Microbiology, Vol. 74, No. 4, 2005, pp. 395–399. Translated from Mikrobiologiya, Vol. 74, No. 4, 2005, pp. 460–465.

EXPERIMENTAL ARTICLES

The Effect of Sodium Salts and pH on the Hydrogenase Activity of Haloalkaliphilic Sulfate-Reducing Bacteria

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Received September 20, 2004; in final form, March 14, 2005

Abstract—Hydrogenase is the main catabolic enzyme of hydrogen-utilizing sulfate-reducing bacteria. In haloalkaliphilic sulfate reducers, hydrogenase, particularly if it is periplasmic, functions at high concentrations of Na^+ ions and low concentrations of H^+ ions. The hydrogenases of the newly isolated sulfate-reducing bacteria *Desulfonatronum thiodismutans, D. lacustre*, and *Desulfonatronovibrio hydrogenovorans* exhibit different sensitivity to Na^+ ions and remain active at NaCl concentrations between 0 and 4.3 M and $NaHCO_3$ concentrations between 0 and 1.2 M. The hydrogenases of *D. lacustre* and *D. thiodismutans* remain active at pH values between 6 and 12. The optimum pH for the hydrogenase of *D. thiodismutans* is 9.5. The optimum pH for the cytoplasmic and periplasmic hydrogenases of *D. lacustre* is 10. Thus, the hydrogenases of *D. thiodismutans, D. lacustre*, and *Dv. hydrogenovorans* are tolerant to high concentrations of sodium salts and extremely tolerant to high pH values, which makes them unique objects for biochemical studies and biotechnological applications.

Key words: haloalkaliphiles, anaerobes, sulfate reducers, *Desulfonatronum, Desulfonatronovibrio*, hydrogenase.

Hypersaline and alkaline environments are not appropriate for higher life forms. However, being enriched in carbon and energy sources, these econiches are populated by well-adapted microbial communities. As a rule, high salt concentrations are very unfavorable to proteins and other biomacromolecules because they (1) enhance hydrophobic interactions, causing the aggregation and structural collapse of proteins; (2) reduce electrostatic interactions inside and between macromolecules; and (3) decrease the availability of free water (as a result of salt ion hydration) below the level that is required for maintenance of important biological processes [1].

In order to maintain their intracellular concentration of salts at a low level, microorganisms that inhabit moderately saline environments use ionic pumps and produce or accumulate organic osmoregulators. Halobacteria that tolerate 3.5–5 M NaCl possess primary ionic pumps, which function at the expense of H^+-ATPase and membrane proteins and can pump protons and chloride ions but not Na^+ ions. At the same time, Na^+ ions can be translocated from a cell due to a secondary Na^+/H^+ antiport [2]. The intracellular concentration of Na^+ and K^+ ions in extreme halophiles may reach 1 and 4–5 M, respectively. For this reason, the proteins of such halophiles are well-adapted to high salt concentrations [3, 4].

Many fermentative reactions are tolerant to high concentrations of NaCl [5]. In some cases, high concentrations of ions are required for neutralization of the charge of proteins and their maintenance in a metabolically active state [3, 6]. The stability of these proteins depends on the conditions under which hydrophobic interactions increase, which occurs due to high concentrations of NaCl or KCl. Such proteins are not only active at high salt concentrations but even require the presence of salt to be stable and active [1].

It should be noted that some intracellular enzymes of halophilic eubacteria exhibit maximum activity in the absence of salts whereas others are stimulated in the presence of moderate NaCl concentrations. Transmembrane enzymes, including transport proteins, are sensitive to both intracellular and extracellular conditions [7].

Hydrogenase is the key enzyme involved in energy metabolism in bacteria (methanogenic, acetogenic, nitrogen-fixing, photosynthesizing, and sulfate-reducing) that can utilize hydrogen for energy purposes or electron sink [8]. Hydrogenases, which contain either iron or iron and nickel in their active centers, catalyze the reversible reaction of hydrogen oxidation and are responsible for the consumption or evolution of hydrogen by bacterial cells.

Although there are an extensive number of publications covering intra- and extracellular enzymes of halophilic bacteria, little information is available on the effect of salts on the activity of hydrogenases. It has

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0026-2617/05/7404-0395

been reported, for instance, that the activity of the periplasmic hydrogenase found in the extremely halophilic eubacterium *Acetohalobium arabaticum* manifoldly increases in the presence of high NaCl and KCl concentrations, up to saturating ones [9].

The haloalkaliphilic sulfate-reducing bacteria *Desulfonatronum lacustre, D. thiodismutans*, and *Desulfonatronovibrio hydrogenovorans* have been isolated from Lakes Khadyn (Tuva, Russia), Mono (United States), and Magadi (Kenya), respectively. The bacterium *D. thiodismutans* can grow in carbonate–bicarbonate media at NaCl concentrations of 1 to 7% and pH values of 8–10 (optimal growth is observed at 3% NaCl and pH 9.5) [10]. *D. lacustre* and *Dv. hydrogenovorans* exhibit the best growth at pH 9.5 and can grow in the absence of NaCl [11, 12].

The aim of this work was to study the effect of sodium salts and pH on the activity of hydrogenases in the aforementioned haloalkaliphilic sulfate-reducing bacteria.

MATERIALS AND METHODS

Organisms and cultivation conditions. Experiments were carried out using type strains of the sulfate-reducing bacteria *Desulfonatronum lacustre* Z-7951^T (= DSM 10312^T), *Desulfonatronum thiodismutans* MLF1^T (= ATCC BAA-395^T = DSM 14708^T), and *Desulfonatronovibrio hydrogenovorans* Z-7935^T (= DSM 9292^T). The last strain was obtained from T.N. Zhilina.

D. lacustre and *Dv. hydrogenovorans* were cultivated under strictly anaerobic conditions at 37°C in a mineral medium (pH 9.5) containing (g/l) KH_2PO_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; $Na_2S \cdot 9H_2O$, 0.5; yeast extract, 1.0; Whitman trace element solution, 1 ml; Wolin trace element solution, 2 ml; and 0.04% resazurin, 2 ml. The medium was supplemented with 5% sodium formate as the substrate [11, 12].

D. thiodismutans was cultivated under strictly anaerobic conditions at 37°C in a mineral medium (pH 9.5) containing (g/l) KH_2PO_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; $NaHCO_3$, 24.0; NaCl, 30.0; $Na_2S \cdot 9H_2O$, 0.4; yeast extract, 0.2; sodium formate, 5.0; Whitman trace element solution, 1 ml; Wolin trace element solution, 2 ml; and 0.04% resazurin, 2 ml [10]. Oxygen was removed from the media by boiling them and bubbling with argon or nitrogen for 15 min. $NaHCO_3$, Na_2S, and the yeast extract were added to the media after cooling. The reduced media were dispensed into Hungate tubes or flasks under a flow of argon or nitrogen and autoclaved at 121°C for 30 min. The media were inoculated with mid-exponential cultures taken in a proportion of 1–3 vol %.

Preparation of cell-free extracts and spheroplasts. Cell-free extracts were prepared by disrupting cells with lysozyme (5 mg/ml) or ultrasound (UZDN-1 ultrasonic disintegrator at 0.4 mA). The cells were disrupted and cellular fractions were obtained under strictly anaerobic conditions using deaerated buffer solutions, which were stored under nitrogen. Cells from a culture (1500 ml) grown to the mid-exponential phase were harvested by centrifugation at 5000 *g* for 60 min at 4°C in a K-23 centrifuge (Germany). The cell pellet was suspended in 10 ml of 0.05 M Tris–HCl buffer (pH 7.8) containing 10 mM sodium formate, 25 mM sodium thioglycolate, 5 mg/ml lysozyme, and 10 μg DNase. The suspension was incubated at 37°C for 12 h. Cell debris was removed by centrifugation at 5000 *g* for 40 min. The supernatant was used for enzyme assay.

To prepare spheroplasts, cells from a mid-exponential culture (1500 ml) were harvested by centrifugation as described above. The cell pellet was suspended in 10 ml of 0.05 M Tris–HCl buffer (pH 7.8) containing 0.5 M sucrose, 5 mM EDTA, 5 mM $MgCl_2$, and 2 mg/ml lysozyme. The suspension was incubated at 37°C for 5 h and then centrifuged at 5000 *g* at 4°C for 30 min. The resulting supernatant represented the "periplasmic" fraction. The precipitated spheroplasts were resuspended in 10 ml of 50 mM Tris–HCl buffer (pH 9.5) containing 5 mM $MgCl_2$ and 2 μg/ml DNase. The suspension was sonicated in a UZDN-1 disintegrator at 0.4 mA in four 4-min bursts. Cell debris was removed by centrifugation. The supernatant corresponded to a "cytoplasmic" fraction [13].

Hydrogenase was assayed at 37°C by measuring the reduction rate of benzylviologen with hydrogen in thermostated anaerobic cuvettes at 600 nm (Specord, Germany) [14]. Protein was quantified according to the method of Lowry *et al.* [15]. One unit of hydrogenase activity (U) was expressed as 1 μmol of benzylviologen reduced per min per mg protein.

RESULTS AND DISCUSSION

The hydrogenase in cell-free extracts of the haloalkaliphilic sulfate-reducing bacterium *D. thiodismutans* was active at NaCl concentrations ranging from 0 to 4.3 M, with the maximum activity (3.1 U) occurring at 1.0 M NaCl. In the absence of NaCl, hydrogenase activity was approximately 20% lower (2.6 U) (Fig. 1, curve *1*). This activity decreased within a range of salt concentrations from 2.1 to 3.4 M and then plateaued.

Study of the effect of $NaHCO_3$ on the hydrogenase activity showed that the activity decreased as the bicarbonate concentration was raised from 0 to 0.47 M (pH 9.5). However, at a $NaHCO_3$ concentration equal to 1.2 M (this bicarbonate concentration is close to being saturating), the activity of the hydrogenase was as high as 60% of its maximum activity (Fig. 2, curve *1*). The hydrogenase of *D. thiodismutans* remained active within the pH range of 7.5 to 11.5 and attained its maximum at pH 9.5. The optimum pH of the enzyme coincided with the optimum pH for growth (Fig. 3, curve *1*). The shoulder observed on the pH dependence curve at

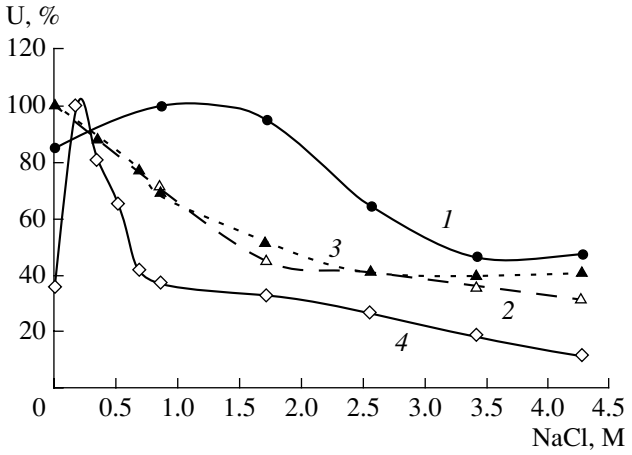

Fig. 1. Dependence of the hydrogenase activity of haloalkaliphilic sulfate-reducing bacteria on the concentration of NaCl: (*1*) the cell extract of *D. thiodismutans*, (*2*) the periplasmic fraction of *D. lacustre*, (*3*) the cytoplasmic fraction of *D. lacustre*, and (*4*) the cell extract of *Dv. hydrogenovorans.* The maximum activity of particular enzymes in μmol/(min mg protein) was taken to be 100%.

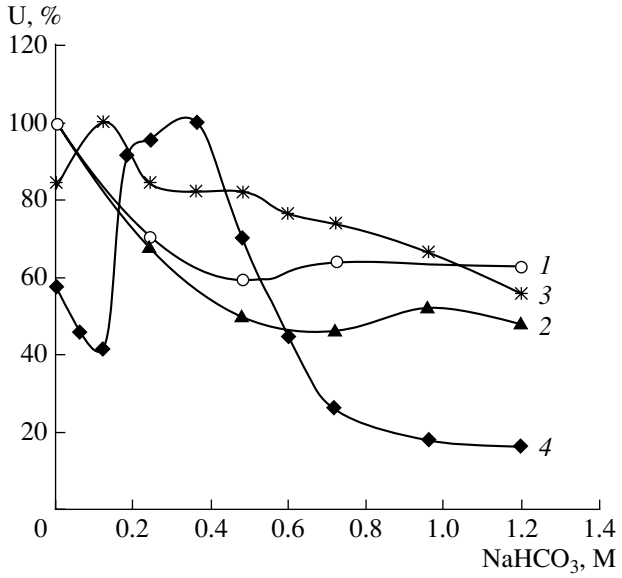

Fig. 2. Dependence of the hydrogenase activity of haloalkaliphilic sulfate-reducing bacteria on the concentration of $NaHCO_3$: (*1*) the cell extract of *D. thiodismutans*, (*2*) the periplasmic fraction of *D. lacustre*, (*3*) the cytoplasmic fraction of *D. lacustre*, and (*4*) the cell extract of *Dv. hydrogenovorans.* The maximum activity of particular enzymes in μmol/(min mg protein) was taken to be 100%.

pH 10–10.5 may indicate the presence of two hydrogenase forms or the existence of cooperative regulation. Thus, the hydrogenase of *D. thiodismutans* is tolerant to high concentrations of sodium salts and a high pH.

According to our previous observations, another haloalkaliphilic sulfate-reducing bacterium, *D. lacustre*, contains periplasmic and intracellular hydrogenases [13]. Periplasmic hydrogenase has also been found in freshwater sulfate-reducing bacteria belonging to the genus *Desulfovibrio* [16]. This form of hydrogenase is believed to be involved in interspecies transfer of hydrogen in anaerobic microbial communities. Casalot *et al.* have reported that the bacterium *Desulfovibrio fructosovorans* has two periplasmic hydrogenases, [NiFe]-hydrogenase and [Fe]-hydrogenase, and one NADP-dependent cytoplasmic hydrogenase [17].

The involvement of molecular hydrogen in the generation of the proton gradient in *Desulfovibrio* bacteria requires the presence of several types of hydrogenase with different cellular locations [18]. Protons are reduced by cytoplasmic hydrogenase. The resultant molecular hydrogen diffuses across the cytoplasmic membrane to the periplasm, where it is oxidized by one or several hydrogenases into protons and electrons. The protons are utilized for ATP synthesis via a reversible ATPase. The electrons pass across the cytoplasmic membrane to reduce endogenous and exogenous electron acceptors. The overall effect of this process is that two or more protons are transferred through the cytoplasmic membrane without the involvement of membrane-bound reductases or proton pumps. The presence of periplasmic hydrogenase in *D. lacustre* suggests that it may play a role in the formation of the proton gradient.

The periplasmic and cytoplasmic hydrogenases found in *D. lacustre* showed different sensitivities to sodium salts. At pH 9, the activity of the periplasmic hydrogenase of this bacterium decreased by 50% (from 0.44 to 0.21 U) as the concentration of $NaHCO_3$ was raised from 0 to 1.2 M (Fig. 2, curve *2*). The activity of the cytoplasmic hydrogenase of this bacterium slightly increased at 0.12 M $NaHCO_3$, followed by an insignificant decrease (Fig. 2, curve *3*). Both hydrogenases were suppressed by NaCl and exhibited their maximum activity in the absence of NaCl. When the concentration of NaCl was raised from 0 to 4.3 M, the activity of the cytoplasmic hydrogenase of this bacterium decreased by approximately 60% (from 0.83 to 0.34 U) and that of the periplasmic hydrogenase declined by about 70% (from 0.3 to 0.09 U) (Fig. 1, curves *2, 3*). The inhibition of the activity of the cytoplasmic (intracellular) hydrogenase can be explained by the fact that the intracellular enzymes of halophilic eubacteria do not usually tolerate the presence of salts in a medium because these enzymes operate at low concentrations of ions in the presence of organic osmoregulators [7].

According to our earlier observations [9], the activity of the periplasmic hydrogenase and some of the catabolic enzymes found in the extremely halophilic bacterium *Acetohalobium arabaticum*, which belongs to the order *Halanaerobiales*, reaches a maximum at high concentrations of NaCl and KCl. For instance, at 4.5 M NaCl or 3.54 M KCl, the hydrogenase activity was, respectively, 5 and 3 times higher than in the absence of these salts. The activity of hydrogenase in cell extracts

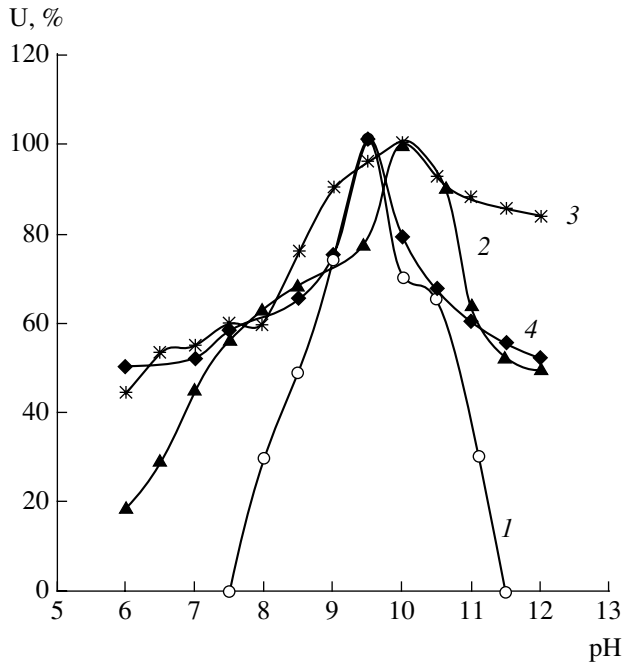

Fig. 3. The pH dependence of the hydrogenase activity of haloalkaliphilic sulfate-reducing bacteria: (*1*) the cell extract of *D. thiodismutans*, (*2*) the periplasmic fraction of *D. lacustre*, (*3*) the cytoplasmic fraction of *D. lacustre*, and (*4*) the cell extract of *Dv. hydrogenovorans.* The maximum activity of particular enzymes in μmol/(min mg protein) was taken to be 100%.

of the moderately halophilic bacterium *Halobacteroides acetoethylicus* also increases (by up to 60%) as the salt concentration is increased [19]. However, at NaCl concentrations higher than 2.3 M, the activity of this hydrogenase decreases. This fact can be accounted for by the good adaptation of *H. acetoethylicus* to moderate salt concentrations (the optimum growth of this bacterium is observed at 10% NaCl).

It is known that the intracellular pH in obligate alkaliphiles is approximately 8.0. This value is about two units lower than outside the cells and somewhat higher than the cytoplasmic pH of neutrophiles [20]. The activity of the cytoplasmic and periplasmic hydrogenases of *D. lacustre* changed very little within the pH range of 7.0 to 8.5, reaching a maximum at a pH 10 (Fig. 3, curves *2, 3*). This suggests that this bacterium is adapted to utilization of molecular hydrogen in alkaline environments.

The hydrogenase in cell extracts of the third haloalkaliphilic sulfate-reducing bacterium, *Dv. hydrogenovorans*, exhibited their maximum activity at $NaHCO_3$ concentrations ranging from 0.18 to 0.36 M (Fig. 2, curve *4*). At $NaHCO_3$ concentrations higher than 0.5 M, the activity of this enzyme declined, reaching 20% of its maximum activity at 1.2 M $NaHCO_3$. As for the effect of NaCl, the activity of the hydrogenase attained its maximum at 0.17 M NaCl and then drastically decreased (Fig. 1, curve *4*). In the presence of 4.3 M NaCl, the hydrogenase activity comprised 12% of the maximum activity.

The hydrogenase of *Dv. hydrogenovorans* remained active within a wide range of pH values. The maximum activity of this hydrogenase was observed at a pH of 9.5, i.e., at the pH that is optimum for the growth of *Dv. hydrogenovorans* (Fig. 3, curve *4*).

Thus, the hydrogenases of representatives of two different genera of haloalkaliphilic sulfate-reducing bacteria proved to be quite tolerant to high concentrations of sodium salts in the medium. Unlike the cytoplasmic and periplasmic hydrogenases of *D. lacustre*, which showed maximum activity in the absence of sodium salts, the hydrogenases of *D. thiodismutans* and *Dv. hydrogenovorans* were stimulated by moderate concentrations of NaCl and can be considered typical halophilic enzymes. The enzymes under study remained active within a wide range of pH values and exhibited their maximum activity at pH 9.5–10.

ACKNOWLEDGMENTS

We are grateful to G.A. Zavarzin for his interest in this study and his valuable contribution to the discussions of the results.

This work was supported by the Presidium of the Russian Academy of Sciences, the program "Molecular and Cell Biology," and the program "Leading Scientific Schools of Russia," grant no. NSh-1101.2003.4

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