JOURNAL OF BACTERIOLOGY, Feb. 1986, p. 579–584
0021-9193/86/020579-06$02.00/0
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Vol. 165, No. 2

Immunological Homology between the Membrane-Bound Uptake Hydrogenases of *Rhizobium japonicum* and *Escherichia coli*†

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Received 30 September 1985/Accepted 18 November 1985

Two polypeptides present in aerobic and anaerobic cultures of *Escherichia coli* HB101 were shown to cross-react with antibodies to the 30- and 60-kilodalton (kDa) subunits of the uptake hydrogenase of *Rhizobium japonicum*. The cross-reactive polypeptides in a series of different *E. coli* strains are of M_rs ca. 60,000 and 30,000, and both polypeptides are present in proportion to measurable hydrogen uptake (Hup) activity (r = 0.95). The 60-kDa polypeptide from *E. coli* HB101 comigrated on native gels with detectable Hup activity. The exact role of the the 30-kDa polypeptide in *E. coli* is unclear. *E. coli* MBM7061, a natural Hup$^-$ variant, grown anaerobically or aerobically lacked detectable Hup activity and failed to cross-react with the antisera against the hydrogenase from *R. japonicum*. Anaerobically cultured *E. coli* MBM7061, however, did express formate hydrogenlyase activity, indicating that the hydrogenases involved in the oxygen-dependent activation of hydrogen and the formate-dependent evolution of hydrogen are biochemically distinct.

The enzyme hydrogenase (EC class 1.12) catalyzes the reversible oxidation of molecular hydrogen. Although all hydrogenases thus far isolated function in vitro to consume and evolve hydrogen, the enzymes which have been well characterized seem to be physiologically constrained to a single function in vivo. The biochemistry and physiological roles of hydrogenase enzymes from a wide variety of microorganisms have been extensively reviewed by Adams et al. (2).

Hydrogenase occurs in anaerobically grown *Escherichia coli* as part of the formate hydrogenlyase pathway. This complex is a membrane-bound multienzyme system which converts formate to H_2 and CO_2 (9, 25). This hydrogenase may likewise transfer electrons from H_2 to fumarate or nitrate via a *b*-type cytochrome and menaquinone (5). Strains of *E. coli* grown aerobically or anaerobically on various carbon substrates also exhibit hydrogen uptake (Hup) activity (1, 22, 32). A hydrogenase from aerobically grown *E. coli* has been purified and shown to consist of a single subunit of M_r 56,000 (1). It exhibits reversible activation of H_2 in the presence of some artificial redox reagents and is located in the cell membrane. Recently, Ballantine and Boxer (4) have demonstrated the presence of two membrane-bound nickel-containing hydrogenase isozymes in anaerobically cultured *E. coli*. The relationship of the enzyme purified from aerobically grown cells or these two isozymes identified in anaerobically grown cells to the diverse hydrogenase activities in *E. coli* has not been clearly demonstrated.

Hydrogenase is also present in some strains of *Rhizobium japonicum*. This enzyme has Hup activity in vivo and participates in the recycling of hydrogen evolved in nodules as an obligate by-product of the nitrogen fixation process. The hydrogenase activity can be derepressed in free-living cultures (23) and under conditions of chemolithotrophic growth when H_2 is the only source of reductant (11, 20).

The uptake hydrogenase has been purified in our laboratory from chemolithotrophically cultured cells of *R. japonicum* (13). The enzyme is composed of two subunits of M_rs 30,000 and 60,000. Antibodies made separately against the two subunits were used to demonstrate that the 30- and 60-kilodalton (kDa) polypeptides are immunologically distinct (13). Similar results have been obtained for hydrogenase purified from *R. japonicum* bacteroids (3). Nickel has been shown to be a constituent of active hydrogenase (3, 13, 29) and necessary for activity in vivo (16).

A gene library of DNA from *R. japonicum* 122DES has been constructed in *E. coli* HB101 by using the broad-host-range cosmid pLAFR1 (6). Several recombinant cosmids have been isolated which complement Hup$^-$ point mutants of *R. japonicum* 122DES (6, 19). The Hup-specific cosmid pHU52 has been transferred into several Hup$^-$ wild-type strains of *R. japonicum*. The cosmid confers limited Hup activity to all transconjugant strains in the free-living state (19), with coordinated synthesis of both hydrogenase subunit polypeptides (12).

The initial objective of this study was to determine if *R. japonicum* hydrogenase activity encoded on pHU52 is expressed in *E. coli* HB101. Hup assays in whole-cell cultures and immunological detection of the subunit polypeptides in crude extracts were used. We report here the detection of polypeptides (M_rs ca. 60,000 and 30,000) in crude extracts of several wild-type strains of *E. coli* which cross-react with the antisera against the hydrogenase subunits from *R. japonicum*. There is a positive correlation in the strains of *E. coli* tested between the amount of detectable cross-reactive protein and the measurable Hup activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains of *E. coli* used in this study are listed in Table 1. All strains were cultured on LB medium at 32°C (15). Liquid cultures (4 ml in a 17- by 150-mm tube) were shaken in air or sealed under an atmosphere of nitrogen. Liquid cultures were grown overnight to an optical density at 550 nm of 1.3 to 1.5 for aerobic cultures and 0.8 for anaerobic cultures. Tetracycline was added at 18 μg/ml to medium used to culture strains carrying the cosmid pHU52.

Protein extraction. A volume of cell suspension containing ca. 10 mg of total protein was centrifuged for 10 min at

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† Technical paper no. 7678 of the Oregon Agricultural Experiment Station.

TABLE 1. Bacterial strains

Strain	Genotype	Source[a]
HB101	*recA hsdR hsdM pro leu str*$^+$	G. Ditta (San Diego)
DC646	C600, λ *c*I$^+$ *hsdR hsdM*$^+$	D. Court (NIH)
LE392	*hsdR hsdM ΔlacY galK2* λ	T. Jacobs (Stanford)
CSH25	*sup-3 B*1	Cold Spring Harbor Laboratory
N100	*galK recA* Str^r	D. Court (NIH)
N99	N100 *recA*$^+$	D. Court (NIH)
SA1943	SA500 *galK*	D. Court (NIH)
C600	*leu thr B*1 *lacY*	D. Court (NIH)
C600 *galK*	C600 *galK*	D. Court (NIH)
MBM7061	*lacY*$^+$ *lacI ΔlacZ* λ *c*I$^+$	D. Court (NIH)

[a] San Diego, University of California at San Diego; Stanford, Stanford University; NIH, National Institutes of Health.

12,000 × *g*, and the supernatant was discarded. The pellet was suspended in 1 ml of lysis buffer containing 0.0625 M Tris hydrochloride (pH 6.8), 2.3% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 10% glycerol. The cell suspensions were heated at 60°C for 20 min and centrifuged at 30,000 × *g* for 1 h at 4°C. The resultant supernatant was retained as the SDS-treated crude extract.

Nondenatured crude extracts were obtained by suspending the initial cell pellet in 1 ml of 0.05 M Tris hydrochloride (pH 8.0) containing 1 mM dithiothreitol, 1 mM sodium dithionite, 0.1 mg of DNase I per ml, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μM antipain (an inhibitor of thiol proteases; Sigma Chemical Co., St. Louis, Mo.). The suspension was frozen in and passed through an Eaton press and collected under hydrogen gas. Triton X-100 was added to a concentration of 1%. The suspension was agitated under hydrogen for 1 h at room temperature and centrifuged at 100,000 × *g* for 1 h at 4°C. The resultant supernatant contained the active solubilized hydrogenase.

Electrophoresis. SDS and native polyacrylamide gel electrophoresis procedures were performed by the methods of Laemmli (18) and Davis (7), respectively. Gels were 90 by 75 mm and 0.75 mm thick. SDS gels were 10% acrylamide. Native gels were 5 to 20% acrylamide gradients. Equal quantities of protein in approximately 10 μl (total volume) were loaded in a 18-well stacking gel and electrophoresed at 20 mA of constant current for ca. 1 h. Proteins separated on SDS gels were electroblotted onto nitrocellulose paper (30) for 1 h at 0.8 A of constant current.

Two-dimensional electrophoresis was performed by excising a band from a native gel and treating it for 1 h at room temperature in the SDS lysis buffer described above. The treated excised band was placed in a single well of an SDS gel and sealed in place with 1% agarose in one-fourth-strength SDS stacking gel buffer (24). The SDS gel was then electrophoresed as described above.

Analytical assays. Total protein of whole cells was measured by solubilization of whole cells by the method of Stickland (28), followed by protein determination by the procedure of Lowry et al. (21). Bovine serum albumin was used as a standard.

Oxygen and hydrogen concentrations over anaerobic liquid cultures were measured by gas chromatography. Hydrogen consumption and evolution by whole cells in liquid culture were measured in a sealed chamber with a hydrogen electrode (10). Formate-dependent hydrogen evolution was initiated by the addition to the electrode chamber of sodium formate to a concentration of 40 mM. Oxygen-dependent hydrogen oxidation was initiated by the addition of 50 μl of oxygen-saturated buffer (50 mM KH_2PO_4, pH 7.5). Hup activity of cell cultures on plates was detected by the methylene blue colony assay of Haugland et al. (14). The in situ detection of Hup activity on native polyacrylamide gels was by the procedure of Schneider and Schlegel (27), in which Nitro Blue Tetrazolium was used as the terminal electron acceptor.

The immunological detection of hydrogenase subunit polypeptides bound to nitrocellulose was performed by published enzyme-linked immunosorbent assay (ELISA) techniques (30) which were modified as described elsewhere (12). The affinity-purified antibody preparations were diluted 1:10,000 for use in the ELISA. Nitrocellulose blots developed by the ELISA were photographed to produce high-quality black-and-white negatives (10 by 12.5 cm). The density of the cross-reacting bands was determined by scanning the negatives on a soft-laser scanning densitometer (Biomed Instruments, Inc., Fullerton, Calif.). These data are reported as relative optical densities referenced to the density of detectable cross-reacting material in crude extracts of *E. coli* HB101.

Isolation of DNA from *R. japonicum* and *E. coli* and isolation of plasmids from *E. coli* were done as described previously (19). Two subclones of the cosmid pHU52 which encode all of the Hup-specific determinants (pMZ513 and pMZ514; M. Zuber, unpublished data) were labeled in vitro to specific activities of 1.2×10^8 cpm/μg by incorporation of [^{32}P]dCTP (New England Nuclear Corp., Boston, Mass.) and used as hybridization probes. Nick translation of the two subclones (26) and DNA hybridization procedures (31) were as described previously. Southern blots and probe mixtures were incubated for 16 h at 42°C. Replicate hybridization blots were washed either for 1 h at 42°C or for 2 h at 68°C.

RESULTS

Screening for Hup-related polypeptides in *E. coli* HB101(pHU52). Aerobically grown cells of *E. coli* HB101 and HB101(pHU52) were lysed and treated with SDS. Samples of these crude extracts were electrophoresed on polyacrylamide gels, electroblotted onto nitrocellulose paper, and probed with the affinity-purified antibodies against the subunits of hydrogenase from *R. japonicum*. Two polypeptides of the expected molecular weights (M_rs 60,000 and 30,000) were detected in HB101(pHU52) but were also present in the parent strain of *E. coli* HB101 (data not shown). Samples of each liquid culture were used to inoculate LB plates containing tetracycline. *E. coli* HB101 exhibited no tetracycline resistance, indicating that the culture of the parent strain was not contaminated with the transconjugant.

Correlation of in vivo hydrogen oxidation and detectable cross-reactive material in wild-type strains of *E. coli*. Numerous strains of *E. coli* were grown on LB plates and screened for hydrogen-dependent methylene blue reduction by the colony assay of Haugland et al. (14). Ten strains representing an apparent range of Hup activities were selected for further study (Table 2). Aerobic cultures were grown overnight, and the Hup activity of each was measured with a hydrogen electrode. Another sample of each culture was treated with the SDS lysis buffer, electrophoresed, and subjected to Western blot-ELISA. Figure 1 illustrates the results obtained for *E. coli* HB101 (lanes 5 and 6). The cross-reacting bands are referenced to the cross-reactive subunit polypeptides of purified hydrogenase from *R. japonicum* (lanes 3 and 4). Lanes 1 and 2 show the corresponding electrophoretic patterns of crude extracts of *E. coli*

MBM7061, which exhibited no detectable cross-reactive material.

Data showing the relative quantity of immunological cross-reactive material and the measured Hup-specific activity are presented in Table 2. There is an apparent positive correlation between the degree of immunological cross-reaction with each antiserum and the Hup-specific activity (r = 0.95 for both the 30- and 60-kDa antisera). *E. coli* HB101 had the highest measured rate of hydrogen consumption, while strain MBM7061 was the only strain that had no detectable Hup activity.

Identification of hydrogen-specific Hup activity bands on native gels. Duplicate samples of Triton X-100-solubilized crude extracts of *E. coli* HB101 and MBM7061 grown aerobically and anaerobically were electrophoresed on a native polyacrylamide gel. After electrophoresis, the gel was divided in half, and duplicate lanes were incubated under H_2 or N_2 in the presence of Nitro Blue Tetrazolium (Fig. 2). A single hydrogen-dependent activity band was evident in the extracts of both aerobically and anaerobically cultured cells of *E. coli* HB101 (lanes 1 and 2). No such band was present in extracts of *E. coli* MBM7061 (lanes 3 and 4). The top band present in all lanes, including those for the negative controls incubated under N_2 (lanes 5 and 6), is probably a protein reduced by dithiothreitol or dithionite in the extraction buffer, which in turn reduces the Nitro Blue Tetrazolium after electrophoresis, irrespective of the incubation atmosphere.

Detection of cross-reactive polypeptides associated with activity bands from native gels. Native polyacrylamide gels identical to those described above were electroblotted onto nitrocellulose and probed with the antibodies against hydrogenase from *R. japonicum*. Reference lanes containing proteins from crude extracts of *R. japonicum* 122DES exhibited a doublet which cross-reacted with both antisera (Fig. 3, lanes 1 and 3). This migration pattern was due perhaps to protein-detergent interactions (13). There was no detectable cross-reaction with any polypeptide in lanes containing native proteins from *E. coli* HB101 (Fig. 3, lanes 2 and 4).

The portion of the native gel containing the uptake hydrogenase from *E. coli* HB101, as determined by activity staining, was excised and treated with SDS sample buffer for second-dimension electrophoresis on an SDS gel. After electrophoresis, replicate SDS gels were electroblotted and probed with the separate antisera. A single polypeptide of M_r ca. 60,000 was detected with the 60-kDa antiserum (Fig. 3, lane 5). No cross-reaction with the 30-kDa antiserum was detected (Fig. 3, lane 6). The implications of such a result are discussed later.

Determination of Hup and formate hydrogenlyase activities in aerobic and anaerobic cultures of *E. coli* HB101 and MBM7061. Aerobic and anaerobic cultures of strains HB101 and MBM7061 were examined with a hydrogen electrode for formate-dependent hydrogen evolution (Fig. 4). Neither strain when grown aerobically evolved hydrogen on the addition of formate (Fig. 4A). Aerobically cultured cells deprived of O_2 in the electrode chamber showed no hydrogenase activity. Consistent with data reported in Table 2,

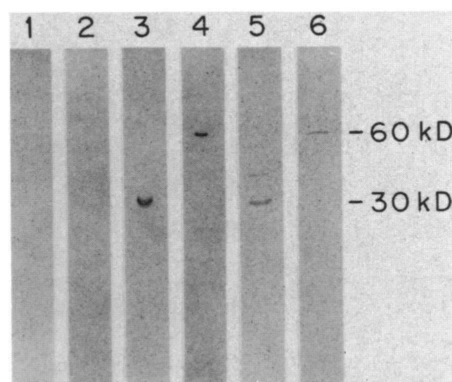

FIG. 1. Immunological cross-reactions of polypeptides in crude extracts of *E. coli* with antisera against the hydrogenase polypeptides from *R. japonicum*. The total protein (150 μg) in the crude extracts was SDS treated, electrophoresed, and subjected to Western blot-ELISA as described in Materials and Methods. Crude extracts of *E. coli* MBM7061 (lanes 1 and 2) and HB101 (lanes 5 and 6) were probed with the 30-kDa antiserum (lanes 1, 3, and 5) and the 60-kDa antiserum (lanes 2, 4, and 6). Lanes 3 and 4 are purified hydrogenase (0.08 μg) from *R. japonicum*.

TABLE 2. Hup activity in whole cells and immunological detection of hydrogenase subunit polypeptides in crude extracts of various strains of *E. coli*

E. coli strain	Methylene blue colony assay	Hup-specific activity[a]	Immunological cross-reaction[b] with antiserum	
			30 kDa	60 kDa
HB101	+	112	1.00	1.00
DC646	+	92	0.84	0.79
LE392	+	85	0.54	0.41
CSH25	+	66	0.27	0.34
N100	+	43	0.20	0.25
N99	+	9	0.00	0.00
SA1943	−	5	0.00	0.00
C600	−	2	0.00	0.00
C600 *galK*	−	1	0.00	0.00
MBM7061	−	0	0.00	0.00

[a] In nanomoles per minute per milligram of protein.

[b] Density of ELISA-detectable cross-reacting material relative to that in *E. coli* HB101 (1.00). 0.00 indicates no detectable cross-reaction.

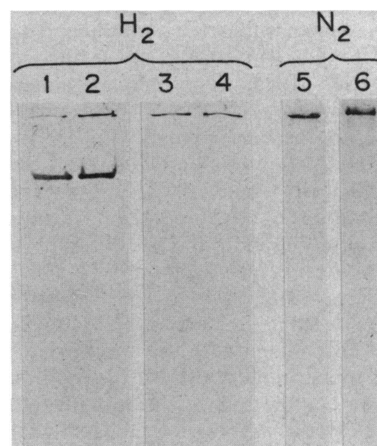

FIG. 2. Native polyacrylamide gel electrophoresis of crude extracts of *E. coli*. Hup activity was detected by hydrogen-dependent reduction of Nitro Blue Tetrazolium. Lanes show aerobic (lane 1) and anaerobic (lane 2) cultures of *E. coli* HB101 and aerobic (lane 3) and anaerobic (lane 4) cultures of *E. coli* MBM7061. Lanes 5 and 6 are identical to lanes 1 and 2, but incubated under N_2 rather than H_2. The appropriate controls for lanes 3 and 4 were identical to lanes 5 and 6.

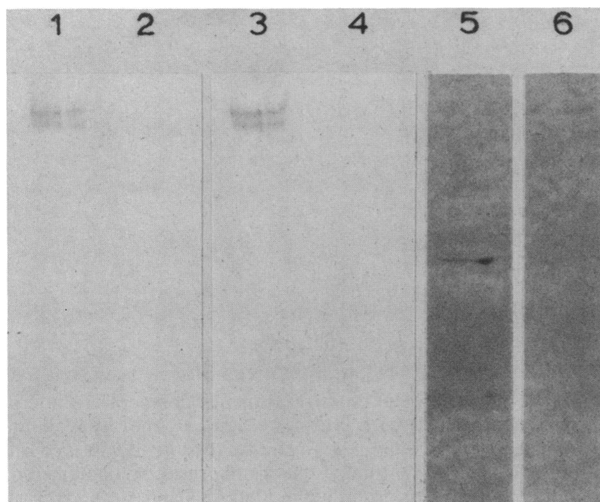

FIG. 3. Immunological detection of hydrogenase-related polypeptides by Western blot-ELISA of native gels (lanes 1 through 4) containing 100 μg of Triton X-100-solubilized protein from autotrophically cultured *R. japonicum* 122DES (lanes 1 and 3) and anaerobically cultured *E. coli* HB101 (lanes 2 and 4). Hydrogen-specific activity bands (lanes 1 and 2) were excised from the native gel, SDS treated, electrophoresed on a second-dimension SDS gel, and subjected to Western blot-ELISA (lanes 5 and 6). Lanes 1, 2, and 5 were probed with the 60-kDa antiserum. Lanes 3, 4, and 6 were probed with the 30-kDa antiserum.

aerobic cultures of *E. coli* HB101 consumed H_2 in the presence of O_2, while aerobic cultures of *E. coli* MBM7061 had no measurable Hup activity (Fig. 4A).

Anaerobic cultures of both strains evolved hydrogen in the presence of added formate (Fig. 4B). When O_2-saturated buffer was added to the electrode chamber, *E. coli* HB101 immediately began to consume the evolved hydrogen. Strain MBM7061 merely ceased to evolve hydrogen until the O_2 was depleted, returning then to formate-dependent hydrogen evolution. SDS-treated crude extracts of each culture represented in Fig. 4 were subjected to SDS-polyacrylamide gel electrophoresis, electroblotting onto nitrocellulose, and ELISA. The results for both aerobic and anaerobic cultures were essentially identical to those shown in Fig. 1.

DNA hybridization experiments. Total DNA from *E. coli* HB101 and MBM7061 was electrophoresed on agarose and blotted onto nitrocellulose. Replicate blots were probed with ^{32}P-labeled subclones of the Hup-specific cosmid pHU52 and were washed under conditions of different stringency as described in Materials and Methods. Total DNA from the Hup^+ wild-type strain *R. japonicum* 122DES was analyzed concurrently as a single-copy positive control. Both of the labeled probes hybridized with the appropriate fragments in the rhizobial controls under both wash conditions. However, there was no detectable hybridization with total DNA from either strain of *E. coli*. Although the specific activities of the probes and the hybridization conditions were sufficient to detect homologous hybridization with a presumptive single copy of the genes encoding Hup in *R. japonicum* 122DES, these conditions may not have been sufficient for heterologous hybridization. Alternatively, the DNA sequences coding for the immunologically homologous polypeptides may not be sufficiently similar for hybridization to occur under these conditions.

DISCUSSION

Immunologically cross-reactive polypeptides were detected in various strains of *E. coli* by using antibodies produced against the subunits of the uptake hydrogenase of *R. japonicum*. A question arises as to the nature and function of the detected polypeptides. The polypeptides detected in *E. coli* have molecular weights similar to those of the hydrogenase subunits in *R. japonicum*. The presence of material cross-reactive to both antisera in crude extracts of *E. coli* is proportional to the measurable rate of hydrogen oxidation in whole cells ($r = 0.95$). Native gel electrophoresis of Triton X-100-solubilized crude extracts of *E. coli* HB101 shows a single band with Hup activity. Although there was no detectable cross-reaction with native protein from this activity band, the protein excised from the native gel and electrophoresed on a second-dimension SDS gel cross-reacted with the 60-kDa antiserum. These data suggest that the two detectable polypeptides in extracts of *E. coli* are components of an uptake hydrogenase system in *E. coli*.

The hydrogenase antisera were originally produced against rhizobial hydrogenase subunits separated on an SDS gel. The absence of detectable cross-reaction with native hydrogenase from *E. coli* may be due to a lack of accessible or recognizable antigenic sites when the protein assumes its native conformation. On SDS treatment of the active protein, only the 60-kDa polypeptide was detected. We have been unable to determine the fate of the 30-kDa polypeptide in Triton X-100-solubilized crude extracts of *E. coli*. The 30-kDa subunit in *R. japonicum* is susceptible to the action of proteases (13), with concomitant diminution of specific activity. However, the uptake hydrogenase from *E. coli* as purified by Adams and Hall (1) contained only one subunit of M_r 56,000. In the protocol outlined for solubilization, deoxycholate and the protease pancreatin were used. The absence of the 30-kDa polypeptide may be due to proteolysis or, alternatively, retention in membrane particles after detergent solubilization.

It is apparent that in *E. coli* the 30-kDa polypeptide is not required for the in vitro activation of molecular hydrogen in

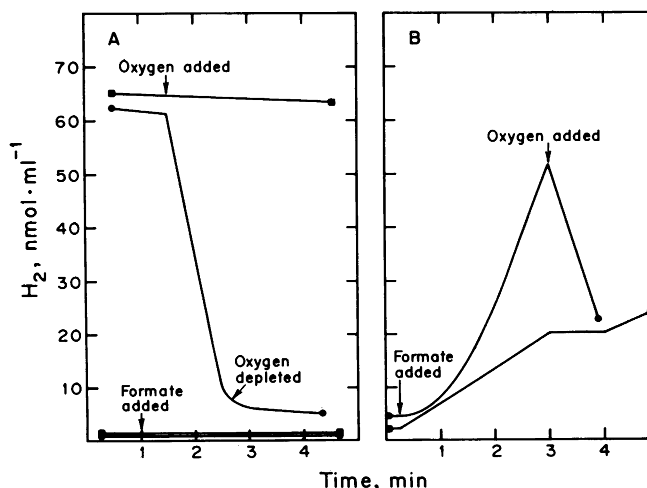

FIG. 4. Hydrogen evolution and uptake by aerobically (A) and anaerobically (B) grown cultures of *E. coli* MBM7061 (■) and HB101 (●) as measured by a hydrogen electrode. Arrows indicate the addition or depletion of O_2 in the presence of H_2 during the measurement of Hup or, alternatively, the addition of formate in the absence of H_2 during the measurement of formate-dependent hydrogen evolution.

the presence of artificial electron acceptors. Adams and Hall (1) noted, however, that the single-subunit hydrogenase was incapable of reducing common physiological acceptors. Other researchers (8, 17) have isolated mutants of *E. coli* with diminished in vivo Hup activity but full in vitro activity in the presence of artificial electron acceptors. It is possible that the 30-kDa polypeptide is required for positioning of the 60-kDa subunit in the membrane or for electron transport from the 60-kDa subunit to proximal physiological acceptors. The close correlation between the detectable levels of the 30-kDa protein and hydrogenase activity in *E. coli* (data presented here) and *R. japonicum* (12) argues for the involvement of this protein in *E. coli* during in vivo hydrogen oxidation.

The biochemistry of hydrogen metabolism in *E. coli* is complex, and data concerning hydrogenase synthesis and activity seem to be dependent on growth conditions, types of reducible carbon substrate, and other variables. Under anaerobic conditions and in the absence of other suitable electron acceptors, formate is converted to H_2 and CO_2 by the formate hydrogenlyase complex. Hydrogenase isozymes have been identified in anaerobically cultured cells (4, 32) and shown to be electrophoretically and immunologically distinct (4). It is unclear from these data (4) whether the uptake hydrogenase purified from aerobically cultured *E. coli* is a species of hydrogenase distinct from that which effects hydrogen evolution in the anaerobically derepressed formate hydrogenlyase complex.

The data presented here indicate that the uptake hydrogenase of *E. coli* is distinct from the hydrogenase involved in formate-dependent hydrogen evolution. The uptake hydrogenase activity was expressed under aerobic and anaerobic growth conditions, whereas the formate hydrogenlyase system operated only when cultures were grown under anaerobiosis. Strains MBM7061 and HB101 expressed comparable levels of formate-dependent hydrogen evolution. Strain MBM7061, however, was devoid of any uptake hydrogenase activity and any detectable polypeptides which cross-reacted with antisera developed against the hydrogenase from *R. japonicum*. It may be argued that the lack of Hup activity in *E. coli* MBM7061 is due to a missing component in electron transport and that the two hydrogenases are the same. If such were the case, on the basis of comparable formate hydrogenlyase activity levels, we would expect detectable levels of immunologically cross-reactive hydrogenase polypeptides in crude extracts of anaerobically cultured *E. coli* MBM7061.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant PCM 8118148 and the Oregon Agricultural Experiment Station.

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