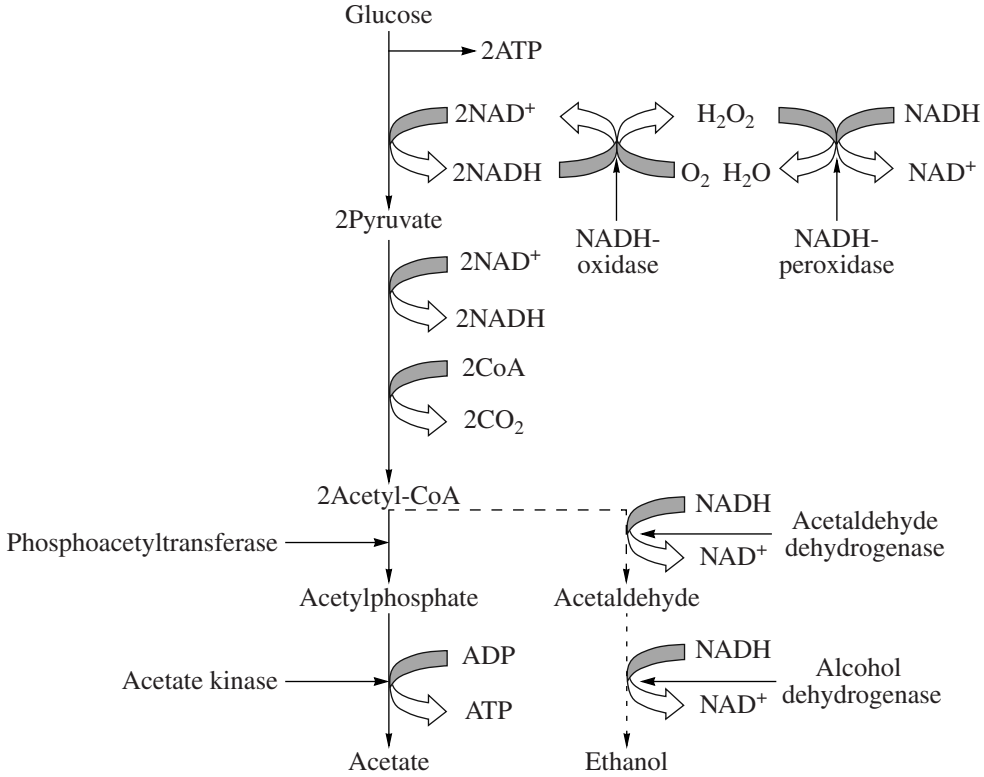

Fig. 5. Scheme of *O. profundus* fermentation on medium with 3.5 mM maltose. Solid arrows: the enzyme activity was determined; broken arrow: the enzyme activity was not determined; dotted arrow: low enzyme activity.

revealed by us are specific features of the genus *Oceanithermus* or are also inherent to related organisms.

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