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Isolation and Characterization of Mutant Strains of *Escherichia coli* Altered in H_2 Metabolism†

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A positive selection procedure is described for the isolation of hydrogenase-defective mutant strains of *Escherichia coli*. Mutant strains isolated by this procedure can be divided into two major classes. Class I mutants produced hydrogenase activity (determined by using a tritium-exchange assay) and formate hydrogenlyase activity but lacked the ability to reduce benzyl viologen or fumarate with H_2 as the electron donor. Class II mutants failed to produce active hydrogenase and hydrogenase-dependent activities. All the mutant strains produced detectable levels of formate dehydrogenase-1 and -2 and fumarate reductase. The mutation in class I mutants mapped near 65 min of the *E. coli* chromosome, whereas the mutation in class II mutants mapped between *srl* and *cys* operons (58 and 59 min, respectively) in the genome. The class II Hyd mutants can be further subdivided into two groups (*hydA* and *hydB*) based on the cotransduction characteristics with *cys* and *srl*. These results indicate that there are two *hyd* operons and one *hup* operon in the *E. coli* chromosome. The two *hyd* operons are needed for the production of active hydrogenase, and all three are essential for hydrogen-dependent growth of the cell.

In 1931, Stephenson and Stickland (36) proposed the name hydrogenase for the enzyme that catalyzes the reversible oxidation of H_2 to protons and electrons. Since that time, hydrogenase activity has been demonstrated in a diverse group of microorganisms (39), including both aerobic and anaerobic bacteria, as well as algae and protozoa.

Hydrogen gas plays a major role in the metabolism of anaerobic bacteria which evolve H_2 as an end product during fermentation. Hydrogen also serves as a source of reductant in the production of CH_4 by archebacteria (5). Reutilization of H_2 in nitrogen-fixing organisms is also known to enhance the energy efficiency of the nitrogen fixation process (13). In spite of the importance of hydrogenase in bacterial metabolism and its potential use in biotechnology for fuel production (23) and nitrogen fixation (13), very little is known about the molecular biology of H_2 metabolism, even in a well-studied organism like *Escherichia coli*.

Pascal and co-workers described the isolation and characterization of a hydrogenase-defective mutant strain of both *E. coli* and *Salmonella typhimurium* (31). These investigators utilized a dye-overlay procedure to identify mutant strains with defects in hydrogenase activity. Using similar procedures, other investigators also isolated mutant strains of *E. coli* with defects in hydrogenase activity (15, 16, 21, 25, 32). The major problem in the analysis of the H_2 metabolism with biochemical genetic tools is the inability to generate a large number of mutants by the dye-overlay procedure initially described by Pascal et al. (31).

To study the molecular biology of H_2 metabolism, we developed a positive selection procedure for the isolation of hydrogenase-defective mutants. Preliminary characteristics on physiological properties and reversion frequencies of these mutants have been presented previously (37). In this and in the accompanying report by Sankar et al. (34), the selection procedure and complete biochemical and genetic characteristics of the mutant strains altered in their H_2 metabolism are presented.

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MATERIALS AND METHODS

Abbreviations. H_2-fumarate (HF) medium, a defined medium used to test the ability of *E. coli* to grow under anaerobic conditions utilizing H_2 as electron donor and fumarate as electron acceptor. HUP, ability to grow in the HF medium. HUP activity, rate of H_2 utilization with fumarate as electron acceptor. Hup, H_2 uptake phenotype (Hup^+ and Hup^- represent the wild-type and mutant phenotypes, respectively); *hup*$^+$, gene(s) essential for hydrogen uptake activity; this includes hydrogenase as well as other electron carriers required for H_2 uptake. Because the gene symbol *hyd* is used for hydrogenase, *hup* is used in this study to designate the electron transport proteins only. *fdv*, a gene coding for formate dehydrogenase (FDH) activity that couples formate oxidation to reduction of artificial electron acceptor, benzyl viologen (BV). This formate dehydrogenase (FDH-2) is a component of the formate hydrogenlyase (FHL) enzyme complex. *fhl* is used to designate genes, the products of which are essential for FHL activity besides the *hyd* and *fdv* gene products.

Bacterial strains and plasmids. All strains are derivatives of *E. coli* K-12 and are listed in Table 1.

Media and growth conditions. Luria broth was prepared as described previously (30). Glucose minimal medium had the following composition: Na_2HPO_4, 6.25 g; KH_2PO_4, 0.75 g; NaCl, 2.00 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $NaMoO_4 \cdot 2H_2O$, 0.01 g; Na_2SeO_3, 0.263 mg in 1 liter of deionized water. The final pH of the medium was 7.5. Glucose was present at a final concentration of 0.3% for aerobic cultures and 1.5% for anaerobic cultures. The HF medium was the same as described by Bernhard and Gottschalk (7). Solid medium contained 15 g of agar per liter of medium.

For growth under H_2, bacterial cultures were spread on the surface of HF medium in petri dishes, and the plates were placed in a vacuum desiccator. The gas phase was removed and replaced with H_2, and the plates were incubated at room temperature. Anaerobic conditions were established in the desiccator within 18 to 24 h, as determined

TABLE 1. Bacterial strains used in this study

Strain	Genotype or phenotype	Source or selection
K-10	Hfr PO2A *relA1 pit-10 tonA22* $T2^r$ λ^+ *spoT*	L. Csonka
Puig 382	*thi-1 thr-1 leu-6 argH1 his-1 pro-33 purE43 lacY1 mtl-2 xyl-7 malA1 ara-13 gal-6 rpsL9 chlA1 tonA2* λ^r λ^- *supE44* (?)	B. Bachmann CGSC[a] 4442
Puig 426	*thi-1 leu-6 suc-10 bioA2* (?) *galT27 rpsL129 chlC3* λ^-	B. Bachmann CGSC 4444
JC10240	Hfr PO45 *srl-300*::Tn*10 recA56 thr-300 ilv-318 thi-1 rpsE-300*	L. Csonka
JC10244	*cysC43 alaS3 srl-300*::Tn*10 thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 rpsL31 tsx-33 supE44*	L. Csonka
BE-117	*leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE-3 str-31 tsx-33 sup-37 recB-21 recC22 sbcB15 hsdS*	R. N. Rao
JM81A	*cysC92 tfr-8* (?)	B. Bachmann
FEJ-1	Hfr PO13 *thi-1 leuB6 lacZ4 srl-1 mtlA9 rpsL8 supE44*	B. Bachmann
LCB850	*thr-1 leu-6 lacY1 tonA21 hyd str supE44*	M. Pascal
H61	*leu thi pro* Str^r *lacY hsdR hsdM hyd*	W. G. Martin
SE-1 to SE-7	Puig 426::Tn*10 hyd-101* to *hyd107*, respectively	This study
SE-8	Puig 426::Tn*10 hup101*	This study
SE-9	JC10244 - *hyd-108* FDH-2$^-$	This study
SE-10	SE-9 - Tc^s	Spontaneous
SE-15	JC10244 - Tc^s	Spontaneous
SE-16	SE-1 - Tc^s	Spontaneous
SE-19	SE-16 - *srl-300*::Tn*10 recA56*	P1(JC10240)
SE-20	SE-10 - *srl-300*::Tn*10 recA56*	P1(JC10240)
SE-24	Puig 382 - *hyd-111*::Tn*10* (Ap *lac*)	This study
SE-31	SE-15 - *hup-102*	This study
SE-32	SE-31 - *srl-300*::Tn*10 recA56*	P1(JC10240)
SE-37	SE-2 - Tc^s	Spontaneous
SE-40	SE-15 - *hup-103*	This study
SE-44	SE-10 - *srl*$^+$	P1(K-10)
SE-46	SE-40 - *alaS*$^+$	Spontaneous
SE-49	SE-46 - *srl-300*::Tn*10 recA56*	P1(JC-10240)
SE-61	SE-20 - *alaS*$^+$	Spontaneous
SE-62	SE-24 - *chlA*$^+$	Spontaneous
SE-1000	JC10244 - *alaS*$^+$	Spontaneous
F'116	*argG metC*	B. Bachmann
F'143-1	*lysA serA*	B. Bachmann

[a] CGSC, Coli Genetic Stock Center.

by anaerobic indicator strip (Gas Pak; BBL Microbiology Systems). Colonies were observed in about 5 days.

Genetic experiments. Complementation analysis with *E. coli* F' elements was carried out as described previously (30). Bacteriophage P1 cm *clr*100 was used in transduction experiments as described previously (30).

Preparation of cells and extracts for enzyme assays. Cells used for enzyme assays were grown in LB medium under anaerobic conditions. Aerobically grown cultures were used as inoculum (5% [vol/vol]) in these experiments, and the cultures were maintained under anaerobic conditions by completely filling the culture vessel. The cells were harvested after 4 h of incubation at 37°C (strains carrying *alaS* mutation were incubated at 30°C for 5 h) by centrifugation. For whole cell assays, the cells from 20 ml of culture were collected after centrifugation at 3,500 × *g* for 10 min at room temperature and washed once with 10 ml of wash buffer (NaK-PO_4 buffer [pH 7.0] 0.1 M containing 1 mM reduced glutathione and 100 μg of chloramphenicol per ml to prevent continued protein synthesis during the assay). The washed cells were maintained in ice before assay.

For preparation of extracts, cells from 1-liter cultures were centrifuged at 8,000 × *g* for 10 min at 4°C. The cells were washed once with 10 ml of wash buffer and centrifuged again at 12,000 × *g* (4°C) for 10 min. The cells were suspended in 1 ml of wash buffer and passed through a French pressure cell at 20,000 lb/in^2. The broken cell suspension was centrifuged at 20,000 × *g* for 20 min at 4°C, and the supernatant was collected. This crude extract was maintained in ice under an N_2 atmosphere before assay.

Protein was determined by previously described procedures (8, 12). Bovine serum albumin was used as the standard.

Hydrogenase. Hydrogenase activity was determined by two different methods, using whole cells. These include H_2-dependent reduction of BV, a method used by other investigators (31), and tritium exchange (3, 26). The tritium exchange reaction ($^3H_2 + H^+ + OH^- \rightleftharpoons {}^3H^+ + OH^- + {}^3H\text{-}H$) is independent of electron transport proteins (3) and provides an actual measure of the hydrogenase activity present in the cell.

For tritium exchange assay, 0.2 ml of cell suspension (50 to 100 μg of cell protein) was placed in a tube (12 by 75 mm), and the tube was sealed with a serum stopper. The gas phase was replaced with helium. Tritium gas (11.2 mCi/mmol; New England Nuclear Corp.) was added (25 μl) to a final concentration of 0.55 μCi per assay. After 1 h of incubation at room temperature, the serum stopper was removed, and the tritium gas was vented out in the hood for 10 min, after vigorous mixing of the tube contents. Tritiated water present in a 50-μl fraction was determined with a scintillation counter in Aquasol-2 scintillation fluid. Hydrogenase activity was expressed as nanomoles of tritiated water produced per hour per milligram of cell protein. Production of tritiated water from $^3H_2$ was linear with time over the entire assay period.

Hydrogenase and hydrogen uptake activities were also determined by monitoring the disappearance of H_2 from the gas phase by using a gas chromatograph in the presence of either BV or fumarate as electron acceptors. The assay mixture for these reactions contained NaK-PO_4 buffer (0.1 M; pH 7.0), BV or fumarate (50 mM), and cell suspension at a final concentration of 150 to 200 μg of cell protein (in a final volume of 1 ml) in a 5-ml wheaton vial (19). The gas phase was replaced with N_2, and H_2 was added to a final concentration of 10%. The amount of H_2 in the gas phase was determined at different time intervals with a Varian gas chromatograph (model 920). The activity was expressed as nanomoles of H_2 consumed per minute per milligram of cell protein.

FDH-1. The assay mixture for FDH-1 contained, in a final volume of 3 ml, phosphate buffer (pH 6.5, 0.33 M); sodium

TABLE 2. Isolation of mutants with defects in hydrogen metabolism

Parent strain	Relevant genotype	Total no. plated ($\times 10^8$)	No. of survivors	No. of survivors tested	No. Hup^-	No. of Hyd^-	Frequency of Hyd^-
K-10	Prototroph	2.8	8,000	100	100	16	5.0×10^{-6}
JC10244	*srl cysC*	1.4	5,500	108	108	25	9.0×10^{-6}
Puig 426	*chlC*	1.6	4,800	200	200	84	1.3×10^{-5}
BE-117	*recB recC*	3.6	150	36	36	5	5.8×10^{-8}
JC10240	*recA*	3.4	110	98	98	32	1.1×10^{-7}

formate (40 mM); phenazine methosulfate (98 μM); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue, MTT; 60 mM) and cell extract (19). The reaction was carried out at room temperature in an anaerobic cuvette under an N_2 atmosphere. The rate of formation of reduced MTT was monitored in a Spectronic 710 spectrophotometer at 560 nm. The amount of reduced formazan produced was calculated by using a molar extinction coefficient of 1.44×10^4 cm^{-1}.

FDH-2. The assay mixture for FDH-2 contained phosphate buffer (pH 7.0, 0.33 M); BV (6.5 mM); sodium formate (40 mM); and cell extract. The final volume was adjusted to 3.0 ml with deionized water (19). The reaction was carried out in an anaerobic cuvette at room temperature in an N_2 atmosphere. The rate of reduction of BV was monitored at 550 nm, and the amount of BV reduced was calculated by using a molar extinction coefficient of 7.78×10^3 cm^{-1}.

Fumarate reductase. The assay mixture for fumarate reductase contained phosphate buffer (pH 7.8, 92.5 mM); BV (350 μM); sodium fumarate (30 mM); and cell extract. The reaction volume was 20 ml (35). The reaction was carried out under an argon atmosphere in an anaerobic cuvette. The reaction was initiated by the reduction of BV by the addition of $S_2O_4^{2-}$ (to a reduced BV absorbance of 2.0 at 550 nm). The rate of oxidation of reduced BV was continuously monitored and converted to the amount of fumarate reduced by the enzyme (35).

FHL. The reaction mixture for FHL contained phosphate buffer (pH 6.5, 0.33 M); sodium formate (40 mM); and cell extract (19). The final volume was 1.0 ml. The reaction was carried out at room temperature with N_2 in the gas phase. The rate of production of H_2 was monitored with a gas chromatograph.

The enzyme activities were expressed as nanomoles of product produced or substrate oxidized per minute per milligram of protein.

Materials. Biochemicals were purchased from Sigma Chemical Co. Inorganic and organic chemicals were obtained from Fisher Scientific Co. and were analytical grade.

RESULTS

Isolation of mutants. The biochemical genetic analysis of metabolic pathways requires the availability of a large number of mutants. The dye-overlay method used previously (31) for the identification of mutant strains with alterations in H_2 metabolism is not specific and requires the analysis of a large number of survivors. We developed a positive selection procedure for the isolation of hydrogenase-defective mutant strains based on the observation by Hassan and Fridovich (18) that *E. coli*, as well as several other organisms, are sensitive to the presence of methyl viologen (paraquat) in the medium. The lethal effect of methyl viologen is presumably mediated through the generation of superoxide radical. These authors also observed that methyl viologen had no effect in an anaerobic culture of *E. coli*.

Whole cells of *E. coli* grown under anaerobic conditions are capable of reducing BV (an analog of methyl viologen) with H_2 as the reductant. Although the actual rates vary, the presence of this reaction in the cells is independent of growth medium composition.

Reduced BV can be oxidized by O_2 to yield semiquinone and the superoxide radical. The ability of reduced BV to generate the superoxide radical readily was the basis for the isolation of mutant strains with defects in H_2 metabolism. By appropriate manipulation of the medium and culture conditions, wild-type cells can be effectively killed, leaving only the mutant strains with defects in hydrogenase or BV reduction. For isolating mutant strains, portions of an actively growing LB culture of *E. coli* appropriately diluted to yield 100 to 200 survivors per plate were plated on LB medium supplemented with 0.5 mM BV. The plates were placed in a vacuum desiccator. An anaerobic indicator strip (Gas Pak) was also placed in the desiccator. The gas phase was replaced with H_2, and the plates were incubated at room temperature. The residual O_2 present in the desiccator after several gas exchanges with H_2 disappeared within 24 h, as indicated by the white color of the anaerobic indicator strip, which was probably a result of O_2 consumption by the inoculum. The plates were removed after 3 to 5 days, and the survivors were transferred to LB plates by replica plating techniques and incubated at 30 or 37°C, depending on the strain requirements. These clones were picked and tested further. Greater than 99% of these clones were found to be defective in H_2 metabolism (Table 2). The frequency at which the Hup^- mutants appeared was about 3×10^{-5} for rec^+ strains and about 3×10^{-7} for strains carrying *recA*, *recB*, and *recC* mutations. The fraction of mutant strains with defects in the hydrogenase activity varied from about 0.14 to 0.42, and the ratio of Hyd^- to Hup^- was independent of the *rec* genotype of the parent strain.

Based on these phenotypic characteristics, the Hup^- mutants can be divided into two major classes. Class I mutants produced both hydrogenase and FHL activities but lacked the ability to reduce BV with H_2 as the electron donor. Class II mutants failed to produce hydrogenase and all hydrogenase-dependent activities.

Biochemical characteristics of the mutants. The amount of hydrogenase activity present in the mutant strains was determined by using two different methods (see above). All the mutant strains assayed lacked H_2-dependent reduction of BV, as well as fumarate-dependent H_2 uptake activity (Table 3). However, the class I mutants (strains SE-8, SE-31, and SE-49) produced $^3H_2$ exchange activity. Tritium exchange activity of the hydrogenase is independent of any accessory electron carriers that may be essential for BV reduction from H_2 (3). Strain SE-8 produced about 50% of the parent hydrogenase activity (506 units versus 1,120 units for strain

TABLE 3. Hydrogenase activities of the BV-resistant mutant strains of *E. coli*

Strain	*hyd/hup* genotype	Hyd$^+$/Hup$^+$ parent	Hydrogenase activity[a]			
			$^3H_2$ exchange	H_2 to BV	H_2 uptake (+fumarate)	FHL
Parent						
Puig 426			1,120	712	178	11.9
JC10244			1,376	559	106	26.3
Puig 382			142	193	95	0.1
Puig 382[b]			1,660	470	141	ND[c]
Mutant						
Class I						
SE-8	*hup-101*	*Puig 426*	506	UD[d]	UD	8
SE-31	*hup-102*	JC10244	1,254	UD	UD	36
SE-49	*hup-103*	JC10244	1,276	UD	UD	42
Class II						
SE-4	*hyd-104*	Puig 426	33	UD	UD	0.9
SE-5	*hyd-105*	Puig 426	19	UD	UD	0.6
SE-6	*hyd-106*	Puig 426	41	UD	UD	0.3
SE-7	*hyd-107*	Puig 426	7	UD	UD	0.1
SE-19	*hyd-101*	Puig 426	78	UD	UD	0.1
SE-24	*hyd-111*	Puig 382	26	UD	UD	0.1
SE-37	*hyd-102*	Puig 426	15	UD	UD	0.1
SE-61	*hyd-108*	JC10244	UD	UD	UD	0.1
LCB850[e]			16	UD	UD	0.1
H61[e]			1,127	458	131	0.1

[a] Units are as follows: $^3H_2$ exchange, nanomoles of ^3H_2O produced per hour per milligram of cell protein; H_2 to BV and H_2 uptake, nanomoles of H_2 consumed per minute per milligram of cell protein; FHL, nanomoles of H_2 produced per minute per milligram of protein.

[b] Induced for H_2 uptake in HF medium.

[c] ND, Not done.

[d] UD, Below the detection limits of the assay (less than 1 unit).

[e] Strain LCB850 was obtained from M. C. Pascal (31), and strain H61 was obtained from W. G. Martin (15).

Puig 426), whereas the other two strains produced hydrogenase at levels comparable to those of the respective parents. The hydrogenase activity in strain SE-8 did not increase, even after preincubation in HF medium. We have no explanation for this low activity. All three strains also produced FHL activity. Fumarate-dependent, but not BV-dependent, activity could be detected at low levels in these three strains after preincubation in the HF medium. Strain SE-8 also lacked the ability to couple hydrogenase to other electron acceptors (methyl viologen and methylene blue). In this strain, reduced methyl viologen-dependent H_2 evolution was found to be about 14% (22 units) of the parent value (158 units – nanomoles of H_2 produced per minute per milligram protein).

The class II mutants contained negligible amounts of hydrogenase activity (Table 3). The tritium exchange activity in these mutant strains varied from 0 to 7% of the parent value. Strain SE-24, which was isolated independently after Mud (Ap *lac*) insertion, falls in this category. Strain LCB 850 described by Pascal et al. (31) also lacked the hydrogenase activity, whereas strain H61 isolated by Glick et al. (15) produced hydrogenase activity but was found to be defective in H_2 uptake. This strain was described by Glick et al. (15) as being defective in anaerobic growth. This defect precluded the determination of FHL activity in this strain, but qualitative experiments indicated a defect in FHL activity also. All other hydrogenase-defective mutants described in Table 3 grew normally under both aerobic and anaerobic growth conditions in minimal and complex media. These results indicate that the Hup$^-$ mutant strains can be divided into two major classes, one producing an active hydrogenase and FHL activity and the other lacking hydrogenase.

Enzymes associated with H_2 metabolism. Formate dehydrogenase and hydrogenase are components of the FHL enzyme complex (33). The mutant strains described by Pascal et al. (31) and Graham et al. (16) have been reported to lack the formate dehydrogenase activity. Strain LCB 850, obtained from M. C. Pascal, produced FDH activities, although at lower levels, under our experimental conditions (Table 4). This raised the possibility that the mutant strains described above may also have defects in formate dehydrogenase. The levels of the two formate dehydrogenases (FDH-1 and FDH-2) and fumarate reductase (the terminal enzyme in the H_2-dependent fumarate reduction) activities of the mutant strains are presented in Table 4.

All strains produced active FDH-1, FDH-2, and fumarate reductase. The levels of these enzyme activities varied among the different mutant strains, and for FDH-2 this was between 16 and 100% of the parent values. The FDH-1 activity in these mutant strains was comparable to that of the parent strain or higher than the parent (as high as three times for strain SE-7). Similar results were also observed with fumarate reductase. Strain SE-62, a *chlA*$^+$ derivative of strain SE-24, produced 1,441 units of fumarate reductase activity compared with 166 units for the parent strain Puig 382 (*chlA*).

TABLE 4. Levels of FDH and fumarate reductase activities in the mutant strains altered in H_2 metabolism

Strain	*hyd/hup* genotype	Hyd$^+$/Hup$^+$ parent	FDH activity[a]		Fumarate reductase
			FDH-1 (+PMS and MTT)	FDH-2 (+BV)	
Parent					
Puig 382			0.1	0.1	166
Puig 426			31	56	237
JC10244			31	86	326
Mutant					
Class I					
SE-8	*hup-101*	Puig 426	42	30	300
SE-32[b]	*hup-102*	JC10244	51	34	547
SE-49	*hup-103*	JC10244	25	20	749
Class II					
SE-4	*hyd-104*	Puig 426	40	16	810
SE-5	*hyd-105*	Puig 426	49	36	220
SE-6	*hyd-106*	Puig 426	47	47	350
SE-7	*hyd-107*	Puig 426	118	20	178
SE-19	*hyd-101*	Puig 426	31	56	862
SE-24	*hyd-111*	Puig 382	0.1	0.1	374
SE-37	*hyd-102*	Puig 426	93	66	ND[c]
SE-61	*hyd-108*	JC10244	56	14	316
SE-62	*hyd-111* (*chlA*$^+$)	Puig 382	27	17	1,441
LCB850$^+$			11	24	844
H61$^+$			8	12	142

[a] Units are as follows: FDH-1, nanomoles of MTT reduced per minute per milligram of protein; FDH-2, nanomoles of BV reduced per minute per milligram of protein; fumarate reductase, nanomoles of fumarate reduced per minute per milligram of protein. PMS, Phenazine methosulfate.

[b] Strains LCB850 and H61 were obtained from M.C. Pascal (31) and W. G. Martin (15), respectively. Strain SE-32, a *srl*::Tn*10 recA* derivative of strain SE-31 produced 1100 units of hydrogenase activity (tritium exchange), which is similar to that strain SE-31, the *recA*$^+$ parent (Table 3).

[c] ND, Not done.

TABLE 5. F' analysis of *E. coli* mutants defective in hydrogen metabolism

Recipient strain (class)	Donor strain	No. of exconjugants/1,000 donor cells in the following mutants:		
		cys$^+$	*srl*$^+$	Hup$^+$
SE-19 (II)	F'143-1			1.1
SE-20 (II)[a]	F'143-1	157	118	240
SE-32 (I)	F'143-1	400	196	0.0
SE-19 (II)	F'116	0.0	0.0	0.0
SE-20 (II)	F'116	0.0	0.0	0.0
SE-32 (I)	F'116	0.0	0.0	467

[a] Strain SE-20, which carries the same *hyd* genotype (*hyd-108*) as strain SE-61, produced enzyme activities that are similar to those of strain SE-61 (Tables 3 and 4).

All the mutant strains were analyzed at the early to mid-exponential phase of growth. The differences in the activities are probably due to the nature of the *hyd* mutation in the mutant strains. Additional information on the *hyd* mutation and the metabolic interactions among hydrogenase, formate dehydrogenases, and fumarate reductase is needed before attempting to explain these differences. However, these results demonstrate that all the mutant strains with defect(s) in the hydrogenase produced the other enzymes associated with the H_2 metabolism.

Based on the FDH-2 (a component of FHL) levels, the class II mutants can be subdivided into two phenotypes. Strains SE-19 and SE-37 produced FDH-2 at the same levels as the parents or higher (class IIA), whereas strains SE-4, SE-5, SE-6, SE-7, SE-61, and SE-62 produced FDH-2 at levels lower than the parent (class IIB).

Genetic analysis. Pascal et al. (31) have mapped the *hyd* mutation in strain LCB 850 near the *srl* gene at 58.5 min on the *E. coli* chromosome (4). To determine whether all the *hyd* mutations described in this study mapped in this area, the Hup mutants described above were tested for complementation with an *E. coli* F' element, F'143-1. This plasmid carries the *E. coli* genes between 57 and 62 min of the chromosome (28). The results obtained with three representative *recA* derivatives are presented in Table 5, and these results indicate that the class II (*hyd*) mutants (strains SE-19 and SE-20, class IIA and IIB, respectively) were complemented by F'143-1 to the Hup$^+$ phenotype, whereas the mutation in class I mutants was not. The frequency at which strain SE-19 was complemented by F'143-1 was lower; this could be a result of some unknown properties of the recipient. However, all the exconjugants tested produced all hydrogenase-dependent activities. The *srl* and *cysC* mutations in class I mutants were complemented by F'143-1, thus ruling out the possibility of plasmid instability in this genetic background. However, the mutation in class I mutants was complemented by the F' element, F'116. Although the results obtained with only three strains are presented, all the other strains that were tested behaved in the same manner. The exconjugants regained the hydrogenase as well as hydrogen uptake activities. These results indicate that the mutation in the class I mutant maps in a different location from the *hyd* operon(s) near the *srl* gene. In all the strains tested, the wild-type allele is dominant over the mutant allele.

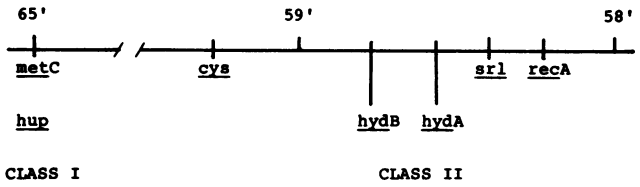

FIG. 1. Genetic map location of the genes essential for H_2 metabolism in *E. coli*.

Bacteriophage P1-mediated transduction analysis. The mutation in class I mutants was cotransduced by phage P1 with *metC* (near 65 min) at a frequency of 76%. The exact location of this mutation in the *E. coli* genome was not determined.

To further distinguish between the two subgroups of class II, the cotransduction frequencies of the *hyd* mutations were determined with respect to *cys* and *srl* with representative strains from each group (Tables 6 and 7).

The *hyd* mutation in strain SE-1 and its derivatives (*hyd* 101; class IIA) was cotransduced with *cys* at a contransduction frequency of about 12% (Table 6). The *srl-hyd*/Hup cotransduction frequency was about 41%. Further transductional analysis was undertaken to determine the position of the *hyd* mutation with respect to *srl* and *recA*. Strain JC10240, which carries the transposon Tn*10* in the *srl* gene, was used as a donor in experiments involving strains SE-16 and SE-37. The *hyd* gene was cotransduced with *srl*::Tn*10* at a frequency of about 75%. The cotransduction frequency between *srl*::Tn*10* and *recA* varied between 65 and 82% in these two strains. A cotransduction frequency of about 85% was reported by Csonka and Clark (10) for these two genes. The *hyd*$^+$/Hup$^+$ *recA*$^-$ cotransduction with *srl*::Tn*10* was lower than the cotransduction frequency observed between *srl*::Tn*10* and *hyd*/Hup or *srl*::Tn*10* and *recA* alone.

The cotransduction frequency between *srl*::Tn*10* and *recA* in the cross involving strain SE-37 is lower than expected (62% versus about 85%). Although this value reduced the percentage of Tc^r transductants that are Hup$^+$ RecA$^-$ to 52%, the ratio of Hup$^+$ clones among the RecA$^-$ population stayed constant in both crosses (83% for strain SE-16 and 79% for strain SE-37). Because the cotransduction frequency between *srl* and *recA* is about 85%, the altered *hyd* gene cannot reside between *recA* and *srl*. These results suggest that the *hyd* mutation in these strains is closer to *srl* and distal to *recA* and lies between *srl* and *cys* (Fig. 1).

TABLE 6. Transductional analysis of hydrogenase-defective mutants (class IIA)

Recipient strain	Relevant genotype of recipient strain	Donor strain	Relevant genotype of donor strain	Selected phenotype	No. tested	Hup	Percent unselected of the following phenotypes:	
							RecA	Hup RecA
FEJ-1	*srl hyd*$^+$	SE-1	*srl*$^+$ *hyd-101*	Srl	119	41		
JM81A	*cysC hyd*$^+$	SE-1		Cys	50	12		
SE-16	*hyd-101* Tc^s	JC10240	*srl*::Tn*10 recA*	Tc^r	195	72	82	69
SE-37	*hyd-102* Tc^s	JC10240		Tc^r	180	77	65	52

TABLE 7. Transductional analysis of hydrogenase-defective mutants (class IIB)

Recipient strain	Relevant genotype of recipient strain	Donor strain	Relevant genotype of donor strain	Selected phenotype	No. tested	Percent unselected of the following phenotypes:		
						Cys	Hup	Srl
SE-9	*cysC srl*::Tn*10* *hyd-108*	K-10	Prototroph	Cys	366		18	7
SE-44	*cysC srl*$^+$ *hyd-108*	FEJ-1	*cys*$^+$ *srl hyd*$^+$	Cys	492		54	17
				Hup	913	47		66
FEJ-1	*cys*$^+$ *srl hyd*$^+$	SE-44	*cysC srl*$^+$ *hyd-108*	Srl	136	1	4	

The location of the gene affected in the class IIA mutants was further confirmed by transformation with recombinant plasmids. Clarke and Carbon (9) have described the construction of an *E. coli* gene bank using ColE1 plasmid as the vector. Plasmids pLC21-33 and pLC22-40 from this collection contain both *srl* and *recA* genes (38). The class IIA mutants were transformed with these plasmids (obtained from the Coli Genetic Stock Center), and the transformants were analyzed for hydrogenase activity. The transformants were found to be *hyd*$^-$, although they carried the *recA*$^+$ gene. These results also demonstrate that the *hyd* mutation in the class IIA mutants is not between *srl* and *recA* operons and thus should be between *cys* and *srl*.

The *hyd* mutation in strain SE-9 (*hyd-108*) and its derivatives was used as a representative of the class IIB mutants, and the results indicate that this mutation is cotransducible with *cysC* at a frequency of about 18% (Table 7). The cotransduction frequency of *cysC* and *srl* was about 7%. Strain SE-44 is a *srl*$^+$ derivative of strain SE-9, and the removal of Tn*10* from the *srl* gene decreased the overall length of the *srl* gene to that of the parent. In this *srl*$^+$ background, the *cysC-hyd* and *cysC-srl* cotransduction frequencies increased to 54 and 17%, respectively. When Hup was the selected phenotype, the *hyd-cysC* and the *hyd-srl* cotransduction frequencies were 47 and 66%, respectively.

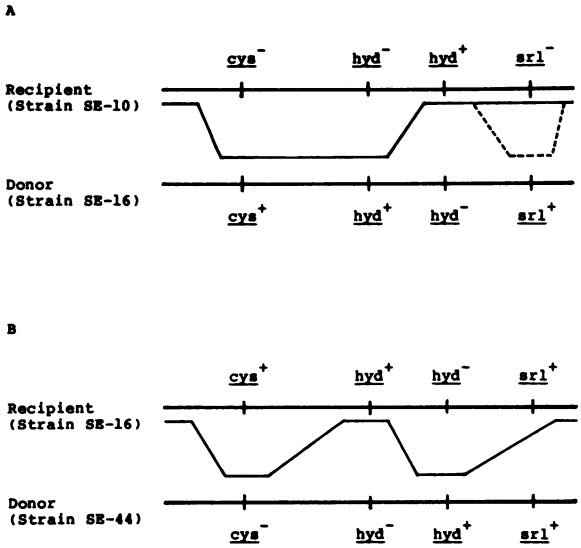

FIG. 2. Diagram of the probable crossover events occurring in the crosses. (A) Strain SE-10 or SE-44 as recipient and strain SE-16 as donor. (B) Strain SE-16 as recipient and strain SE-44 as donor. Data were obtained from Table 8.

srl as the selected phenotype in a reciprocal cross provided very low cotransduction frequencies for the *hyd*/Hup character.

These results demonstrate that the *hyd* gene altered in strain SE-9 also maps between *cysC* and *srl* genes and the mutation lies closer to *srl* than to *cysC* (66 versus 47% cotransduction, respectively). Similar results were obtained with other mutants as well (data not shown). A number of *hyd*$^+$ transductants from each experiment were assayed for tritium exchange activity, and all *hyd*$^+$ strains also regained the hydrogenase and FHL activities. None of the *hyd* mutants of this class was complemented by plasmids pLC21-33 and pLC22-40 carrying *srl*$^+$ and *recA*$^+$ genes.

If the two genes essential for hydrogenase (altered in strains SE-16 and SE-44) are very near each other, in crosses between the two strains a recombination event needs to occur between the two altered genes to restore the Hup$^+$ phenotype (Fig. 2). The cotransduction frequency for such a Hup$^+$ phenotype with *cysC* will be lower because of this constraint and will depend on the distance between the two *hyd* mutations (donor and recipient). The cotransduction frequency between *cys* and *hyd*/Hup$^+$ is reduced from 50% (Table 7; transduction involving strain SE-44) to about 12% (Table 8). Similar results were obtained with strain SE-10 as recipient and strain SE-16 as donor, and none of the *cys*$^+$ or Hup$^+$ transductants was *srl*$^+$ because this would require a quadruple crossover (Fig. 2A). The cotransduction frequency in the reverse direction is about 2% because of the need for a double recombination event to generate Hup$^+$ and *cys* transductants (Fig. 2B).

These results indicate that the two class II phenotypes (IIA and IIB) represent two *hyd* genes (operons). These genes are close to each other, between the *srl* and *cys*

TABLE 8. Transductional analysis of the two subgroups of hydrogenase-defective mutants (classes IIA and B)

Recipient (class; relevant genotype)	Donor (class; relevant genotype)	Selected phenotype	No. tested	Percent unselected of the following phenotypes:		
				Cys	Hup	Srl
SE-10 (IIB; *cysC srl hyd-108*)	SE-16 (IIA; *cys*$^+$ *srl*$^+$ *hyd-101*)	Cys	521		13	0
		Hup	179	9		0
SE-44 (IIB; *cysC srl*$^+$ *hyd-108*)		Cys	1,911		12	
		Hup	793	8		
SE-16 (IIA; *cys*$^+$ *srl*$^+$ *hyd-101*)	SE-44 (IIB; *cysC srl*$^+$ *hyd-108*)	Hup	142	2		

operons, but separate enough not to provide negative interference in the transduction experiments (Fig. 1).

DISCUSSION

E. coli is capable of utilizing several different terminal electron acceptors during growth (17). These include O_2, NO_3^-, H^+, organic compounds, etc. The end products of glucose catabolism depend on the availability of these compounds. In the presence of O_2, the major end products are carbon dioxide and water. Under conditions of O_2 limitation, the glucose catabolism is altered at the level of pyruvate metabolism. The pyruvate dehydrogenase activity decreases with a concomitant increase in the pyruvate formatelyase activity. The major end products of glucose catabolism under anaerobic conditions are acetate, lactate, ethanol, H_2, and CO_2. The two gaseous products H_2 and CO_2 are produced from formate by the FHL, enzyme complex which contains FDH-2, hydrogenase, and electron carriers between the two enzymes (17, 33, 39). It is generally believed that the excess reducing power generated under O_2 limitation is released as H_2 (39). The physiological, biochemical, and genetic mechanism by which the cell switches the pyruvate metabolism in response to changing internal redox state (redox control?) is poorly understood. The major difficulties generally encountered in these studies include the lack of good selection procedures for the isolation of mutants, the inherent difficulty of working with proteins that are O_2 sensitive, and the elucidation of electron transport proteins with no enzymatic activity (capable of interacting with small molecules).

To understand the mechanism of redox control, the enzyme hydrogenase was chosen in the present study, because it plays a critical role in the overall reductant metabolism in the cell-hydrogen evolution and hydrogen uptake (14, 29). Hydrogenase is produced in anaerobic cells (17, 32), but the activity (synthesis) is enhanced under conditions of H_2 uptake (24). Hydrogenase is also regulated by formate and H_2. Strain Puig 382, a *chlA* mutant, is defective in both FDH-1 and FDH-2 (Table 4) and produced low levels of hydrogenase (142 units) in LB medium (Table 3). Addition of formate, the substrate for FHL, or H_2 (substrate for HUP system) to the LB medium increased the hydrogenase levels of cells cultured in this medium to 1,245 and 1,660 units, respectively. This indicates that formate and H_2 act as inducers of hydrogenase in *E. coli*, although the mutant strain cannot utilize formate because of the *chlA* mutation.

In spite of the control of hydrogenase by formate, the enzyme is not essential for growth in glucose minimal or LB medium under anaerobic conditions. The positive selection procedure described above also allows the selection of a large number of mutants that are affected by H_2 metabolism in *E. coli* for detailed studies by biochemical genetic methods. Because of these characteristics, study of this enzyme will provide the necessary information on the mechanism of biosynthesis of anaerobic proteins in *E. coli* with potential applications to other organisms.

Analysis of the Hup mutants demonstrates the presence of at least three operons that are involved in H_2 metabolism. The gene(s) defective in the class I mutants produces an electron transport protein that couples the hydrogenase to the electron transport chain, probably to quinone (20). The phenotype of these mutant strains (Table 3) suggests that the same protein is also capable of interacting and reducing BV. This BV-reducing protein is different from the electron transport protein employed by FDH-2 (to BV) in the cell because these mutant strains were FDH-2 positive. This Hup-dependent, BV-reducing protein plays no role in the FHL complex. However, interestingly, the stability of hydrogenase during purification declines rapidly in the absence of this protein in a functional form (unpublished data). Additional experiments are needed to determine the biochemical defect of this mutation.

The other two operons code for hydrogenase protein or activity. The results, although presented for only a few strains, are corroborated by complementation experiments described in the accompanying paper (34). Hydrogenase is regulated by several metabolic processes, including (i) redox environment; (ii) formate-FHL, and (iii) energy-Hup. Hydrogenase is also a nonheme iron protein (1), and the presence of cofactors in the protein would indicate a requirement for activation of the hydrogenase apoprotein. The two clusters of *hyd* genes and the genes between the two may play a major role in the synthesis, regulation, and activation of the hydrogenase protein in the cell. Additional experimental results are necessary before the gene-product-function relationships for these various *hyd* genes can be established.

The basic principle used in the isolation of *hyd* and *hup* mutants of *E. coli* can be applied for the selection of Hup^- mutant strains of other organisms, as well as mutants with defects in other low-potential electron transport proteins, by the use of appropriate electron donors and culture conditions. The experimental conditions used in this study yielded 100% of the Hup^- mutants in the surviving population (Table 2). The very high frequency of Hup^- mutants in the population indicates that either the Hup^- mutants are present in a normal *E. coli* population at this frequency and generally have no selective advantage under normal growth conditions or that the BV superoxide actually acts as a mutagen. The frequency of this mutation is about 10^{-7}.

The requirement for *rec* genes for survival (Table 2) shows that the superoxide generated from reoxidation of reduced BV leads to DNA damage. Loewen (27) has noted that catalase-defective mutants also exhibit an enhancement of lethal effect of H_2O_2 in the *rec* genotype. Both superoxide and H_2O_2 generated from superoxide are known to cause single-strand breaks in the DNA that are repaired by *rec* gene products (2, 11). Among the several hundred survivors tested, auxotrophic mutants comprised about 2% of the population, indicating that the superoxide radical did act as a mutagen. When strain Puig 426 was used as starting strain, 25% of the auxotrophs were found to be *pyrA*. We have no explanation for the preponderance of *pyrA* mutants. Barrett et al. (6) have also isolated *pyrA* mutants as FHL conditional mutants of *S. typhimurium* and proposed that a gene for carbonic anhydrase is probably a part of the *pyrA* operon and *pyrA* mutants are defective in CO_2 metabolism. However, the presence of greater than 99% of Hup^- strains in the surviving population (Table 2) suggests that the primary lethal effect of BV is induced by membrane damage initiated by the superoxide radical (22) rather than by DNA damage.

In summary, the positive selection procedure developed for the isolation of Hup^- mutants yielded several *hyd* mutants. Analysis of these mutants demonstrates the presence of at least two *hyd* operons (*hydA* and *hydB*) in *E. coli*. Both of these operons map between *srl* and *cysC* in the *E. coli* genetic linkage map (Fig. 1).

The *E. coli* genetic linkage map (4) shows that the *hyd* gene resides between *srl* and *nalB*, counterclockwise from *srl*. This map location was based on the results reported by Pascal et al. (31). Recent genetic analysis of *hyd* mutants of *E. coli* indicated that the *hyd* gene resides between *srl* and

cys (16, 32). The mutation in strain H61 was not mapped by Glick et al. (15). The gene altered in strain H61 is not in the same genetic location where the *hydA* and *hydB* genes reside (34). Glick et al. (15) have reported that hydrogenase activity can be detected in strain H61 when isolated membranes are assayed for hydrogenase. Strain H61 produced hydrogenase activity under the conditions used in this study as well (Table 3). However, strain H61 failed to produce FHL activity (Table 3), although FDH-2 activity could be detected (Table 4). Strain H61 grew very poorly under anaerobic conditions (15), a property that is not shared by the *hyd* mutants isolated during this study. It is possible that strain H61 carries a defect in membrane protein(s), as suggested by Glick et al. (15).

We used the term *hydA* to denote the mutation in the class IIA mutants, because the first *hyd* mutant, strain LCB 850, described by Pascal et al. (31) falls in this class (34). The second gene is *hydB*. The *hydA* mutant strains isolated and described in this study produced normal or higher levels of FDH-2, whereas the *hydB* mutant strains produced lower levels of FDH-2 activity. The presence of at least three genes for hydrogenase activity in the cell (*hydA*, *hydB*, and *hup*; Fig. 1) and the differences in FDH-1, FDH-2, and fumarate reductase activities of the *hyd* mutants (Table 4) indicate that the H_2 metabolism in *E. coli* is complex and a large body of information is needed for a thorough understanding of this process.

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