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Characterization and Physiological Roles of Membrane-Bound Hydrogenase Isoenzymes from *Salmonella typhimurium*

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We found that *Salmonella typhimurium* strain LT2 (Z) possessed two immunologically distinct, membrane-bound hydrogenase isoenzymes, which were similar in electrophoretic mobilities and apoprotein contents to hydrogenase isoenzymes 1 and 2 of *Escherichia coli*. The *S. typhimurium* enzymes cross-reacted with antibodies raised to the respective hydrogenase isoenzymes of *E. coli*. As for *E. coli*, an additional membrane-bound hydrogenase activity (termed hydrogenase 3), which did not cross-react with antibodies raised against either hydrogenase 1 or 2, was also present in detergent-dispersed membrane preparations. The physiological role of each of the three isoenzymes in *E. coli* has remained unclear owing to the lack of mutants specifically defective for individual isoenzymes. However, analysis of two additional wild-type isolates of *S. typhimurium* revealed specific defects in their hydrogenase isoenzyme contents. *S. typhimurium* LT2 (A) lacked isoenzyme 2 but possessed normal levels of hydrogenases 1 and 3. *S. typhimurium* LT7 lacked both isoenzymes 1 and 2 but retained normal hydrogenase 3 activity. Characterization of hydrogen metabolism by these hydrogenase-defective isolates allowed us to identify the physiological role of each of the three isoenzymes. Hydrogenase 3 activity correlated closely with formate hydrogenlyase-dependent hydrogen evolution, whereas isoenzyme 2 catalyzed hydrogen uptake (oxidation) during anaerobic, respiration-dependent growth. Isoenzyme 1 also functioned as an uptake hydrogenase but only during fermentative growth. We postulate that this enzyme functions in a hydrogen-recycling reaction which operates during fermentative growth.

Two modes of hydrogen metabolism are apparent in enterobacteria. Hydrogen evolution occurs during fermentative growth as a result of the activity of the formate hydrogenlyase pathway. Endogenously produced formate is oxidized to carbon dioxide, coupled to the reduction of protons to form molecular hydrogen (1). This pathway consists of two enzymes, a formate dehydrogenase and a hydrogenase (24), neither of which have been characterized in molecular terms. The formate hydrogenlyase reaction is thought to relieve the buildup of reducing equivalents and, in addition, to offset acidification of the growth medium during fermentative growth (7). The formate hydrogenlyase-linked formate dehydrogenase (FDH-BV) is able to use benzyl viologen as an oxidizing substrate, which distinguishes it from the respiratory formate dehydrogenase which is present during respiration-dependent anaerobic growth (9, 11). In the second mode of hydrogen metabolism, hydrogen uptake, hydrogen is oxidized and the energy so liberated is used to support anaerobic growth (19, 29). In the hydrogen uptake reaction, a membrane-bound hydrogenase catalyzes the hydrogen-dependent reduction of the quinone pool, with concomitant energy conservation by transmembrane proton translocation (15).

We recently demonstrated the existence of multiple membrane-bound hydrogenase isoenzymes in anaerobically grown *Escherichia coli* cells (2, 25). Two nickel-containing isoenzymes, hydrogenases 1 and 2, can be readily distinguished by immunological analysis and have been purified and characterized (3, 26). However, an appreciable proportion of the hydrogenase activity of detergent-dispersed membranes is independent of these two hydrogenases (25). At least one additional isoenzyme (hydrogenase 3) must, therefore, be present. Although correlations between isoenzyme content and the two modes of hydrogen metabolism have been made (25), the specific role of each of the hydrogenase isoenzymes remains uncertain. This is primarily due to a lack of mutants specifically defective for the individual isoenzymes. All the characterized *hyd* mutants which are presently available possess lesions which map near 59 min on the chromosome, lack all known hydrogen-linked activities, and are pleiotropically defective in all three hydrogenase isoenzymes (4, 14, 17, 23, 28).

In the preceding study (13) we identified two novel anaerobic regulatory loci in *Salmonella typhimurium*. To ascertain the roles of these loci in the anaerobic expression of hydrogenase activity (14), we have characterized the hydrogenase isoenzymes of *S. typhimurium*. We show here that the spectrum of hydrogenase isoenzymes in this species was found to be similar to that in *E. coli*. However, two wild-type isolates of *S. typhimurium* were found to be deficient in one or more of the hydrogenase isoenzymes. A detailed analysis of hydrogen metabolism in these natural mutants enabled us to ascertain the specific physiological role of each of the three hydrogenase isoenzymes.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* wild-type isolates LT2 (Z), LT2 (A), and LT7 were obtained from the laboratory stocks of B. N. Ames. *E. coli* P4X (Hfr *metB*) was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. *E. coli* DB10 (P4X *hydB*) has been described (28).

Growth of bacteria. Anaerobic growth was achieved in stoppered 500-ml bottles completely filled with a medium based on that of Cohen and Rickenberg (6) containing glucose (0.4% [wt/vol]), bacteriological peptone (0.5% [wt/vol]), casein hydrolysate (0.1% [wt/vol]), thiamine (0.001% [wt/vol]), $MgCl_2$ (1 mM), $(NH_4)_6Mo_7O_{24}$ (1 μM), and $KSeO_3$ (1 μM) adjusted to pH 6.4. Where indicated, sodium formate (0.2% [wt/vol]) or KNO_3 (1% [wt/vol]) was added as

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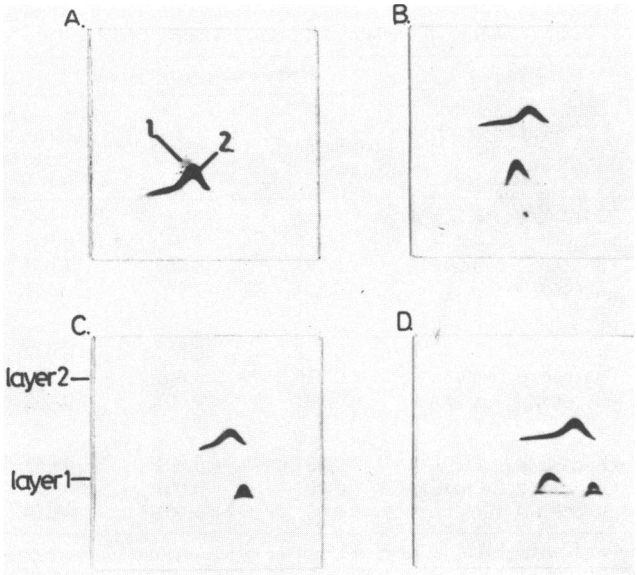

FIG. 1. Analysis of membranes of *S. typhimurium* LT2 (Z) with antibodies raised to *E. coli* hydrogenases 1 and 2. Crossed immunoelectrophoresis of Triton-X-100-dispersed membrane samples (40 μg of protein) from cells grown with glucose plus formate. Immunoplates were stained to detect hydrogenase activity. (A) Membranes from LT2 (Z) against anti-total *E. coli* membrane vesicle serum (200 μl). (B) Membranes from LT2 (Z) against anti-*E. coli* hydrogenase 1 immunoglobulin fraction (10 μl; layer 1); anti-*E. coli* hydrogenase 2 immunoglobulin fraction (10 μl; layer 2). (C) Membranes from *E. coli* (P4X) analyzed with the same antibody samples as in panel B. (D) Membranes from *E. coli* (P4X) (20 μg of protein) and *S. typhimurium* LT2 (Z) (20 μg of protein) were mixed and electrophoresed against the same antibody samples as in panels B and C. Arcs 1 and 2 depict *S. typhimurium* hydrogenases 1 and 2, respectively.

a terminal electron acceptor. For growths with glycerol and fumarate, the glucose was replaced by glycerol (0.5% [wt/vol]) and sodium fumarate (0.5% [wt/vol]). The media were inoculated with 1 ml of an overnight anaerobic culture of cells and incubated at 37°C without shaking for 4 to 5 h, by which time the cultures had reached mid- to late-exponential phase. Growth in the presence of hydrogen and fumarate was carried out in the same medium, but with the glucose replaced by sodium fumarate (0.5% [wt/vol]), the casein hydrolysate increased (to 0.2% [wt/vol]), and the cells shaken under a 100% hydrogen atmosphere.

The cells were harvested by centrifugation at 5,000 × *g* for 15 min (4°C) and washed once in 100 mM potassium phosphate buffer (pH 6.8). The cells were either stored as pellets at −80°C or resuspended in the above buffer for immediate enzyme assay. The bacterial cells were broken in a French press, and the soluble (cytoplasmic) and particulate (membrane) fractions were prepared as described previously (2) except that sodium dithionite was omitted from the buffers. Membrane samples were dispersed by suspending them (10 mg of protein ml^{-1}) in 50 mM Tris hydrochloride (pH 7.5) containing 4% (wt/vol) Triton X-100 and incubating them on ice for 15 min. Triton X-100-insoluble material was subsequently removed by sedimentation at 145,000 × *g* for 20 min.

Enzyme assays. All enzyme assays were performed at 25°C. Hydrogenase was measured spectrophotometrically by the H_2-dependent reduction of benzyl viologen as described previously (2). A unit of activity represents 1 μmol of benzyl viologen reduced per min. FDH-BV activity was monitored spectrophotometrically at 600 nm as the formate-dependent reduction of benzyl viologen (25). A unit of activity represents 1 μmol of formate oxidized per min. Fumarate reductase activity (reduced benzyl viologen:fumarate oxidoreductase) was assayed spectrophotometrically by monitoring fumarate-dependent oxidation of reduced benzyl viologen (8). A unit of activity represents 1 μmol of fumarate reduced per min. Formate hydrogenlyase was assayed polarographically as formate-dependent hydrogen evolution using a Clark-type electrode modified for H_2 measurement (25). A unit of activity represents 1 μmol of H_2 evolved per min. H_2 oxidation, also measured by using an H_2 electrode, was monitored as fumarate-dependent H_2 uptake (25). A unit of activity represents 1 μmol of H_2 consumed per min. All measurements using the H_2 electrode required the reaction chamber of the vessel (Hansatech Ltd.) to be free of oxygen. Protein was measured by the method of Lowry et al. (18).

Immunoelectrophoresis. Crossed immunoelectrophoresis was performed essentially as described previously (2). Triton-X-100-solubilized membrane samples (10 mg of protein ml^{-1}) were centrifuged (145,000 × *g* for 20 min) to remove insoluble material, and the supernatants were analyzed directly. Samples (about 40 μg of protein) were placed in a small circular well cut near a corner of a 1% (wt/vol) agarose gel (5 by 5 by 0.15 cm), made up in 20 mM barbital hydrochloride (pH 8.6) containing 1% (wt/vol) Triton X-100, and electrophoresed in the first dimension at 4.5 mA per gel for 1.5 h. Electrophoresis in the second dimension, at right angles to the first, was overnight at 2.5 mA per gel into an agarose layer (identical in composition to that described above) containing the appropriate antiserum (18 μl per gel). Alternatively, the antiserum-containing agarose for the second dimension was divided into two equal layers containing different specific antisera. Rocket immunoelectrophoresis (12) was performed as described above with a single antibody layer, omitting the first dimension and placing a series of wells directly in the antibody-containing agarose near the edge of the gel and using the hydrogenase activity stain described previously (2). The plates were immersed in 100 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM benzyl viologen (Sigma Chemical Co.) and 1 mM triphenyltetrazolium chloride (purchased from BDH) and incubated under a hydrogen atmosphere for up to 24 h. Antisera raised to purified *E. coli* hydrogenase isoenzymes 1 and 2 were described previously (3, 26). For certain experiments, antiserum was used which had been raised to the entire membrane fraction prepared from *E. coli* cells grown anaerobically on glucose-containing medium.

Polyacrylamide gel electrophoresis. Nondenaturing polyacrylamide (5.5% [wt/vol]) gel electrophoresis was performed in the presence of 1% (wt/vol) Triton X-100 by using the neutral pH system described earlier (2). After electrophoresis in sodium dodecyl sulfate-polyacrylamide gels, Western immunoblot analysis was performed exactly as described previously (26).

Immunoprecipitation. The quantitation of H_2:benzyl viologen oxidoreductase activity catalyzed by each specific hydrogenase isoenzyme was accomplished by assaying the activity removed after immunoprecipitation with antiserum specific for either hydrogenase isoenzyme 1 or isoenzyme 2. An excess of *E. coli* hydrogenase isoenzyme 1 antiserum (40 μl), of *E. coli* hydrogenase isoenzyme 2 antiserum (40 μl), or of a mixture of both (40 μl of each) was added to aliquots of Triton-X-100-dispersed membranes (500 μg of protein) in 50 mM Tris hydrochloride (pH 7.5). The mixtures were incu-

bated on ice for 20 min, after which time an excess (80 μl) of *Staphylococcus* protein A preparation was added (25); the mixtures were vortexed and placed on ice for a further 20 min before centrifugation (5,000 × *g* for 15 min) to remove the immune complexes and immunoglobulins. The supernatants were assayed for hydrogenase activity. As for the *E. coli* enzymes, neither antiserum nor protein A caused any inhibition of hydrogenase activity and no activity was lost in control incubations performed without antibody addition (25). Hydrogenase 1 and 2 activities were taken as the activity precipitated from the supernatant by the respective antiserum. Hydrogenase 3 (isoenzyme 1 and 2 independent) activity was taken as that remaining in the supernatant after immunoprecipitation with a mixture of antibodies to isoenzymes 1 and 2. Quantitative rocket immunoelectrophoresis confirmed that hydrogenase 1 and hydrogenase 2 antigens were absent from the supernatant after immunoprecipitation with their respective antibodies.

RESULTS

Immunochemical characterization of hydrogenase isoenzymes from *S. typhimurium* LT2 (Z). A preliminary characterization of the hydrogenase activity of *S. typhimurium* membranes showed that the specific activity (about 2.5 μg/mg of protein) was about two- to threefold higher than that of *E. coli*. As expected, over 85% of the total hydrogenase activity was membrane associated.

Antibodies were raised against preparations of hydrogenases 1 and 2 purified from *E. coli* (3, 26). To determine whether the hydrogenase isoenzyme complement of *S. typhimurium* LT2 (Z) is similar to that of *E. coli*, membrane preparations of *S. typhimurium* were examined by crossed immunoelectrophoresis by using antisera raised against the *E. coli* enzymes (Fig. 1). Two distinct precipitin arcs which stained for hydrogenase activity were observed when membranes from LT2 (Z) were electrophoresed against anti-*E. coli* membrane vesicle antiserum (Fig. 1A). One of these antigens cross-reacted specifically with antiserum against *E. coli* hydrogenase 1, and the other cross-reacted with antiserum against hydrogenase 2 (Fig. 1B). Thus, *S. typhimurium* appears to have two hydrogenase isoenzymes immunologically equivalent to hydrogenases 1 and 2 of *E. coli*. Mixing membrane samples from LT2 (Z) and *E. coli* (P4X) showed that the electrophoretic mobility of the hydrogenase isoenzyme 2 equivalent from *S. typhimurium* was identical to that of *E. coli* hydrogenase 2. However, the *S. typhimurium* antigen which cross-reacted with *E. coli* anti-hydrogenase 1 serum had a somewhat slower electrophoretic mobility than that of *E. coli* hydrogenase 1 (Fig. 1D).

To further characterize the hydrogenases of *S. typhimurium*, samples of detergent-dispersed membranes of LT2 (Z) were electrophoresed on nondenaturing polyacrylamide gels and stained for hydrogenase activity (Fig. 2). Membranes of *S. typhimurium* had a band which corresponded in staining intensity and mobility to hydrogenase 2 of *E. coli* (Fig. 2, lanes a and b). A Western immunoblot analysis of this gel by using anti-*E. coli* hydrogenase 2 showed that this band was immunologically related to hydrogenase 2 of *E. coli* (Fig. 2, lanes e and f). *S. typhimurium* membranes showed no activity band with the same electrophoretic mobility as *E. coli* hydrogenase 1, but they did have an activity band which migrated somewhat more slowly. Western immunoblotting showed that this slower migrating band cross-reacted specifically with anti-hydrogenase 1 antibodies from *E. coli* (Fig. 2, lanes c and d). It is, therefore, clear that *S. typhimurium* LT2 (Z) possesses hydrogenase isoenzymes equivalent to hydrogenases 1 and 2 of *E. coli*.

Sodium dodecyl sulfate-polyacrylamide gel electrophore-

TABLE 1. Variation in hydrogenase isoenzyme activity among *S. typhimurium* isolates LT2 (Z), LT2 (A), and LT7

Strain and growth condition[a]	Hydrogenase sp act (U mg^{-1})[b]		
	Isoenzyme 1	Isoenzyme 2	Nonimmunoprecipitable hydrogenase activity
LT2 (Z)			
Glucose	0.156	0.84	0.967
Glucose + formate	0.245	0.367	1.662
Glycerol + fumarate	<0.001	1.30	0.144
LT2 (A)			
Glucose	0.112	0.017	0.472
Glucose + formate	0.235	0.005	1.225
Glycerol + fumarate	<0.001	<0.001	0.025
LT7			
Glucose	<0.001	<0.001	0.252
Glucose + formate	<0.001	<0.001	0.701
Glycerol + fumarate	<0.001	<0.001	0.059

[a] Cells were grown and harvested, and the membrane fractions were made as described in Materials and Methods. The Triton-X-100-dispersed membranes (10 mg of protein per ml) were challenged with antisera raised to the purified *E. coli* hydrogenase isoenzymes 1 and 2 and the activity associated with each, and the nonassignable activity was determined as described in Materials and Methods.

[b] H_2:benzyl viologen oxidoreductase is expressed as units per milligram of protein present in the Triton-X-100-dispersed membranes.

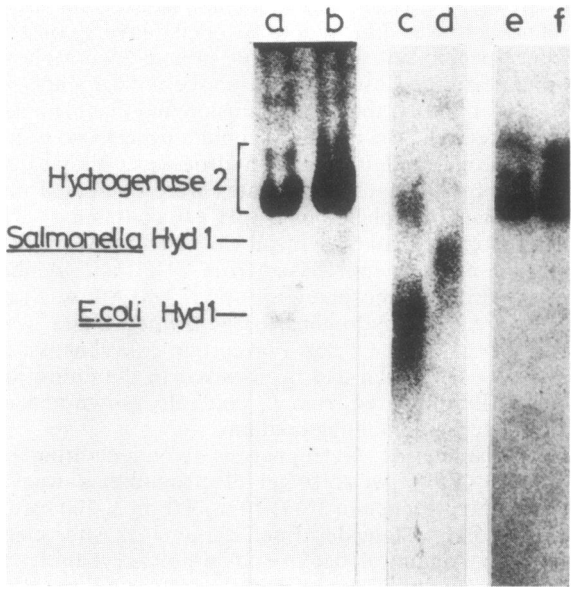

FIG. 2. Electrophoretic analysis of *S. typhimurium* LT2 (Z) hydrogenases. Nondenaturing 5.5% (wt/vol) polyacrylamide gel electrophoresis of Triton-X-100-dispersed membrane samples (100 μg of protein). Lanes: a, *E. coli* (P4X) membrane fraction prepared from a culture grown anaerobically with glucose plus formate. The gel was stained to detect hydrogenase activity. b, As for lane a, but with *S. typhimurium* LT2 (Z) membranes. c, As for lane a, but the gel was analyzed by Western immunoblot analysis with antiserum raised to *E. coli* hydrogenase isoenzyme 1 (autoradiograph). d, As for lane b, but analyzed as in lane c. e, As for lane c, but with antiserum raised to *E. coli* hydrogenase isoenzyme 2. f, As for lane d, but with antiserum raised to *E. coli* hydrogenase isoenzyme 2.

TABLE 2. H_2 metabolism of intact cells of *S. typhimurium* LT2 (Z) grown under various conditions

Growth condition[a]	Sp act (U mg^{-1})				
	Formate hydrogenlyase	Hydrogenase[c]	FDH-BV	Hydrogen uptake[d]	Fumarate reductase
Glucose	0.447	0.141	0.063	0.050	0.097
Glucose + formate	0.588	0.048	0.114	0.049	0.133
Glucose + nitrate	0.014	<0.001	<0.001	<0.001	0.006
Glycerol + fumarate	0.016	0.080	0.015	0.081	0.247
Hydrogen + fumarate	<0.001	0.029	<0.001	0.171	0.088

[a] Cells were harvested, washed, suspended in 400 mM potassium phosphate buffer (pH 6.8), and assayed immediately.
[b] For unit definitions, see Materials and Methods.
[c] Hydrogenase (H_2:benzyl viologen oxidoreductase).
[d] Fumarate-dependent H_2 uptake.

sis of *S. typhimurium* membranes followed by immunoblotting with the appropriate *E. coli* anti-hydrogenase antibodies showed that, like their *E. coli* counterparts (3, 26), the *S. typhimurium* hydrogenases each consist of two subunits of molecular weights of about 60,000 and 35,000 (data not shown). The electrophoretic mobilities on sodium dodecyl sulfate gels of the larger subunits of the two *S. typhimurium* isoenzymes were distinct from one another but indistinguishable from those of their *E. coli* counterparts, corresponding to M_rs of 61,000 and 63,000 for isoenzymes 1 and 2, respectively. However, the 35,000-dalton subunit of *S. typhimurium* hydrogenase 1 appeared somewhat heterogeneous. This may account for the observed differences in the electrophoretic mobilities on native gels between hydrogenase 1 from *E. coli* and the equivalent isoenzyme from *S. typhimurium*.

***S. typhimurium* possesses hydrogenase activity distinct from hydrogenases 1 and 2.** We have previously shown that membranes from anaerobically grown *E. coli* cells possess a hydrogenase activity which cannot be precipitated by antibodies against either hydrogenase 1 or 2 (25). This activity, designated hydrogenase 3, was also present in membrane fractions of anaerobically grown cells of *S. typhimurium* LT2 (Z) (Table 1). Hydrogenase 3 activity constituted a high proportion of total hydrogenase activity, particularly in cells grown in the presence of formate. We were unable to correlate this activity with an immunoprecipitable antigen by using antisera against total *E. coli* membrane preparations or as a hydrogenase activity-staining band on nondenaturing polyacrylamide gels. This was also the case for the *E. coli* enzyme(s) and probably reflects an instability of this activity during analysis. Interestingly, the nonimmunoprecipitable hydrogenase (hydrogenase 3) had a greater specific activity in *S. typhimurium* than in *E. coli*.

It is clear from the above data that *S. typhimurium* possesses three hydrogenase isoenzymes analogous to those present in *E. coli*. These three enzymes will be referred to as *S. typhimurium* hydrogenase isoenzyme 1, isoenzyme 2, and nonimmunoprecipitable hydrogenase activity (hydrogenase 3).

Expression of hydrogenase isoenzymes of *S. typhimurium*. The expression of the hydrogenase isoenzymes of *E. coli* is greatly influenced by growth conditions, as are the hydrogen uptake and evolution capacities of the whole cell (25). *S. typhimurium* exhibited a similar regulation of its H_2 uptake and H_2 evolution activities (Table 2). H_2 evolution occurred during fermentative growth and was enhanced if the cells were grown in the presence of formate. However, H_2 evolution was virtually absent if a terminal respiratory electron acceptor was present. As was noted previously for *E. coli*, growth with nitrate led to the virtual abolition of all H_2-linked functions. H_2 uptake (fumarate-dependent H_2 uptake) was detected under all growth conditions tested (except with nitrate) but, as anticipated, was greatest during H_2-dependent growth.

To attempt to correlate these hydrogenase activities with the various hydrogenase isoenzymes, the changes in the cellular contents of isoenzyme 1 and 2 antigens under these various growth conditions were analyzed by rocket immunoelectrophoresis (Fig. 3A and B). Growth with formate led to an increase in hydrogenase 1, but this isoenzyme was virtually absent from cells grown in the presence of a terminal respiratory electron acceptor. In contrast, isoenzyme 2 was present during growth under all conditions examined except when cells were grown in the presence of nitrate. A quantitative assessment of the activities of each of the hydrogenase isoenzymes was made by immunoprecipitation by using the antisera raised against isoenzymes 1 and

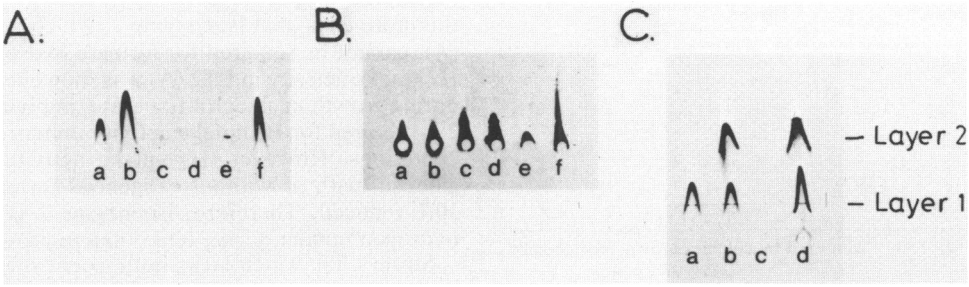

FIG. 3. Expression of hydrogenase isoenzymes 1 and 2 of *S. typhimurium*. (A) Rocket immunoelectrophoresis of Triton-X-100-dispersed membranes (40 μg of protein) of *S. typhimurium* LT2 (Z) with antibodies raised to *E. coli* hydrogenase isoenzyme 1 (stained to reveal hydrogenase activity). Membrane samples were obtained after anaerobic growth with the following additions. Lanes: a, glucose; b, glucose plus formate; c, glycerol plus fumarate; d, H_2 plus fumarate; e, glucose plus nitrate; f, *E. coli* (P4X) grown with glucose. (B) As for panel A, but with antibodies raised to *E. coli* hydrogenase isoenzyme 2. (C) Rocket immunoelectrophoresis of Triton-X-100-dispersed membrane samples (40 μg of protein) electrophoresed into agarose containing 20 μl of anti-*E. coli* hydrogenase 1 immunoglobulin (layer 1) and 20 μl of anti-*E. coli* hydrogenase 2 immunoglobulin (layer 2). The immunoplate was stained to reveal hydrogenase activity. Membrane samples were obtained after anaerobic growth with glucose from the following strains. Lanes: a, *S. typhimurium* LT2 (A); b, *S. typhimurium* LT2 (Z); c, *S. typhimurium* LT7; d, *E. coli* (P4X).

2. For the growth conditions examined, the variation in isoenzyme 1 and 2 activities closely followed the variation in the cellular content of the respective antigens. Thus, regulation of hydrogenase activity seemed to be at the level of transcription/translation rather than by the direct modulation of enzyme activity. The nonimmunoprecipitable hydrogenase activity (hydrogenase 3) was found to correlate closely with cellular formate hydrogenlyase activity, being greatest in cells grown with formate and very much reduced after growth with glycerol and fumarate. The pattern of expression of the three *S. typhimurium* isoenzymes is very similar to that found for their *E. coli* counterparts (25), further substantiating their structural and functional equivalence.

Hydrogenase isoenzyme content of *S. typhimurium* strains LT2 (A) and LT7. We examined the hydrogenase isoenzyme contents of two further *S. typhimurium* wild-type isolates. Strain LT2 (A) had no detectable isoenzyme 2 when analyzed by rocket immunoelectrophoresis but had normal levels of isoenzyme 1 (Fig. 3C, lane a). Isolate LT7, however, lacked both isoenzymes 1 and 2 (Fig. 3C, lane c). Both LT2 (A) and LT7, however, showed apparently normal hydrogenase 3 activity (Table 1). Native polyacrylamide gel analysis confirmed these conclusions (data not shown). Immunoprecipitation showed that inactive antigens corresponding to the missing isoenzymes were absent from the respective strains. Thus, the defects were due to a failure to synthesize the relevant proteins (data not shown). Quantitative immunoprecipitation confirmed the absence of isoenzyme 2 from LT2 (A) and of both isoenzymes 1 and 2 from LT7 under all growth conditions examined (Table 1).

Extensive analysis of LT2 (A) and LT7 failed to reveal any additional defects in anaerobic metabolism (14; Table 3). Apart from a reduced response to the formate:benzyl viologen overlay test (20) for cellular formate-dependent benzyl viologen reduction on agar plates (Table 3), LT2 (A) and LT7 appeared indistinguishable from LT2 (Z). LT2 (A) and LT7 are readily distinguishable from the pleiotropic hydrogenase mutants previously described (4, 17, 28), which lack all hydrogenase activity. Furthermore, despite some similarities among the hydrogenase phenotypes of LT7 (lacking both hydrogenases 1 and 2) and those of *fnr* (*oxrA*) strains of *E. coli* (25) and *S. typhimurium* (14), LT7 is otherwise distinguishable from *fnr* strains by its growth patterns on glycerol-nitrate and glycerol-fumarate media (16; Table 3). Thus, LT2 (A) and LT7 contain specific defects in hydrogenase synthesis rather than in any of the known pleiotropic anaerobic regulatory loci. These strains provide the first available mutants specifically defective in one or two rather than all three of the hydrogenase isoenzymes.

TABLE 3. Characterization of the phenotypes of *S. typhimurium* isolates LT2 (Z), LT2 (A), and LT7

Strain	Nitrate reductase[a]	Respiratory formate dehydrogenase[b]	FDH-BV[c]	Growth on glycerol and nitrate[d]	Growth on glycerol and fumarate[e]	Gas[f]	LB-BV[g]
LT2 (Z)	+	+	+ +	+	+	+	−
LT2 (A)	+	+	+	+	+	+	−
LT7	+	+	+	+	+	+	−
P4X	+	+	+ +	+	+	+	−
DB10	+	+	−	+	+	−	+

[a] Reduction of NO_3^- to NO_2^{-1} catalyzed by colonies grown anaerobically on LB plates (10).
[b] Phenazine methosulphate-mediated reduction of triphenyl tetrazolium chloride by formate, catalyzed by colonies grown anaerobically on LB plates (5).
[c] Ability to catalyze reduction of oxidized benzyl viologen by formate after anaerobic growth on LB plates (20).
[d] Ability to grow anaerobically on minimal agar containing glycerol and nitrate (16).
[e] As for footnote *d*, but containing glycerol and fumarate (16).
[f] Production of gas during anaerobic growth on LB medium (21).
[g] Ability to grow anaerobically and form white colonies on LB plates containing benzyl viologen (17, 28).

TABLE 4. H_2 metabolism of intact cells of *S. typhimurium* isolates LT2 (Z), LT2 (A), and LT7 grown under various conditions

Growth condition and strain[a]	Sp act (U mg^{-1})[b]				
	Formate hydrogenlyase	Hydrogenase[c]	FDH-BV	Hydrogen uptake[d]	Fumarate reductase
Glucose					
LT2 (Z)	0.611	0.212	0.094	0.078	0.135
LT2 (A)	0.330	0.205	0.112	0.048	0.126
LT7	0.339	0.020	0.115	0.005	0.126
Glucose + Formate					
LT2 (Z)	0.733	0.330	0.110	0.066	0.114
LT2 (A)	0.771	0.203	0.130	0.044	0.085
LT7	0.300	0.016	0.112	<0.001	0.130
Glycerol + Fumarate					
LT2 (Z)	0.001	0.418	<0.001	0.131	0.221
LT2 (A)	0.002	0.001	<0.001	0.007	0.192
LT7	<0.001	<0.001	<0.001	0.025	0.059

[a] Cells were harvested at late exponential phase, washed once, and suspended in 100 mM potassium phosphate. Assays were performed immediately.
[b] For unit definitions, see Materials and Methods.
[c] H_2:benzyl viologen oxidoreductase.
[d] Fumarate-dependent H_2 uptake.

H_2 metabolism by *S. typhimurium* strains LT2 (A) and LT7. With the surprising finding that various wild-type *S. typhimurium* strains specifically lack particular hydrogenase isoenzymes, we examined the H_2-related activities of these strains in order to clarify the physiological roles of the various isoenzymes. Assay of the formate hydrogenlyase activity of LT2 (A) revealed that it was similar to that of LT2 (Z) under all growth conditions examined (Table 4). It is therefore clear that isoenzyme 2, which is absent from LT2 (A), cannot be required for formate hydrogenlyase activity. H_2 uptake activity in LT2 (A) was, however, greatly reduced during growth in glycerol-fumerate, implying that isoenzyme 2 is required for H_2 uptake activity under respiratory growth conditions. However, H_2 uptake activity after growth on glucose, with or without exogenous formate, is only some 30% reduced. Therefore, isoenzyme 2 is not essential for hydrogen uptake during fermentative growth.

Strain LT7, which lacks both isoenzymes 1 and 2, exhibited near normal [i.e., LT2 (Z)] levels of formate hydrogenlyase activity (Table 4). Although isoenzyme 1, like cellular formate hydrogenlyase activity, is induced by formate, this isoenzyme clearly cannot function in the formate hydrogenlyase pathway. However, H_2 uptake under fermentative growth conditions was virtually absent in LT7. Isoenzyme 1 must therefore be responsible for H_2 uptake during fermentative growth. The nonimmunoprecipitable hydrogenase activity (hydrogenase 3) was unimpaired in

both LT2 (A), and LT7 and its activity correlated closely with formate hydrogenlyase activity. Thus, all or part of this activity must be required for fermentative, formate hydrogenlyase-dependent H_2 evolution.

DISCUSSION

E. coli possesses three hydrogenase isoenzymes (25). Two of these enzymes, hydrogenase 1 and hydrogenase 2, have been purified and characterized at a molecular level (3, 26). The third hydrogenase isoenzyme, defined by that activity which is immunologically distinct from isoenzymes 1 and 2, is somewhat enigmatic, since it has not been identified after electrophoretic or immunoelectrophoretic analysis (25). Presumably, this reflects its instability under the conditions of analysis. Its molecular nature is therefore unknown, and the activity may in fact be catalyzed by more than one enzyme. Both hydrogenases 1 and 2 are Ni-containing enzymes (3, 26); indirect evidence suggests that hydrogenase 3 activity is also Ni dependent (28). Unfortunately, the absence of *E. coli* mutants possessing specific defects in the individual hydrogenase isoenzymes has precluded the establishment of their function in this species. In this study we describe the characterization of the hydrogenase isoenzymes of *S. typhimurium*. This species possesses a similar complement of membrane-bound hydrogenase isoenzymes to that of *E. coli*. The enzymes of the two species are immunologically related, and their synthesis is similarly regulated (14). The somewhat higher hydrogenase activity of *S. typhimurium* LT2 (Z) membranes compared with that of *E. coli* membranes appears to be due to an increased content of hydrogenase 3. It is therefore clear that, although respiratory hydrogen uptake and formate-hydrogenlyase-dependent hydrogen evolution are the only two modes of hydrogen metabolism in these two species, both *E. coli* and *S. typhimurium* possess a minimum of three distinct hydrogenase isoenzymes. This raises the question of which isoenzyme is responsible for which aspect of H_2 metabolism. The most important aspect of this study is the somewhat surprising finding that various wild-type isolates of *S. typhimurium* possess specific defects in their hydrogenase isoenzyme contents. This has enabled us to establish the physiological role of each of the three isoenzymes. The role ascribed to each of the *S. typhimurium* isoenzymes is entirely consistent with the correlations which have been made previously between isoenzyme expression and hydrogen metabolism in *E. coli* (25) and is also consistent with the anaerobic regulation of their expression (14).

S. typhimurium LT2 (Z), like *E. coli*, contains all three hydrogenases. However, strain LT2 (A) was found to lack hydrogenase 2 under all growth conditions, whereas LT7 lacks both hydrogenases 1 and 2. Both strains have essentially normal hydrogenase 3 activity. An analysis of the effects of various anaerobic regulatory mutations on hydrogenase expression (14), the finding that the hydrogenase activities in these isolates are absent under all growth conditions, and the absence of any other obvious defects in anaerobic metabolism suggest that the lesions in these strains are in the hydrogenase structural genes or, alternatively, in previously unidentified regulatory loci specific for the hydrogenases.

The absence of isoenzyme 2 from strain LT2 (A) led to the complete loss of respiration-dependent hydrogen uptake. Thus, hydrogenase 2 must catalyze hydrogen uptake, presumably reducing the quinone pool in an energy-conserving manner (15). On the other hand, both LT2 (A) and LT7 showed normal hydrogen evolution. Thus, hydrogenases 1 and 2 cannot play a significant role in H_2 evolution; hydrogenase 3 activity must be responsible for the hydrogenase component of the formate hydrogen lyase pathway.

The role of hydrogenase 1 is less straightforward. A comparison of hydrogen metabolism in strains LT2 (A) (lacking isoenzyme 2) and LT7 (lacking both isoenzymes 1 and 2) indicated that isoenzyme 1 catalyzed hydrogen uptake but only during fermentative growth. Our data also indicated that isoenzyme 2, although present during fermentative growth, did not contribute significantly to hydrogen uptake when cells were growing fermentatively. Presumably, an unidentified component of the isoenzyme-2-dependent hydrogen uptake mechanism was absent under these growth conditions. Thus, hydrogenases 1 and 2 both catalyze H_2 uptake but function under different growth conditions; hydrogenase 2 catalyzes respiration-linked hydrogen uptake, whereas hydrogenase 1 functions only under fermentative conditions in the absence of an exogenous terminal electron acceptor. Interestingly, expression of hydrogenase 1, like that of formate hydrogenlyase and unlike that of hydrogenase 2, is induced under fermentative growth conditions and is formate dependent (14). Thus, we propose that hydrogenase 1 functions to recycle hydrogen produced by formate hydrogenlyase during fermentative growth. Hydrogen uptake under these growth conditions would be dependent on the availability of endogenously generated terminal respiratory electron acceptors (i.e., fumarate). If this hydrogenase-1-catalyzed H_2 uptake was energy conserving, then clearly such a system of hydrogen recycling would be beneficial for growth. Similar schemes involving hydrogen recycling were proposed for both N_2-fixing (27) and sulfidogenic bacteria (22). It should be pointed out that, although we ascribe hydrogen uptake roles to both hydrogenases 1 and 2, neither has been shown directly to catalyze the hydrogen-dependent reduction of quinone (3, 26). Indeed, purified preparations (both proteolytic derivatives) of these isoenzymes are unable to use quinone as an oxidizing substrate. Clearly, this point requires careful examination in the future. Paradoxically, both the formate dehydrogenase and the hydrogenase components of formate hydrogenlyase presently lack even preliminary molecular characterization.

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