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EXPERIMENTAL ARTICLES

Investigation of the Catabolism of Acetate and Peptides in the New Anaerobic Thermophilic Bacterium *Caldithrix abyssi*

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Abstract—This work is concerned with the metabolism of *Caldithrix abyssi*—an anaerobic, moderately thermophilic bacterium isolated from deep-sea hydrothermal vents of the Mid-Atlantic Ridge and representing a new, deeply deviated branch within the domain *Bacteria.* Cells of *C. abyssi* grown on acetate and nitrate, which was reduced to ammonium, possessed nitrate reductase activity and contained cytochromes of the *b* and *c* types. Utilization of acetate occurred as a result of the operation of the TCA and glyoxylate cycles. During growth of *C. abyssi* on yeast extract, fermentation with the formation of acetate, propionate, hydrogen, and CO_2 occurred. In extracts of cells grown on yeast extract, acetate was produced from pyruvate with the involvement of the following enzymes: pyruvate:ferredoxin oxidoreductase (2.6 μmol/(min mg protein)), phosphate acetyltransferase (0.46 μmol/(min mg protein)), and acetate kinase (0.3 μmol/(min mg protein)). The activity of fumarate reductase (0.14 μmol/(min mg protein)), malate dehydrogenase (0.17 μmol/(min mg protein)), and fumarate hydratase (1.2 μmol/(min mg protein)), as well as the presence of cytochrome *b*, points to the formation of propionate via the methyl-malonyl-CoA pathway. The activity of antioxidant enzymes (catalase and superoxide dismutase) was detected. Thus, enzymatic mechanisms have been elucidated that allow *C. abyssi* to switch from fermentation to anaerobic respiration and to exist in the gradient of redox conditions characteristic of deep-sea hydrothermal vents.

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Key words: deep-sea hydrothermal vents, thermophilic bacterium, *Caldithrix abyssi*, dissimilatory nitrate reduction, pyruvate:ferredoxin oxidoreductase, methyl-malonyl-CoA pathway.

The thermophilic bacterium *Caldithrix abyssi* was isolated from deep-sea hydrothermal vents of the Mid-Atlantic Ridge [1]. It is an obligately anaerobic organism capable of organotrophic growth at the expense of peptide fermentation. In addition, in the presence of nitrate, this bacterium can grow at the expense of utilization of molecular hydrogen or acetate as electron donors for nitrate reduction to ammonium. Phylogenetic analysis has shown that *C. abyssi* is the first cultivated representative of a new phylogenetic lineage within the domain *Bacteria.* Earlier, analysis of a library of environmental clones of 16S rRNA genes obtained from the DNA isolated from shallow-water hydrothermal vents of Greece showed that organisms belonging to the same group massively occur in subsurface layers of hydrothermal sediments [2]; however, due to the absence of laboratory cultures, their functions in the microbial community remained unknown. The aim of the present work was to investigate the organotrophic and lithotrophic metabolisms of *C. abyssi*, which is the first cultivated representative of the new phylogenetic lineage.

MATERIALS AND METHODS

Subject of the study and cultivation conditions. The subject of this study was the thermophilic anaerobic bacterium *Caldithrix abyssi* DSM 13497^T. *C. abyssi* cells were grown in an anaerobically prepared medium whose mineral composition mimicked seawater [1]. For investigation of anaerobic respiration, medium with 2 g/l of sodium acetate, 2 g/l of sodium nitrate, and 0.1 g/l of yeast extract (Difco) (medium 1) was used. The free space in the flask was filled with nitrogen purified from oxygen traces. If hydrogen was the substrate of anaerobic respiration, acetate was omitted from medium 1 and the free space in the cultivation flask was filled with 100% hydrogen (medium 1 contains 0.3 g/l of sodium bicarbonate [1]). To ensure growth at the expense of fermentation, medium 2 was used, which had the same composition as medium 1 except that it was devoid of acetate and nitrate, and the content of yeast extract, meant to be the energy source, was 2 g/l.

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A pH value of 6.8–7.0 was adjusted by titration with 10% NaOH or 10% HCl. The anaerobically prepared medium was dispensed in 300-ml portions into 500-ml flasks and sterilized at 120°C. Inoculated flasks were incubated at 60°C for 24 h, during which the culture attained the late exponential phase of growth.

Analytical methods. The ammonium formed was determined with the Nessler reagent. Hydrogen, gaseous products of denitrification, and volatile fatty acids were determined by gas and gas–liquid chromatography according to methods described in [1, 3].

Obtaining cell suspensions. Cells were harvested by centrifugation at 4500 *g* and 4°C for 1 h. The sediment was washed three times with 20 mM Tris–HCl or MES buffer (pH 7.0). To obtain cell homogenates, cell suspensions were subjected to disruption in a UZDN-2T ultrasonic disintegrator at 22 kHz for 3 min under cooling and with pauses. The supernatant obtained after centrifugation of the homogenate at 15000 *g* and 4°C for 30 min was stored at –18°C or used immediately for enzyme activity determinations; it was introduced into a cuvette in an amount of 0.05–0.1 ml. Protein was determined by the method of Lowry et al. [4]; the protein concentration was 1.5 mg/ml in the cell homogenate and 0.02–0.08 mg/ml in the supernatant.

Investigation of the enzyme activity. All determinations of enzyme activity were performed spectrophotometrically on a Specord UV-VIS in 3-ml quartz cuvettes at 60°C. The activity of all enzymes was determined in the supernatant; an exception was succinate dehydrogenase, whose activity was determined in the homogenate.

The activity of nitrate reductase was determined anaerobically in a nitrogen atmosphere with benzyl viologen as the electron donor. The reaction mixture had the following composition (mM): anaerobically prepared MES (pH 7.0), 20; benzyl viologen, 2; $NaNO_3$, 10; the mixture was reduced with 40 mM $Na_2S_2O_4$. The temperature dependence of the nitrate reductase activity was determined in a temperature range of 25–85°C; the pH dependence was determined in a pH range of 2.5–10.0, using 10 mM citrate–phosphate buffer (pH 2.5–6.0), 20 mM MES (pH 6.5–8.0), and 100 mM glycine buffer (pH 8.5–10.0). The activity of aminotransferases was determined with the use of a Lahema Diagnostica (Czechia) biotest. References to the methods for determination of the other enzymes are indicated in the table.

Determination of the composition of cytochromes. To determine cytochrome composition, disrupted cells were centrifuged for 1 min at 15000 *g*, and 3 ml of the extract obtained were oxidized with air, whereas the same volume was reduced with sodium dithionite. Difference absorption spectra were recorded on an SF-56 spectrophotometer.

RESULTS

Nitrate respiration of *C. abyssi* during growth with acetate and nitrate. The growth of *C. abyssi* in medium 1 was accompanied by consumption of acetate and nitrate and the formation of ammonium (data not shown). No other products of nitrate reduction, including gaseous ones, were detected.

The activity of a number of key enzymes of the carbon metabolism were determined in extracts of *C. abyssi* cells grown on medium 1 with acetate and nitrate (see table). The activity of acetate kinase and phosphate acetyltransferase was found, as well as the activity of all enzymes of the TCA and glyoxylate cycles.

In cells of *C. abyssi* grown on medium 1 without acetate under hydrogen, the activity of hydrogenase was found, which was 6.8 μmol/(min mg protein).

An assay with benzyl viologen as the electron donor revealed nitrate reductase activity in the cell extracts of *C. abyssi* (table). The activity optima of this enzyme were found to be 70°C and pH 7.68.

Investigation of the extract of *C. abyssi* cells grown with acetate and nitrate for the presence of cytochromes revealed their *b*- and *c*-types, with absorption peaks at 430, 559 (cytochrome *b*), 433, 525, and 552 (cytochrome *c*).

Metabolism of *C. abyssi* during growth at the expense of yeast extract fermentation. Anaerobic growth of *C. abyssi* on medium 2 with 2 g/l of yeast extract was accompanied by the production of acetate (0.6 ± 0.05 mM) and propionate (2.27 ± 0.19 mM), as well as hydrogen and carbon dioxide. The addition of nitrate (20 mM) produced no effect on either the growth rate or the cell yield.

In extracts of *C. abyssi* cells grown on yeast extract, we detected proteolytic activity, apparently related to peptide hydrolysis. We also found the activity of aspartate aminotransferase and alanine aminotransferase, which are responsible for the formation of pyruvate and oxalacetate.

Extracts of yeast extract–grown cells of *C. abyssi* also exhibited the activity of a number of key enzymes of propionic acid fermentation (see table and figure): malate dehydrogenase, fumarate hydratase, and fumarate reductase. The detection of the activity of these enzymes suggests operation of the methyl-malonyl-CoA pathway of propionate formation and correlates with the production of large amounts of propionate. No activity of lactate dehydrogenase (the enzyme of the acryloyl-CoA pathway of propionate formation) was found. The production of hydrogen correlates with the detection of activity of pyruvate:ferredoxin oxidoreductase and hydrogenase in organotrophically grown cells.

The presence of the activity of phosphate acetyltransferase and acetate kinase, which are consecutively involved in the transformation of acetyl-CoA to acetate,

Activity of *C. abyssi* enzymes during nitrate respiration and fermentation

Designation in the figure	Enzyme	Activity, μmol/(min mg protein)		Reference to determination method
		during growth on acetate with nitrate	during fermentation of yeast extract	
8	Acetate kinase (EC 2.7.2.1)	0.3	0.3	[11]
–	Aconitate hydratase (EC 4.2.1.3)	1.4	0.9	[12]
–	Isocitrate dehydrogenase (NADP) (EC 1.1.1.42)	0.24	0.06	[13]
–	Succinate dehydrogenase (EC 1.3.99.1)	2.9	0.7	[14]
11	Fumarate reductase (EC 1.3.1.6)	ND	0.14	[15]
9	Malate dehydrogenase (NAD) (EC 1.1.1.37)	0.24	0.17	[13]
–	Isocitrate lyase (EC 4.1.3.1)	0.18	ND	[16]
10	Fumarate hydratase (EC 4.2.1.2)	1.6	1.2	[17]
–	Malate synthase (EC 4.1.3.2)	0.03	ND	[13]
–	Citrate synthase (EC 4.1.3.7)	0.17	ND	[13]
–	2-Oxoglutarate dehydrogenase (NAD) (EC 1.2.4.2)	0.48	ND	[13]
7	Phosphate acetyltransferase (EC 2.3.1.8)	1.8	0.46	[11]
6	Hydrogenase (EC 1.12.1.2)	6.8*	4.8	[11]
–	Nitrate reductase (EC 1.7.99.4)	1.4–1.8	ND	see Materials and Methods
2	Glutamate dehydrogenase (NADP) (EC 1.4.1.3)	0.4	0.4	[11]
5	Pyruvate: ferredoxin oxidoreductase (EC 1.6.6.4)	ND	2.6	[18]
–	Lactate dehydrogenase (EC 1.1.1.27)	ND	0.0	[19]
1	Protease** (EC 3.4)***	ND	5.0	[11]
3	Alanine aminotransferase (EC 2.6.1.2)	0.06	0.14	see Materials and Methods
4	Aspartate aminotransferase (EC 2.6.1.1)	0.02	0.05	see Materials and Methods
–	Superoxide dismutase	4.3	2.4	[20]
–	Catalase (EC 1.11.1.6)	ND	4.7	[10]

Note: ND stands for "not determined".
* Cells were grown on medium with hydrogen, nitrate, and bicarbonate.
** Proteolytic activity is given in arb. units/(h mg protein).
*** Cumulative activity of peptidases (exopeptidases; EC 3.4.11–15) and proteinases (endopeptidases; EC 3.4.21–25) was determined.

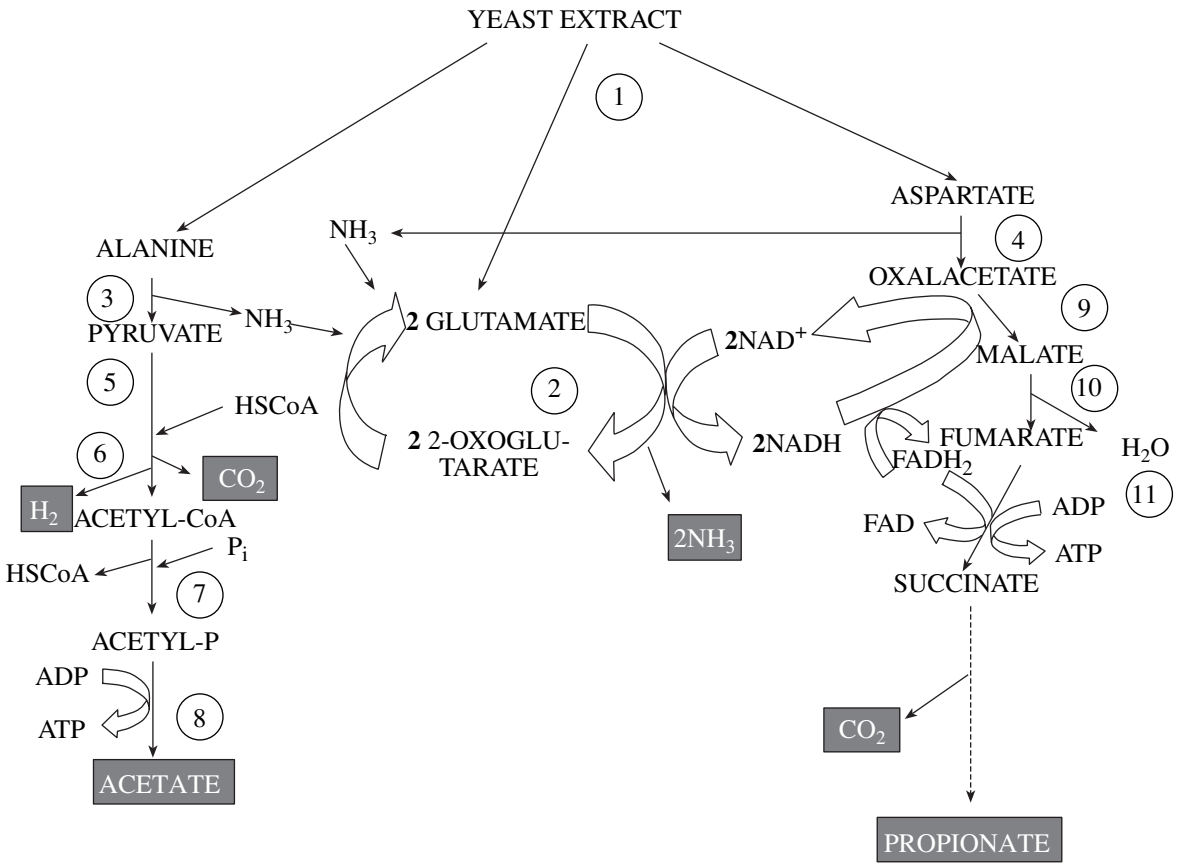
indicates that pyruvate oxidation to acetate proceeds via acetyl-CoA and acetyl phosphate.

In *C. abyssi* cells grown at the expense of yeast extract fermentation, the presence of cytochrome *b* was detected, with the α-band peak at 429 nm and the β-band peak at 560 nm.

Antioxidant defense enzymes in *C. abyssi.* Anaerobically grown *C. abyssi* cells were inspected for the presence of the activity of enzymes involved in the elimination of toxic oxygen species, namely, catalase, which catalyzes decomposition of hydrogen peroxide, and superoxide dismutase, involved in elimination of the superoxide anion radical O_2^-. The activity of superoxide dismutase was found in cells grown on both medium 1 and medium 2 (table). The activity of catalase was detected only in cells grown on medium 2.

DISCUSSION

Deep-sea hydrothermal vents are environments characterized by high temperature and pronounced gradients of pH, oxygen, and hydrogen sulfide, as well as of other inorganic and organic compounds that can be used by microorganisms as electron donors and acceptors. Dissimilatory reduction of inorganic electron acceptors under anaerobic conditions is one of the key metabolic properties of prokaryotic organisms, especially thermophilic ones [5]. The capacity for dissimilatory nitrate reduction is widespread among prokaryotes of diverse phylogenetic origins; however, for a long time, no thermophilic nitrate reducers were known. It was not long ago that the capacity for nitrate reduction was found in three genera of hyperthermophilic archaea—*Pyrobaculum, Ferroglobus*, and *Pyrolobus*—and in representatives of the new bacterial



Fermentation of yeast extract by *C. abyssi*: (1) proteolytic activity; (2) glutamate dehydrogenase; (3) alanine aminotransferase; (4) aspartate aminotransferase; (5) pyruvate:ferredoxin oxidoreductase; (6) hydrogenase; (7) phosphate acetyltransferase; (8) acetate kinase; (9) malate dehydrogenase; (10) fumarate hydratase; (11) fumarate reductase.

genera *Aquifex, Ammonifex, Caminibacter*, and *Persephonella* [6]. These organisms are diverse in terms of their phylogenetic origin and the energy sources used, as well as in the products of nitrate reduction, which suggests different types of nitrate respiration. Thus, *Ammonifex, Caminibacter*, and *Pyrolobus* carry out dissimilatory reduction of nitrate to ammonium, whereas *Aquifex* and *Pyrobaculum* are genuine denitrifiers that reduce nitrate to molecular nitrogen. All of these organisms are obligate or facultative anaerobes and lithoauto- or lithoheterotrophs that use molecular hydrogen as the energy substrate for denitrification. The ability to oxidize acetate in the course of nitrate reduction has been only found in the hyperthermophilic archaeon *Pyrobaculum aerophilum* [7].

Like all currently known thermophilic nitrate reducers, *C. abyssi*, representing a novel phylogenetic lineage of the domain *Bacteria*, can use molecular hydrogen for nitrate reduction, which is due to the presence of hydrogenase activity; like *P. aerophilum*, it can also use acetate in this process. However, *C. abyssi* differs from *P. aerophilum* by producing ammonium rather than molecular nitrogen as the nitrate reduction product. Utilization of acetate occurs via the TCA and glyoxylate cycles: we managed to detect all of the corresponding enzymes.

Another important distinction of *C. abyssi* from other known thermophilic nitrate reducers is its capacity for peptide fermentation, which proceeds with the production of hydrogen (as the source of peptides and amino acids, we used yeast extract, since its basis is formed by these compounds). Several mesophilic bacteria are also known to produce hydrogen as a result of amino acid degradation: *Clostridium tetanomorphum* [8]. *Peptostreptococcus asaccharolyticus, Peptostreptococcus prevotii* [9], and others; some of them, like *C. abyssi*, are incapable of using carbohydrates as the energy source. Interestingly, nitrate does not stimulate growth of *C. abyssi* on yeast extract; thus, it is not used as an electron sink during amino acid fermentation.

For at least three amino acids, we can suggest a scheme of enzymatic reactions leading to the formation of fermentation products detected in the culture liquid of *C. abyssi* grown on yeast extract (see figure). Hydrogen formation by *C. abyssi* involves pyruvate:ferredoxin oxidoreductase, whose activity we have detected. For the operation of this enzyme, a low redox potential of the medium is needed; under such conditions, cleav-

age of pyruvate with the formation of acetyl-CoA is catalyzed by pyruvate:ferredoxin oxidoreductase rather than by the pyruvate dehydrogenase complex. In this case, as distinct from the pyruvate dehydrogenase reaction, two hydrogen atoms are used to reduce ferredoxin instead of being transferred to NAD. The reduced ferredoxin provides electrons for the formation of hydrogen by hydrogenase. The activity of acetate kinase and phosphate acetyltransferase that we have detected suggests that, during organotrophic growth, acetate formation occurs via acetyl phosphate and acetyl-CoA in reactions catalyzed by the above-mentioned enzymes. As a result of these reactions, an ATP molecule is formed. The presence of fumarate reductase activity and cytochrome *b* in organotrophically grown cells of *C. abyssi* is indicative of the operation of the fumarate–succinate segment of the methyl-malonyl-CoA pathway and suggests that propionate formation by *C. abyssi* occurs via this pathway. Lack of lactate dehydrogenase activity indicates that *C. abyssi* cells lack the alternative pathway of propionate formation.

Detection of the enzymes involved in elimination of the toxic oxygen species H_2O_2 and O_2^-, namely, of catalase and superoxide dismutase, in *C. abyssi* cells grown at the expense of fermentation suggests the constitutive nature of these enzymes. However, the activity of catalase was not recorded in cells of *C. abyssi* grown in the presence of nitrate. This can be explained by the fact that the active center of catalase contains an iron-containing heme, which is affected by products of nitrate reduction, including the intermediate product nitrite [10]. On the other hand, nitrite can be oxidized by hydrogen peroxide to nitrate, which results in the elimination of the toxic hydrogen peroxide.

Thus, the representative of a new bacterial phylogenetic lineage, *C. abyssi*, implements diverse metabolic reactions and, as distinct from other thermophilic nitrate-reducing bacteria, is capable not only of lithotrophic growth with hydrogen and nitrate but also of peptide fermentation, as well as of acetate oxidation in the course of nitrate respiration. In the course of peptide fermentation, nitrate reduction does not occur. It can be inferred that, in the presence of organic matter in hydrothermal vents, *C. abyssi* is involved in its degradation; if nitrate is available, *C. abyssi* oxidizes acetate, completing the mineralization of organic matter. In the absence of organic substrates, *C. abyssi* can act as an organic matter producer, utilizing the energy of molecular hydrogen of volcanic origin. Possession of enzymes that defend it from toxic oxygen species allows the anaerobic metabolism of *C. abyssi* to operate under conditions of a steep oxygen gradient characteristic of deep-sea hydrothermal vents. Assuming that the relatives of *C. abyssi* that were detected in shallow-water hydrothermal vents of Greece exhibit the same metabolism, it is appropriate to search for the nitrate source that may be the factor promoting their massive development in subsurface layers of sediments.

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