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EXPERIMENTAL ARTICLES

Carbonic Anhydrase Activity in Halophilic Anaerobes from Soda Lakes

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Abstract—The activity and cellular localization of carbonic anhydrase (CA) in two alkaliphilic anaerobes growing in soda lakes at pH 9–10 were studied. CA activity in the cell extracts of the acetogenic bacterium *Natroniella acetigena* was comparable to that of neutrophilic acetogens. Hydrogenotrophically grown cells of *Desulfonatronum lacustre* exhibited higher CA activity compared to the cells grown on medium with formate. High CA activity in the cytoplasmic fraction and the absence of high activity in the extracellular fraction were demonstrated. We propose that the cytoplasmic CA in alkaliphilic sulfate-reducers participates in conversion of bicarbonate to CO_2, which is reduced in the cell to acetate via the acetyl-CoA pathway.

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Carbonic anhydrases, zinc-containing enzymes present in animal, plant, bacterial, and archaeal cells, catalyze reversible CO_2 hydration ($CO_2 + H_2O \longleftrightarrow HCO_3^- + H^+$). Carbonic anhydrase plays a major role in the metabolism of various prokaryotic species; it participates in maintaining pH homeostasis, in CO_2 and HCO_3^--fixation, in mutual conversion of CO_2 and HCO_3^-, and in ion transport coupled to bicarbonate transport [1]. Carbon in soda lakes is mostly present as carbonate and bicarbonate ions; the study of carbonic anhydrase in the cells of alkaliphilic anaerobes is therefore of interest as one aspect of their adaptation to extreme conditions.

Extremely alkaliphilic anaerobes with growth optimum at pH 9.5–9.7 were isolated from the alkaline continental lakes of Equatorial Africa and the Transbaikal region. For our purpose, two organisms were of special interest: the extremely haloalkaliphilic acetogenic bacterium *Natroniella acetigena*, which grows at 1.57% NaCl, total Na^+ concentration 2 M, and pH 9.7 on media with ethanol or lactate [2]; and the extremely alkaliphilic sulfate-reducing bacterium *Desulfonatronum lacustre*, which reduces sulfate, uses H_2, formate, or ethanol as electron acceptors, and grows without NaCl at pH 9.5 and a total Na^+ concentration of 0.2 M [3]. These organisms use the reductive acetyl-CoA/Co dehydrogenase pathway of CO_2 fixation for anabolic and katabolic cellular processes [4, 5].

The goal of the present work was comparative investigation of carbonic anhydrase activity and of its localization in the cells of these representatives of extremely alkaliphilic acetogenic and sulfate-reducing bacteria.

MATERIALS AND METHODS

The objects of investigation were *Natroniella acetigena* Z-7937^T and *Desulfonatronum lacustre* Z-7951^T from the RAS Vinogradskii Institute of Microbiology's laboratory collection of relic microbial communities. *N. acetigena* was grown under strictly anaerobic conditions at 37°C and pH 9.7 on a mineral medium with ethanol (0.5%) containing (g/l) KH_2PO_4, 0.2; $MgCl_2 \cdot 6H_20$, 0.1; NH_4Cl, 1.0; KCl, 0.2; NaCl, 15.7; Na_2CO_3, 68.3; $NaHCO_3$, 38.3; yeast extract, 0.2; $Na_2S \cdot 9H_2O$), 1.0; Lippert mineral solution, 1 ml; 0.04% resazurin, 2 ml; Volin vitamin solution, 2 ml [2]. *D. lacustre* was grown under strictly anaerobic conditions at 37°C and pH 9.5 on a mineral medium of the following composition (g/l): K_2HPO_4, 0.2; KCl, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; NH_4Cl, 1.0; Na_2SO_4, 3.0; Na_2CO_3, 2.76; $NaHCO_3$, 10.0; yeast extract, 1.0; $Na_2S \cdot 9H_20$, 0.5; Witman mineral solution, 1 ml; Volin vitamin solution, 2 ml; 0.04% resazurin, 2 ml [3]. Formate (0.5%) or hydrogen (100%

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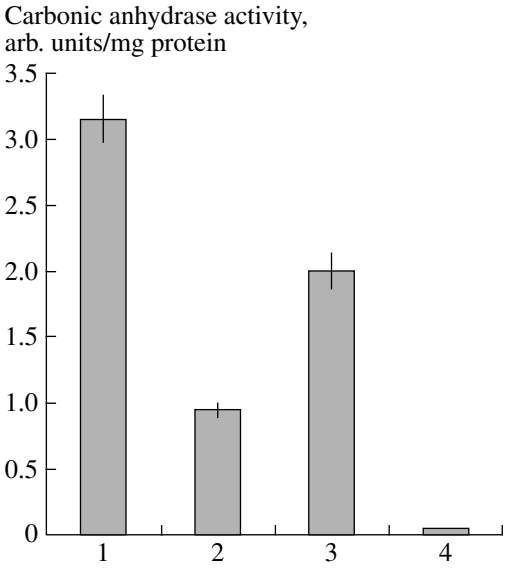

Fig. 1. Carbonic anhydrase activity in the cells of *N. acetigena* and *D. lacustre*: (1) cell extract of *N. acetigena* (cultured on ethanol); (2) cell extract of *N. acetigena* in the presence of 1 mM EZA; (3) cell extract of *D. lacustre* (cultured on formate); (4) cell extract of *D. lacustre* in the presence of 1 mM EZA.

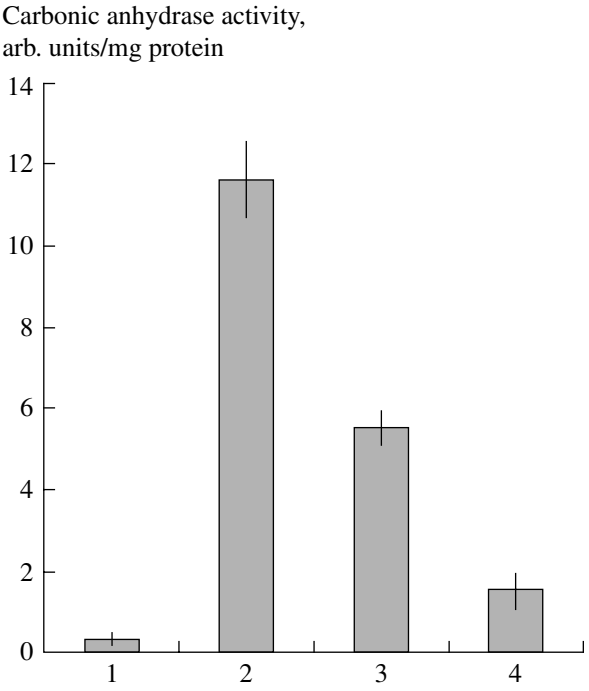

Fig. 2. The distribution of carbonic anhydrase activity in the fractions of hydrogen-grown *D. lacustre* cells: (1) intact cells; (2) cell extract; (3) cytoplasmic fraction; (4) periplasmic fraction.

in the gas phase) were used as electron donors for the culture of *D. lacustre*.

Cell extracts were obtained under anaerobic conditions. The cells from 5 l of the culture were precipitated by centrifugation at 4550 g for 1 h and resuspended in 10 ml of 0.05 M Tris–HCl buffer, pH 9.5 with 5 mM EDTA, 25 mM sodium thioglycollate, 5 mM $MgCl_2$, and 1 mg DNase. The cells were disintegrated three times by ultrasound in a UZDN-1device for 1 min at 0.4 mA under refrigeration and centrifuged for 1 h at 4550 g. The cell extract was used for determination of enzymatic activity.

To obtain the periplasmic and the cytoplasmic fractions, the cells from *D. lacustre* culture were refrigerated and suspended in 20 ml of 0.05 M Tris–HCl buffer, pH 7.8 with 0.75 M sucrose, 1 μg/ml lysozyme, and 1 M EDTA. The formation of spheroplasts was monitored microscopically. The spheroplasts were precipitated (20 min at 4550 g), and the supernatant was used as a rough periplasmic fraction. The spheroplast precipitate was washed with Tris–HCl buffer, pH 7.8 with 0.25 M sucrose and 1 mM $MgCl_2$, resuspended in the same buffer with DNase, and lysed by addition of cold water. The lysate was used as a cytoplasmic fraction [5].

Carbonic anhydrase activity was determined electrometrically as changes in H^+ concentration in the reaction of carbon dioxide hydration by a pH meter equipped with a sensitive electrode and a self-recorder. The reaction mixture (2 ml) contained intact washed cells, cell extract, or its fractions (0.1–0.5 mg protein) in phosphate buffer. The reaction was performed at 2°C; it was initiated by the rapid addition of the saturated CO_2 solution into an equal amount of the reaction medium. The time required to decrease pH from 8.0 to 7.0 was recorded. Carbonic anhydrase activity was calculated in Wilburg–Andersen arbitrary units (a.u.) per milligram of protein. The nonenzymatic reaction was used as the control [6]. Reaction rates were determined in three replicate experiments. Ethoxyzolamide was used as a carbonic anhydrase inhibitor. Protein concentrations were determined by the Lowry method.

RESULTS AND DISCUSSION

The activity of carbonic anhydrase (CA) in the cell extracts of the acetogenic bacterium *N. acetigena* grown on ethanol was 3.2 a.u./mg protein. The enzymatic activity was confirmed by the reaction in the presence of the specific carbonic anhydrase inhibitor, ethoxyzolamide (EZA), which decreased the activity of the enzyme by 70% (Fig. 1). According to published data, CA activity in *N. acetigena* was somewhat higher than the activities characteristic for the previously studied neutrophilic acetogens, which varied from the threshold values to 1.5–2.1 a.u/mg protein; only *Acetobacterium woodii* exhibited the highest value of 15.2 a.u./mg protein [7]. The authors of this work believe that one of the physiological functions of CA is to increase the intracellular CO_2 concentration.

For the alkaliphilic sulfate-reducer *D. lacustre* grown on formate, CA activity in cell extracts was

2.0 a.u./mg protein; it was almost completely suppressed by 1 mM EZA (Fig. 1). When grown on H_2, CA activity increased almost sixfold, to 11.6 a.u./mg protein (Fig. 2); this finding indicated the increased role of CA during hydrogenotrophic growth. The activity of CA in the cell extracts of the neutrophilic methanogenic and sulfate-reducing bacteria capable of utilizing acetate as a substrate was reported to be no higher than 0.42 a.u./mg protein [8]. In acetate-utilizing anaerobes, it is supposed that CA participates in acetate transport via the acetate-HCO_3^- antiporter.

Bacterial CA is known to be localized either in the periplasm, in the cytoplasm, or extracellularly [1]. To determine the localization of CA in the cells of the gram-negative *D. lacustre*, the culture grown on hydrogen was used. Activity was determined for whole cells, cell extracts, and of the cytoplasmic and periplasmic fractions. The entire *D. lacustre* cells were shown to have low CA activity (0.33 a.u./mg protein) compared to cell extracts (11.6 a.u./mg protein) (Fig. 2). In the cytoplasm, CA activity was 5.52 a.u/mg protein; in the periplasmic fraction, 1.55 a.u./mg protein; CA was therefore localized mostly in the cytoplasm of *D. lacustre* cells.

Low CA activity in the intact *D. lacustre* cells indicated the absence of the extracellular enzyme, unlike alkaliphilic cyanobacteria from soda lakes [6].

Our results allow us to suggest that the cytoplasmic CA in the cells of alkaliphilic sulfate-reducers can convert HCO_3^- to CO_2; the latter is the substrate for formate dehydrogenase, which catalyzes the first stage of CO_2 reduction to acetate via the acetyl-CoA pathway.

The presence of CA in the cells of extremely alkaliphilic acetogens and sulfate-reducers at high pH values and therefore under CO_2 deficiency in the medium was thus demonstrated for the first time.

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