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EXPERIMENTAL ARTICLES

The Involvement of Hydrogenases 1 and 2 in the Hydrogen-Dependent Nitrate Respiration of *Escherichia coli*

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Abstract—The study of *Escherichia coli* mutants synthesizing either hydrogenase 1 (HDK203) or hydrogenase 2 (HDK103) showed that the nitrate-dependent uptake of hydrogen by *E. coli* cells can be accomplished through the action of either of these hydrogenases. The capability of the cells for hydrogen-dependent nitrate respiration was found to depend on the growth conditions. *E. coli* cells grown anaerobically without nitrate in the presence of glucose were potentially capable of nitrate-dependent hydrogen consumption. The cells grown anaerobically in the presence of nitrate exhibited a much lower capability for nitrate-dependent hydrogen consumption. The inhibitory effect of nitrate on this capability of bacterial cells was either weak (the mutant HDK203) or almost absent (the mutant HDK103) when the cells were grown in the presence of peptone and hydrogen. Hydrogen stimulated the growth of the wild-type strain and the mutant HDK103 (but not the mutant HDK203) cultivated in the medium with nitrate and peptone. These data suggest that hydrogenase 2 is much more active in catalyzing nitrate-dependent hydrogen consumption than hydrogenase 1.

Key words: hydrogenase 1, hydrogenase 2, nitrate respiration, hydrogen uptake.

Depending on the presence of particular electron acceptors in the cultivation medium, the bacterium *Escherichia coli* can grow either by aerobic respiration, fermentation, or anaerobic respiration (in the presence of such electron acceptors as nitrate, nitrite, fumarate, dimethylsulfoxide, and trimethylaminoxide). Molecular hydrogen can be either produced in the course of fermentation or consumed in the course of anaerobic respiration. In both cases, hydrogen molecules are reversibly activated by hydrogenases. The bacterium *E. coli* has at least three [Ni,Fe] hydrogenases. Hydrogenase 3, encoded by the *hyc* gene, is involved in hydrogen production by the formate hydrogenlyase complex, whereas hydrogenases 1 (*hya*) and 2 (*hyb*) are involved in hydrogen uptake. Hydrogenases 1 and 2 are membrane-bound enzymes, which differ not only genetically but also immunologically and biochemically [1–6]. Both of these hydrogenases are synthesized under anaerobic conditions (namely, either fermentation conditions or the conditions of anaerobic respiration), the expression of the hydrogenases being greater in the absence of oxygen than in the presence of an alternative electron acceptor, such as fumarate [7, 8]. Nitrate suppresses the synthesis of both hydrogenases [3, 5–8] through the action of the two-component system NarL/NarX and NarP/NarQ [8]. There is evidence that the anaerobic syntheses of hydrogenases 1 and 2 are regulated by different regulators. ArcA, the global transcriptional regulator of aerobic metabolism, enhances the anaerobic induction of hydrogenase 1 and diminishes the anaerobic induction of hydrogenase 2 [7, 8]. The *hya* operon is under the control of the anaerobic regulator AppY [7–9].

In the case of nitrate respiration, nitrate is reduced to nitrite with the involvement of three genetically and biochemically different nitrate reductases. The resulting nitrite is reduced to ammonium through the action of two nitrite reductases. The expression of nitrate and nitrite reductases depends on the growth phase, medium composition, concentrations of nitrate and nitrite, and on other factors [10–12]. Formate is considered to be the main physiological electron donor for nitrate reduction [10]. The hydrogen-dependent reduction of nitrate in *E. coli* spheroplasts was found to be coupled with proton translocation through the membrane [13]. Yamamoto and Ishimoto [14] showed that the hydrogen-dependent anaerobic growth of *E. coli* is associated with the reduction of nitrate to nitrite in equimolar amounts [14]. High nitrate concentrations induce the synthesis of nitrite reductase encoded by the *nirB* gene [11]; however, hydrogen cannot be used as an electron donor for the reduction of nitrite [15].

The hydrogen-dependent reduction of fumarate occurs with the involvement of hydrogenase 2 [3]. The occurrence of this hydrogenase in bacterial cells is a necessary condition of their growth at the expense of fumarate respiration [6]. The hydrogen-dependent

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reduction of nitrate in suspensions of *E. coli* cells requires the presence of either of the two hydrogenases [15]. Hydrogen-dependent growth in the presence of nitrate was shown only for the wild-type *E. coli* strain possessing the complete set of hydrogenases [14].

The aim of this work was to study the nitrate-dependent uptake of hydrogen with the involvement of hydrogenases 1 and 2 and to elucidate their role in the growth of *E. coli* at the expense of nitrate reduction by hydrogen.

MATERIALS AND METHODS

Experiments were carried out with the wild-type strain *Escherichia coli* MC4100, the mutants HDK103 (Δ*hya*, Δ*hyc*) and HDK203 (Δ*hyb*, Δ*hyc*), and the MC4100 derivatives (F^-, *araD139*, Δ(*argF-lac*)U169, *ptsF25*, *relA1*, *flbB5301*, *rpsL150*, *deoC1*, *rbsR*, λ^-) described by Jacobi *et al.* [16]. The bacteria were grown anaerobically at 37°C in M63 medium containing (g/l) KH_2PO_4, 13.6; $(NH_4)_2SO_4$, 2.0; $MgSO_4$, 0.2; $FeSO_4$, 0.5; NaCl, 0.5; glucose, 2.0; kanamycin, 0.025 (only in the case of the mutants); and KNO_3 (when required). The pH of the medium was 7.0. In some experiments, glucose was substituted by 0.5% peptone. The cultivation medium was boiled to remove dissolved gases and then dispensed into 42-ml vials in 5-ml aliquots. After the gas was evacuated from the vials, they were filled with either argon or hydrogen, sterilized by heating, and inoculated with bacterial cells using a syringe. Growth was monitored by measuring the optical density of cultures at 650 nm (OD_{650}) in a 1-cm cuvette. Dry biomass was calculated by the formula $X = 0.71\ OD_{650}$.

Hydrogen consumption was determined by gas chromatography. Reactions were carried out in flasks from 14.5 to 15.0 ml in volume. The reaction mixture (2 ml) contained 50 mM Tris–HCl buffer (pH 8.0), 20 mM nitrate, and an appropriate volume of the cell suspension prepared as described earlier [15]. The flasks were evacuated, filled with argon, supplemented with 170 μl hydrogen, and incubated at 37°C with shaking. The gas phase of the flasks was analyzed for hydrogen content at the beginning of the experiment and after 3 h. The hydrogen consumption rate was calculated from the decrease in the hydrogen content of the gas phase and expressed either in μl H_2/h or in μl H_2/(h mg dry cell wt.). The hydrogen used in experiments was purified from residual oxygen by passing the gas through a palladium-coated asbestos catalyst.

RESULTS AND DISCUSSION

The Dependence of Hydrogen Consumption in the Presence of Nitrate on the Concentration of the Mutant HDK103 and HDK203 Cells

According to earlier observations [15], the wild-type and mutant *E. coli* cells grown anaerobically in the presence of glucose without nitrate exhibit a high level of hydrogenase activity (hydrogenase 1 in the mutant HDK203, hydrogenase 2 in the mutant HDK103, and both hydrogenases in the wild-type strain) and are capable of nitrate-dependent hydrogen consumption.

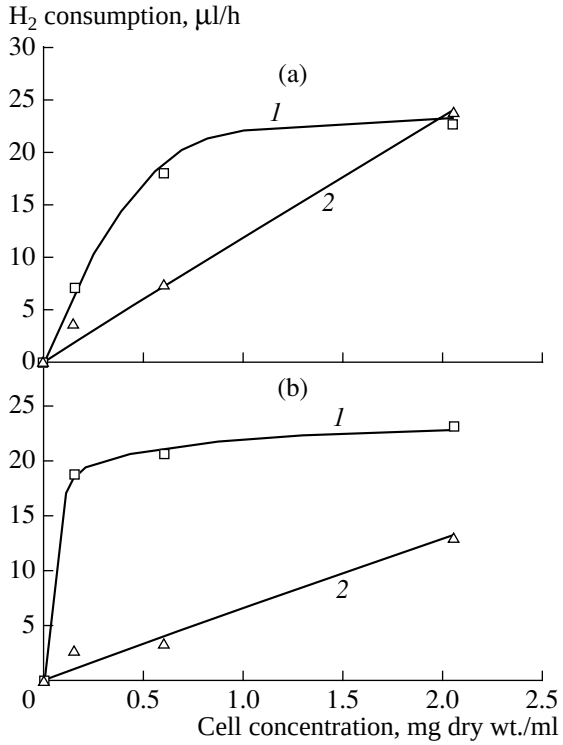

Fig. 1. The nitrate-dependent consumption of hydrogen by (*1*) the HDK103 and (*2*) HDK203 mutant cells grown anaerobically on (a) glucose or (b) 0.5% peptone, 0.2% nitrate, and hydrogen as a function of the cell concentration in the reaction mixture. Experimental data are presented with the subtraction of the nitrate-independent consumption of hydrogen, which comprised no more than 6 μl H_2/h.

The concentration of cells in the reaction mixture differently influenced the rates of nitrate-dependent hydrogen consumption by the mutants (Fig. 1a). At a high cell concentration (2.1 mg/ml), the mutants consumed hydrogen at almost the same rate. At lower cell concentrations, the mutant HDK103 consumed hydrogen at a higher rate than the mutant HDK203, the difference being maximum at cell concentrations between 0.3 and 1.0 mg/ml. Unlike the mutant HDK203, which showed a linear dependence of the hydrogen consumption rate within the entire range of cell concentrations studied, the rate of hydrogen consumption by the mutant HDK103 plateaued at cell concentrations higher than 0.6 mg/ml (Fig. 1a). This can be explained by the fact that, at high hydrogen consumption rates (more than 20 μl H_2/h), hydrogen consumption is limited not by the hydrogenase activity of cells but by some other factors, such as the rate of hydrogen diffusion into the liquid phase. The concentration of hydrogen in the gas phase (about 0.6 mM) is likely saturating, since the K_m for hydrogen of hydrogen-consuming

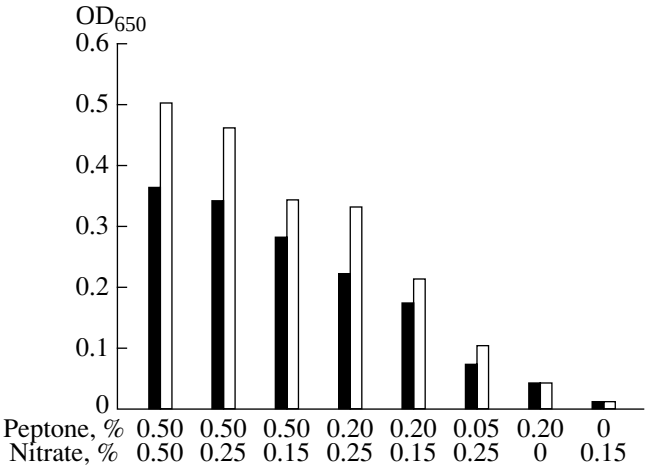

Fig. 2. The effect of hydrogen on the growth of the wild-type MC4100 strain at different concentrations of peptone and nitrate. Black and open bars correspond to bacterial growth in atmospheres of argon and hydrogen, respectively.

hydrogenases is lower than 1 μM [17]. At low rates of hydrogen consumption (below 8 μl H_2/h), the correction for nitrate-independent hydrogen consumption (about 6 μl H_2/h) became essential. For this reason, the range of hydrogen consumption rates between 8 and 20 μl H_2/h (this corresponds to a cell concentration of about 0.6 mg/l for both mutants) seemed to be the most appropriate for relevant experiments. At this cell concentration, the rate of hydrogen consumption by the mutant HDK103 was about 2.5-fold higher than in the case of the mutant HDK203, which is in agreement with the earlier measurements [15].

The Repression of Hydrogenase Synthesis by Nitrate: Does Such Repression Contradict the Capability of E. coli Cells to Grow at the Expense of Hydrogen Utilization and Nitrate Respiration?

E. coli cells incubated anaerobically in the absence or deficiency of organic electron donors could presumably grow due to their ability to take up hydrogen in the presence of nitrate as an electron acceptor. However, experimental data available in the literature indicate that nitrate suppresses the synthesis of hydrogenases. For instance, Sawers *et al.* [3] showed that nitrate in the medium completely suppresses the synthesis of hydrogenase 1 and partially suppresses the synthesis of hydrogenase 2. Menon *et al.* [5] found that nitrate inhibits the synthesis of the small subunit of hydrogenase 2, so that this hydrogenase becomes inactive. According to the data of Richard *et al.* [8], 0.2% nitrate in the medium diminishes the level of the anaerobic expression of *hya* and *hyb* by 80 and 70%, respectively.

Other factors may also influence the expression of hydrogenases. For instance, at a concentration of 0.5%, nitrate diminished the expression of *hya* in the medium with glucose by 90%, whereas the expressions of *hya* in the medium with glycerol and nitrate or with formate and nitrate were almost the same as in the medium with glucose alone [7]. Nitrate (0.5%) lowered the total hydrogenase activity of *E. coli* cells grown anaerobically on glucose by 98.7%, and by 91.7% when *E. coli* cells were grown on peptone in the presence of hydrogen. In the presence of hydrogen, the biomass yield was almost three times higher and proportional to the amount of nitrite produced [14]. These data suggest that *E. coli* cells grew anaerobically at the expense of hydrogen oxidation coupled with the reduction of nitrate. This implies that even low levels of hydrogenase activity are sufficient to provide for bacterial growth on hydrogen. To answer the question of which of the hydrogenases is responsible for the hydrogenase activity of bacterial cells and their growth, wild-type and mutant strains of *E. coli* were grown in the presence of nitrate and hydrogen.

The yield of the wild-type *E. coli* cells grown anaerobically in the presence of peptone and nitrate was found to be dependent on their concentrations (Fig. 2). In the absence of either nitrate or peptone the wild-type strain did not grow. The addition of hydrogen to the medium containing 0.2–0.5% peptone and nitrate resulted in a 35–50% increase (by 0.11–0.14 OD units) in the cell concentration, the increase being statistically significant at a 99% confidence level. At lower concentrations of nitrate and peptone, both the final turbidity of the culture and the hydrogen-induced increase in the culture turbidity were lower. Thus, as opposed to Yamamoto and Ishimoto [14], we failed to show a 3-fold increase in bacterial growth induced by hydrogen. This failure can be explained as follows: in the cited work [14], the bacterium *E. coli* was grown at a constant hydrogen flow of 300 ml/(min l medium). In our study, however, this bacterium was grown under the stationary gas phase at a gas/liquid ratio equal to 7.5/1 and a hydrogen/nitrate molar ratio equal to 13/1. Raising the latter proportion to 52/1 did not improve bacterial growth, indicating that the growth was not limited by hydrogen. It should also be noted that the *E. coli* biomass accumulated in our experiments was 0.26 mg/ml, compared to only 0.1 mg/ml in the experiments of Yamamoto and Ishimoto. This can be explained by the fact that the peptone that we used contained more reduced compounds than did the peptone used by the Japanese researchers.

The experimental data presented in Fig. 2 were obtained with 15- to 16-h-old cultures. The investigation of cultures grown in the medium with 0.5% peptone and 0.25% nitrate (Fig. 3a) showed that hydrogen did not augment the specific growth rate of bacterial cells (the doubling time of cells was about 1 h irrespective of the presence of hydrogen). Nor was hydrogen able to reduce the lag phase (this is clearly seen from the experiment presented in Fig. 3b, in which the lag phase was intentionally extended by using a 0.3% inoculum instead of the 4% inoculum used in the other experiments). At the same time, hydrogen enhanced the

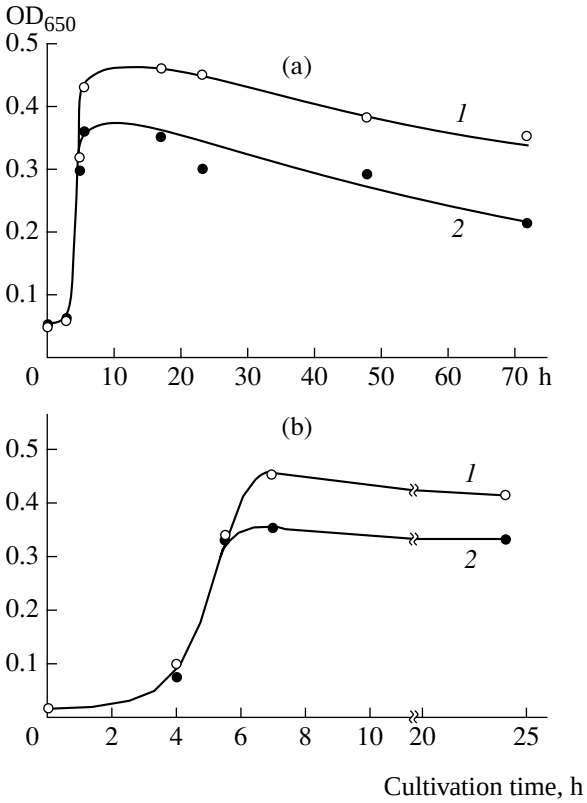

Fig. 3. Growth of the wild-type MC4100 strain in medium with 0.5% peptone and 0.25% nitrate in (*1*) the presence and (*2*) absence of hydrogen. The inoculum size was either (a) 4% or (b) 0.3%.

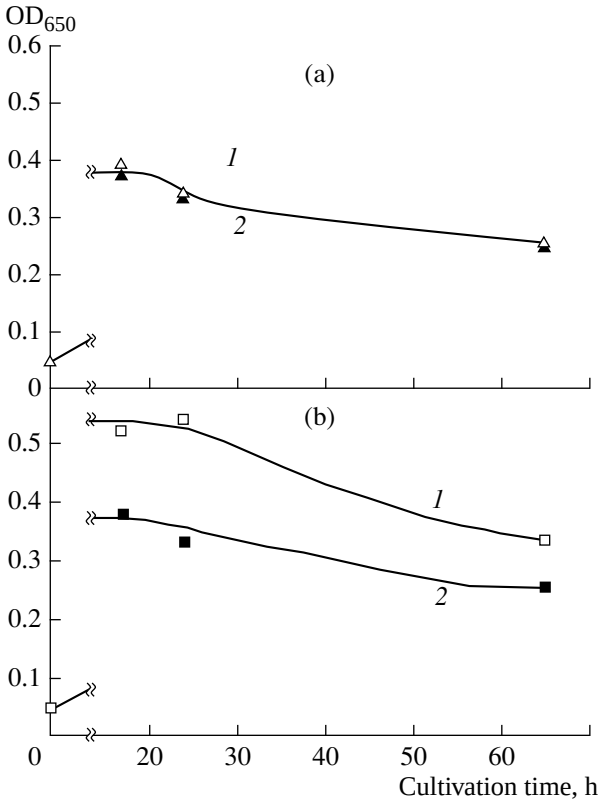

Fig. 4. Growth of the mutant (a) HDK203 and (b) HDK103 cells in medium with 0.5% peptone and 0.25% nitrate in (*1*) the presence and (*2*) absence of hydrogen.

biomass yield, the effect being profound in the late exponential, stationary, and decline growth phases. These data suggest that bacterial cells accomplish hydrogen-dependent nitrate respiration as an alternative source of energy after the reduced compounds of peptone have been exhausted.

Similar experiments with the mutants HDK103 and HDK203 synthesizing one of the hydrogenases showed that hydrogen did not stimulate the anaerobic growth of HDK203 in the presence of nitrate (Fig. 4a). At the same time, the effect of hydrogen on the growth of HDK103 was the same as in the case of the wild-type *E. coli* strain (Fig. 4b). These data indicate that hydrogen-dependent nitrate respiration is able to provide for the growth of bacterial cells only if they contain hydrogenase 2 (the wild-type and mutant HDK103 cells).

The Effect of Cultivation Conditions on the Nitrate-Dependent Hydrogen Consumption by the Mutant HDK103 and HDK203 Cells

It will be of interest to clarify why hydrogenase 1 is able to catalyze the nitrate-dependent consumption of hydrogen but fails to stimulate the growth of the mutant HDK203. Our speculations as to this point are given below.

It is known that hydrogenase 1 has a low activity, so that most of the hydrogenase activity of the wild-type *E. coli* cells, estimated by the reduction of benzyl viologen, is due to hydrogenase 2, although its content in the cells is lower than that of hydrogenase 1 [5, 8]. Furthermore, the rate of nitrate-dependent hydrogen consumption can be limited not only by hydrogenase activity but also by the activities of nitrate reductase and the electron carriers involved in this process. Nitrate, which suppresses the synthesis of hydrogenases, can concurrently induce the synthesis of the *narGHJI*-encoded nitrate reductase A, which adds to the activity of the *narZYWV*-encoded constitutive nitrate reductase Z [10]. For this reason, the resulting total effect of nitrate on hydrogen consumption by *E. coli* cells is difficult to predict.

According to our data, the nitrate-dependent hydrogen consumption by HDK203 cells grown in the absence of nitrate is slower than in the case of HDK103 cells (Fig. 1a). The addition of nitrate to the glucose-containing cultivation medium drastically decreased hydrogen consumption due to hydrogenase 1 or 2 to a level typical of the reaction mixture con-

The nitrate-dependent consumption of hydrogen (in μl H_2 (h mg cells)) by the HDK103 and HDK203 mutant cells grown anaerobically on different substrates

Growth substrates	HDK103	HDK203
Glucose	16.3	7.4
Glucose + 0.25% nitrate	3.5	1.3
0.5% peptone + 0.25% nitrate + Ar	23.2	2.1
0.5% peptone + 0.25% nitrate + H_2	18.6	4.1
Control (measurements without nitrate)	3.3	1.4

Note: The concentration of cells in the reaction mixture was 0.6 mg/ml.

taining no nitrate (table). At the same time, the mutant HDK103 cells grown in the medium with peptone and nitrate but without glucose and hydrogen exhibited a higher rate of hydrogen consumption than did the glucose-grown cells of the mutant (table). In contrast, the mutant HDK203 (containing only hydrogenase 1) grown under the same conditions exhibited a rate of nitrate-dependent hydrogen consumption that comprised only about 30% of that of the glucose-grown HDK203 cells. Similar data were obtained for the mutant cells grown in the medium with peptone, nitrate, and hydrogen (without glucose). In this case, the rate of nitrate-dependent hydrogen consumption by the mutant HDK203 comprised no more than 22% of that exhibited by the mutant HDK103 (containing only hydrogenase 2) (table). It should be noted that the data under discussion were obtained when the cell concentration in the reaction mixture was 0.6 mg/ml. Based on the data presented in Fig. 1b (from which it is clearly seen that the rate of hydrogen consumption by cells grown in the presence of nitrate and hydrogen plateaus at cell concentrations lower than 0.6 mg/ml), we may assume that the difference between the hydrogen consumption abilities of the HDK103 and HDK203 mutant cells is even greater when the cell concentration in the reaction mixture corresponds to that observed in the growing cultures. Thus, it is likely that the hydrogen consumption ability of HDK203 is insufficient to stimulate the growth of this mutant on hydrogen. Our earlier observation that HDK203 has a low rate of fumarate-dependent hydrogen consumption [15] is in agreement with the observations of other researchers that hydrogenase 1 is unable to provide for bacterial growth at the expense of anaerobic fumarate respiration [1, 3, 6].

The hypothesis that the oxidation of hydrogen by nitrate in *E. coli* cells with the involvement of hydrogenases 1 and 2 may differ not only at the level of these hydrogenases but also at the level of both nitrate reductases and the electron transport chain needs a strong experimental underpinning.

To conclude, either hydrogenase 1 or 2 can catalyze the nitrate-dependent consumption of hydrogen, but only hydrogenase 2 is able to provide for bacterial growth at the expense of hydrogen oxidation with nitrate as the terminal electron acceptor.

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