JOURNAL OF BACTERIOLOGY, Apr. 1985, p. 353–360
0021-9193/85/040353-08$02.00/0
Copyright © 1985, American Society for Microbiology
Vol. 162, No. 1

Cloning of Hydrogenase Genes and Fine Structure Analysis of an Operon Essential for H_2 Metabolism in *Escherichia coli*†

PUSHPAM SANKAR, JONG HO LEE, AND K. T. SHANMUGAM*

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611

Received 23 July 1984/Accepted 4 January 1985

Escherichia coli **has two unlinked genes that code for hydrogenase synthesis and activity. The DNA fragments containing the two genes (*hydA* and *hydB*) were cloned into a plasmid vector, pBR322. The plasmids containing the *hyd* genes (pSE-290 and pSE-111 carrying the *hydA* and *hydB* genes, respectively) were used to genetically map a total of 51 mutant strains with defects in hydrogenase activity. A total of 37 mutants carried a mutation in the *hydB* gene, whereas the remaining 14 *hyd* were *hydA*. This complementation analysis also established the presence of two new genes, so far unidentified, one coding for formate dehydrogenase-2 (*fdv*) and another producing an electron transport protein (*fhl*) coupling formate dehydrogenase-2 to hydrogenase. Three of the four genes, *hydB*, *fhl*, and *fdv*, may constitute a single operon, and all three genes are carried by a 5.6-kilobase-pair chromosomal DNA insert in plasmid pSE-128. Plasmids carrying a part of this 5.6-kilobase-pair DNA (pSE-130) or fragments derived from this DNA in different orientations (pSE-126 and pSE-129) inhibited the production of active formate hydrogenlyase. This inhibition occurred even in a prototrophic *E. coli*, strain K-10, but only during an early induction period. These results, based on complementation analysis with cloned DNA fragments, show that both *hydA* and *hydB* genes are essential for the production of active hydrogenase. For the expression of active formate hydrogenlyase, two other gene products, *fhl* and *fdv* are also needed. All four genes map between 58 and 59 min in the *E. coli* chromosome.**

The enzyme hydrogenase plays a major role in the metabolism of several bacteria, including *Escherichia coli* (17, 18, 30). *E. coli* is capable of utilizing H_2 as a sole source of reductant and energy under appropriate growth conditions (19, 23, 28). In this organism, as well as in several enteric bacteria, hydrogenase is also a component of formate hydrogenlyase (FHL), an enzyme system involved in removing formate, produced during fermentation (26). It is not known at present whether the two hydrogenase activities (H_2 evolution and H_2 uptake) are catalyzed by two enzymes or by one enzyme in conjunction with different electron transport proteins. Although several investigators have isolated and characterized hydrogenase-defective mutant strains of *E. coli* (12, 15, 20, 21, 24, 25), the number of genes present in the cell has not been elucidated because of the limited number of mutants isolated by these investigators. Some of these strains also exhibited other defects (12, 15, 24).

To understand the molecular biology of H_2 metabolism, we have isolated and described a large number of hydrogenase-defective mutant strains of *E. coli* (22). Genetic analysis of these *hyd* mutants indicated the presence of two closely linked operons, and both of these operons were found to be essential for the production of active hydrogenase. In this report, additional physical evidence is presented for the existence of the two *hyd* operons, based on the analysis of cloned DNA fragments. The fine-structure analysis of one of the two operons, presented below, identified the presence of genes for hydrogenase, formate dehydrogenase-2, and an intermediary electron transport protein in this operon.

* Corresponding author.
† Florida Agricultural Experiment Station Publication no. 5755.

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 activity that couples formate oxidation to reduction of artificial electron acceptor, benzyl viologen (BV). This formate dehydrogenase (FDH-2) is a component of formate hydrogenlyase enzyme complex (FHL). *fhl* is used to designate genes, the products of which are essential for FHL activity besides the *hyd* and *fdv* gene products.

Bacterial strains. All bacterial strains are derivatives of *E. coli* K-12 and have been described previously or derived from these strains (22). Strains SE-38, SE-53, SE-64, SE-65, SE-66, SE-67, and SE-68 were obtained as Tc^s derivatives by fusaric acid selection (5) from strains SE-3, SE-19, SE-4, SE-5, SE-6, SE-7, and SE-61, respectively. Table 1 provides the pedigree and *hyd* genotype of the hydrogenase-defective mutant strains of *E. coli* used in this study, and these mutant strains were isolated by BV selection (22).

Media and growth conditions. Luria broth, glucose minimal medium, and HF have been described previously (22). Ampicillin and tetracycline, when present, were added to the medium after autoclaving to a final concentration of 100 and 15 μg/ml, respectively. Cultures for enzyme assays were

TABLE 1. Pedigree of mutant strains of *E. coli* with *hyd* mutation

Strain	*hyd* genotype	Parent strain
SE-1[a], SE-2[a]	*hydA101* -102	Puig 426
SE-3 to SE-7[a]	*hydB103* to *hydB107*	Puig 426
SE-9[a]	*hydB108*, *fdv-101*	JC 10244
SE-24[a]	*hydB111*	Puig 382
SE-88, SE-90	*hydA112*, *hydB113*	K-10
SE-92, SE-94 to SE-97	*hyd-114* to *hyd-121*	JC 10244
SE-99, SE-100, SE-101		
SE-102 to SE-110	*hyd-122* to *hyd-130*	JC 10240
SE-111 to SE-132	*hyd-131* to *hyd-152*	Puig 426

[a] These strains have been described previously. All other mutations were obtained by previously described methods (22). *hyd* genotypes *-116*, *-118*, *-119*, *-123*, *-127*, *-128*, *-130*, *-142*, *-144*, and *-147* belong to *hydA* gene, whereas the remainder of the undefined *hyd* mutations reside in the *hyd*B gene. Genotypes of the parent strains have been described previously (22) (see text for details).

grown in Luria broth supplemented with 0.15% glucose for 4 h under anaerobic conditions as previously described (22).

Enzyme assays. Hydrogenase, hydrogen uptake activity, FDH-1 and FDH-2 and formate hydrogenlyase activities were determined as described before (22).

Cloning the *hyd* gene. Total chromosomal DNA was isolated from a prototrophic strain of *E. coli*, K-10 (27). Plasmid pBR322 (6) was used as a vector in the cloning experiments. Plasmid DNA was isolated as described by Davis et al. (9). A gene bank containing *E. coli* chromosomal DNA fragments was constructed by the general procedures described by Ditta et al. (10). Chromosomal DNA, partially digested with the restriction endonuclease *Sau*3A was ligated with an endonuclease *Bam*HI-digested, calf intestine alkaline phosphatase-treated vector with bacteriophage T4 DNA ligase. The hydrogenase-defective *recA* strain of *E. coli* SE-61 was transformed with the ligation mixture, and the ampicillin-resistant colonies were selected with LB medium supplemented with ampicillin. To isolate the plasmids containing hydrogenase genes, the Ap^r clones were transferred to HF medium by replica plating methods. The Hup^+ Ap^r clones were selected and maintained. The plasmid present in these clones was extracted and tested for the presence of *hyd* and other relevant characters. Total plasmid DNA from the pool of Ap^r transformants was isolated and maintained as an *E. coli* gene bank. Starting with this *E. coli* gene bank, plasmids capable of complementing the *hyd*/*hup* mutants belonging to other classes and not represented by strain SE-61 (e.g., strain SE-53) were also isolated by the procedures described above.

Manipulation of *hyd* plasmids. A physical map of the plasmids, based on restriction endonuclease digestion, was constructed by using previously published procedures (6, 9, 27). Restriction endonuclease digestion conditions were either described by Davis et al. (9) or as recommended by the manufacturer of the enzyme. Plasmid pBR322 was used as the vector in all subcloning experiments, and the procedures used were as described by Rodriguez et al. (27).

Materials. Biochemicals were purchased from Sigma Chemical Co. Inorganic and organic chemicals were obtained from Fisher Scientific Co. and were analytical grade. Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Laboratories, Inc.; Enzo Biochem, Inc.; or Miles Laboratories, Inc. Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals.

RESULTS

Cloning the *hyd* genes. The hydrogenase-defective mutant strains of *E. coli* can be divided into two groups based on bacteriophage P1-mediated transduction analysis (22). To further establish the presence of two independent hydrogenase operons, cloned DNA fragments containing hydrogenase genes were used. In these experiments, mutant strains that are representative of the two groups (strain SE-53 *hydA101* and SE-68 *hydB108*) were transformed with plasmid DNA from the gene bank, and the Hup^+ transformants in the population were selected. The presence of *hyd*/*hup* genes in the plasmid DNA was confirmed by transformation of the same recipient strains with the isolated pure plasmid DNA. These transformants were analyzed for the presence of hydrogenase activity by three assay procedures (tritium exchange, H_2-dependent reduction of BV, and fumarate-dependent H_2 uptake). The results of a typical experiment are presented in Table 2. The hydrogenase defect in strain SE-68, which carried a *hydB108* mutation, was complemented by plasmid pSE-111 and not by plasmid pSE-290. On the other hand, the defects in strains SE-2 and SE-53, both carrying a mutation in the *hydA* gene (*hydA102* and *hydA101*, respectively), were complemented by plasmid pSE-290 and not by plasmid pSE-111. The *hyd* mutation in strain LCB850, described by Pascal et al. (24), was also complemented by plasmid pSE-290. In the presence of the appropriate plasmid, the mutant strains produced normal levels of hydrogenase, hydrogen uptake, as well as H_2-dependent BV reduction activities.

The physical map of the two plasmids (pSE-111 and pSE-290) based on the restriction endonuclease digestion is presented in Fig. 1. This figure also contains the restriction map of two other smaller plasmids, pSE-201 and pSE-22, capable of complementing *hydA* and *hydB* genes, respectively. Plasmid pSE-22 contains a 9-kilobase-pair (kb) chro-

TABLE 2. Complementation analysis of *hyd* mutants by recombinant plasmids

Strain	*hyd* genotype	Plasmid	Hydrogenase activity in[a]:		
			$^3H_2$ exchange	H_2 to BV	H_2 uptake (+ fumarate)
Puig 426	*hyd*$^+$		1110	427	144
		pSE-111	1110	340	175
		pSE-290	1198	446	158
SE-68	*hydB108*		18	UD	UD
		pSE-111	1030	703	202
		pSE-290	15	ND	ND
SE-2	*hydA102*		1	UD	UD
		pSE-111	1	ND	ND
		pSE-290	1228	506	82
SE-53	*hydA101*		5	UD	UD
		pSE-111	9	ND	ND
		pSE-290	1135	742	183
LCB850[b]			16	UD	UD
		pSE-111	7	ND	ND
		pSE-290	1020	538	128

[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 in the presence of BV or fumarate as electron acceptors; UD, Undetectable levels of activity—below 1 unit; ND, Not done. None of the strains lacking the tritium exchange activity was found to produce other H_2-dependent activities (22).

[b] Strain LCB850 was obtained from M. C. Pascal (24).

mosomal DNA insert that is present within the 14-kb insert observed in plasmid pSE-111. Plasmid pSE-201 contains a 5.5-kb insert, whereas plasmid pSE-290 carries a 12.3-kb chromosomal DNA insert. The inserts in these two plasmids (pSE-201 and pSE-290) overlap in one end to a total length of about 4.8 kb. Based on the restriction map (Fig. 1), no overlap between the two groups of plasmids (plasmid pSE-111 versus plasmids pSE-201 and pSE-290) was detected.

Complementation analysis of *hyd* mutants. The presence of these recombinant *hyd* plasmids capable of complementing either the *hydA* or the *hydB* gene provided a convenient method for the separation of the *hyd* mutants (Table 1) into two major groups. Because the number of genes in each *hyd* operon (corresponding to *hydA* and *hydB*) is unknown at present, the *hyd* mutation in the mutant population can potentially reside in any of the genes within the operon. Because of this concern, two of the large plasmids, pSE-111 and pSE-290, were used in the complementation experiments as representative plasmids for *hydB* and *hydA*, respectively, to help identify as many mutants as possible. The Ap^r transformants were selected and tested for FHL and HUP activities. The results obtained from these experiments were used to identify the location of the hydrogenase mutation present in the mutant strains (Fig. 2). Most of the *hyd* mutant strains lacking tritium exchange activity (51 of 60) were complemented by one of the two plasmids. Neither plasmid complemented the remaining 9 of 60 mutants. Seven of these nine mutants produced hydrogenase activity that was detectable by the tritium exchange assay but lacked both FHL and HUP activities. These seven mutants were not studied further. A total of 2 *hyd* mutant strains (SE-93 and SE-98), of a total of 60 mutants analyzed, were not complemented by any of the *hyd* plasmids tested for hydrogenase, FHL, or HUP activities. These mutant strains may carry a deletion that removes both *hydA* and *hydB* genes. The other possibility is that the mutation in the two mutant strains may be dominant over the wild-type allele. Additional experiments are needed to distinguish between these two alternatives. None of the mutant strains tested was complemented by both plasmids.

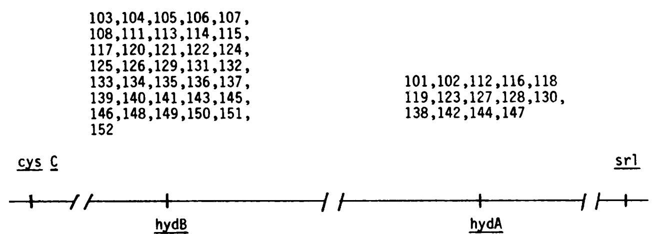

FIG. 2. Genetic map location of *hyd* mutations in *E. coli*, based on complementation analysis (see text for details).

Plasmid pSE-111 ($hydB^+$) restored the parental hydrogenase phenotype in 37 of 51 mutant strains (72%). The remaining 14 strains (28%) carried the mutation in the *hydA* gene. Some Hyd^- mutants (e.g., strain SE-68) carried a mutation in formate dehydrogenase-2 gene (*fdv*). This defect in the *fdv* gene was also restored by the plasmid pSE-22. Plasmid pSE-22 also complemented an *fdv* hyd^+ strain to fdv^+. These results obtained with the cloned DNA fragments are in complete agreement with the genetic evidence on the presence of two operons for hydrogenase in the cell (22).

Plasmid pSE-22 contains the genes needed for hydrogenase (*hydB*) and formate dehydrogenase-2 (*fdv*). These are the two enzymes that constitute the formate hydrogenlyase complex (26). To understand the organization of the two genes and the formate hydrogenlyase operon in the *E. coli* chromosome, fine-structure analysis of plasmid pSE-22 was carried out.

Subcloning the *hyd* and *fdv* genes. Plasmid pSE-22 was digested with restriction endonuclease *Sal*I or *Pst*I, and the fragments produced were cloned into an appropriately digested vector (pBR322). The construction of these plasmid derivatives are presented in Fig. 3.

The chromosomal DNA insert in plasmid pSE-22 contains three cleavage sites for the enzyme *Sal*I and two sites for *Pst*I (Fig. 1). The vector has one site each for *Sal*I, *Pst*I, and *Cla*I (6, 9). Complete digestion of plasmid pSE-22 by *Sal*I produced four fragments (1.15, 2.0, 2.8, and 7.85 kb), and

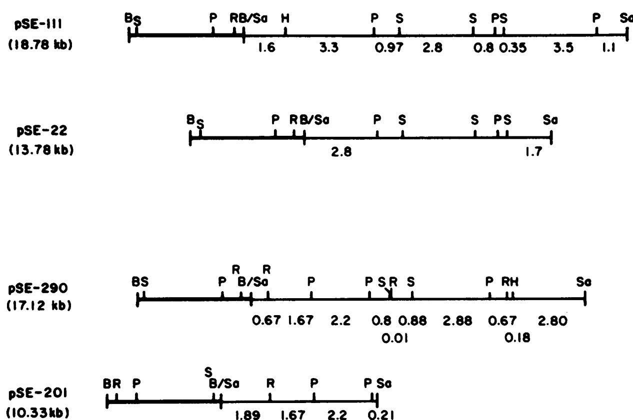

FIG. 1. Restriction map of *E. coli* recombinant plasmids containing the two different hydrogenase operons. Plasmid pBR322 (heavy lines) is the vector. Only the relevant restriction sites are marked for the vector. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; B/Sa, *Bam*HI-*Sau*3A junction of the vector and chromosomal DNA insert. The numbers indicate the size of the DNA fragment between two restriction sites in kb (see text for details).

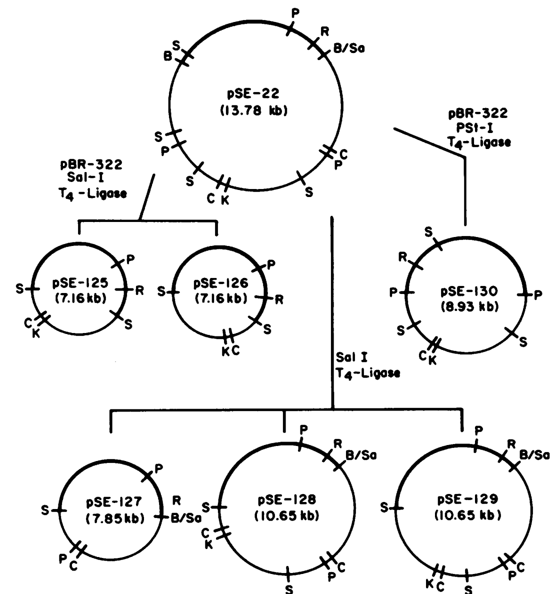

FIG. 3. Isolation of plasmid derivatives from plasmid pSE-22 (see text for details).

TABLE 3. Hydrogenase activity in mutant strains carrying subclones derived from plasmid pSE-22

Strain	*hyd* genotype	Hydrogenase Activity in strains with the following plasmids[a]:						
		Control	+pSE-125	+pSE-126	+pSE-127	+pSE-128	+pSE-129	+pSE-130
SE-38	*hydB103*	<1	1574	551	1	447	605	719
SE-64	*hydB104*	1	1009	818	<1	1152	1448	916
SE-66	*hydB106*	1	1140	1012	<1	1169	1913	1011
SE-67	*hydB107*	9	1196	1084	5	1820	1987	1331
SE-68	*hydB108*	17	448	867	2	1987	1237	1596

[a] Units are as follows: nanomoles of ^3H_2O produced per hour per milligram of cell protein.

the large fragment also carried most of the vector (plasmid pSE-127). The chromosomal DNA insert in pSE-22 also has two restriction sites for *Cla*I and one for *Kpn*I (Fig. 3). The 2.8-kb *Sal*I fragment carries the *Kpn*I site and one of the two *Cla*I sites, and the location of these restriction sites can be used to orient the 2.8-kb *Sal*I fragment in relation to the vector. Based on these experiments, plasmids pSE-125 and pSE-126 were found to contain the 2.8-kb fragment in opposite orientations. Plasmids pSE-128 and pSE-129 contain two of the *Sal*I fragments (2.8 and 7.85 kb). The 2.8-kb fragment is in the native orientation in plasmid pSE-128 and in the opposite orientation in plasmid pSE-129. Plasmid pSE-130 carries the 4.6-kb *Pst*I chromosomal DNA fragment from pSE-22 and the vector pBR322.

These plasmids, carrying different parts of the chromosomal DNA from pSE-22, were analyzed for the presence of the *hydB* gene by complementation analysis (Table 3).

Plasmids pSE-125 and pSE-126 complemented the *hydB* mutations tested for hydrogenase activity, indicating that the hydrogenase gene resides completely within this 2.8-kb fragment. Other plasmid subclones containing this 2.8-kb *Sal*I fragment (pSE-128, pSE-129, and pSE-130) restored the Hyd phenotype upon transformation, whereas other plasmid derivatives lacking this 2.8-kb fragment (e.g., pSE-127) failed to do so. There were significant differences in the levels of enzyme activities among the mutant strains with different plasmids. These differences may be a consequence of different chromosomal mutations that interact with the gene product(s) produced by the multicopy plasmids.

Based on the complementation characteristics of the plasmids and the biochemical properties of the transformants, the analyzed strains can be separated into three distinct groups (Tables 4, 5, and 6). Strains SE-38, SE-65, and SE-66 produced FHL activity in the presence of plasmid pSE-125 at a level lower than or comparable to that of the parent (Puig 426; Table 4). The levels of FHL activity observed with plasmid pSE-128 was always greater than that of the parent. This high FHL activity may be a consequence of the high copy number of the *E. coli* genes present in plasmid pSE-128, a result that is similar to the values reported by Karube et al. (20).

The strains carrying plasmid pSE-129, with the 2.8-kb *Sal*I fragment in an opposite orientation as compared with pSE-128 (Fig. 3), produced enzyme activities that are qualitatively similar to the strains with plasmid pSE-125, although the FDH-2 activity was higher in these strains. The FHL activity in all three strains was also lower in the presence of plasmid pSE-130, which carried the *Pst*I fragment. The FHL activity was not detected in the presence of plasmid pSE-127 because this plasmid lacks the 2.8-kb *Sal*I fragment needed for hydrogenase.

In these three strains, an inverse relationship between the FHL and HUP activities could also be observed. The presence of plasmid pSE-125 led to the production of higher

TABLE 4. FHL, FDH-2, and HUP activities in *hyd* mutant strains SE-38, SE-65, and SE-66 in the presence of different subclones of plasmid pSE-22

Strain	*hyd* genotype	Plasmid	Enzyme activity[a]:		
			HUP	FDH-2	FHL
Puig 426	*hyd*[+]		178	56	12
SE-38	*hydB103*		UD	28	UD
		pSE-125	162	14	4
		pSE-127	UD	15	UD
		pSE-128	71	23	20
		pSE-129	97	26	4
		pSE-130	109	15	1
SE-65[b]	*hydB105*		UD	16	UD
		pSE-125	211	12	12
		pSE-127	UD	8	UD
		pSE-128	111	15	39
		pSE-129	174	31	8
		pSE-130	124	8	8
SE-66	*hydB106*		UD	17	UD
		pSE-125	196	11	7
		pSE-127	UD	14	UD
		pSE-128	113	13	31
		pSE-129	145	30	5
		pSE-130	116	11	3

[a] HUP activity was determined with fumarate as the electron acceptor (nanomoles of fumarate reduced per minute per milligram of cell protein). Units for FDH-2 and FHL are nanomoles of BV reduced or H_2 produced, respectively, per minute per milligram of protein. UD, Undetectable.

[b] Strain SE-65 produced hydrogenase activity in the presence of all the plasmids tested, except pSE-127.

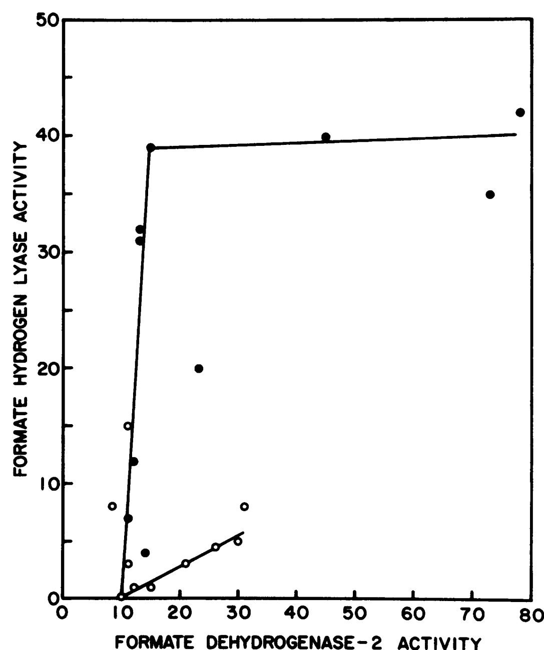

FIG. 4. The interrelation between FDH-2 and FHL activities in *E. coli*. The results reported in Tables 4, 5, and 6 are plotted here. Closed circles represent the values from strains containing plasmid pSE-125 and pSE-128. All other FDH-2 and FHL values are represented by open circles. The values for SE-64(pSE-130) fall in the line drawn through the closed circles.

levels of HUP activity, whereas the levels of HUP activities in the presence of plasmid pSE-128 were only about 50% of those with pSE-125. Interestingly, in the presence of plasmid pSE-130, the HUP activities were similar to the strains with plasmid pSE-128, and the FHL activities were similar to the levels observed with plasmid pSE-125.

The results presented in Fig. 4 indicate that the FHL activity, which requires FDH-2, hydrogenase, and unknown electron carriers (18), was saturated, under normal conditions, at an FDH-2 level of about 15 to 20 units. Between 8 and 15 units, the FHL activity increased with increasing FDH-2 levels. In the presence of plasmids pSE-125, pSE-129, and pSE-130 (except for SE-64 [pSE-130]), the FHL activity, although increased with increasing FDH-2 activity, did not exhibit the same rate of increase and failed to saturate even at 30 units of FDH-2 activity, indicating a deficiency for some unknown component needed for coupling FDH-2 and hydrogenase or production of an inhibitory component of formate hydrogenlyase activity.

Strains SE-64 and SE-68 were Hup$^+$ in the presence of the plasmid pSE-125 (Table 5). In both strains, FHL activity was not complemented by plasmid pSE-125, which is in contrast to the results presented in Table 4.

Strain SE-64 produced 12 units of FDH-2 activity which was unaltered in the presence of all plasmids tested, except with pSE-128. The FHL activity in SE-64(pSE-125) was 0.1 unit. This value increased to 15 units (similar to the parent value of 12 units) in the presence of plasmid pSE-130 without a concomitant increase in FDH-2 activity. These results suggest that the plasmid pSE-130 produced an electron transport component that is essential for FHL activity (besides FDH-2 and hydrogenase) in strain SE-64. The lack of FHL activity in strain SE-64(pSE-129) indicates that the inversion of the 2.8 kb *Sal*I fragment (Fig. 3) disrupted the production of this component. These results also indicate that strain SE-64 carries an additional defect, besides the *hydB104* mutation, which affects the production of FHL activity. Strain SE-64 reverts to the Hup$^+$ phenotype at a frequency of about 10^{-5}. The Hup$^+$ revertants were FHL positive, indicating that the genes coding for hydrogenase and the electron transport protein constitute one operon. The *hydB104* mutation may have a polar effect on the second gene. The requirement for plasmid pSE-128 in the production of FDH-2 activity in strains SE-64 and SE-68 suggests that this plasmid codes for a protein needed for FDH-2 activity.

TABLE 5. FHL, FDH-2, and HUP activities of *hyd* mutant strains SE-64 and SE-68 in the presence of different subclones of plasmid pSE-22

Strain	*hyd* genotype	Plasmid	Enzyme activity[a]:		
			HUP	FDH-2	FHL
Puig 426	*hyd*$^+$		178	56	12
SE-64	*hydB104*		UD	12	UD
		pSE-125	219	13	0.1
		pSE-127	UD	14	UD
		pSE-128	110	78	42
		pSE-129	154	10	0.03
		pSE-130	147	11	15
JC10244	*hyd*$^+$		113	86	26
SE-68	*hydB108*		UD	6	UD
		pSE-125	235	6	UD
		pSE-127	UD	3	UD
		pSE-128	191	45	40
		pSE-129	225	11	UD
		pSE-130	82	3	0.01

[a] Units are as defined in footnote *a* in Table 4.

TABLE 6. Inhibition of FHL activity in strain SE-67 (*hydB107*) by recombinant plasmids

Plasmid	Enzyme activity[a]			
	$^3H_2$ exchange	HUP	FDH-2	FHL
None	9	UD	20	0.3
pSE-125	1196	147	13	32
pSE-126	1084	46	15	1
pSE-130	1331	167	12	1
pSE-128	1820	128	73	35
pSE-129	1987	128	21	3
Puig 426 (*hyd*$^+$ parent)	1110	178	56	12

[a] Units of enzyme activities are as defined in footnotes *a* in Tables 3 and 4.

Inhibition of FHL activity by cloned DNA fragments. Strain SE-67 produced high level of FHL activity even in the presence of plasmid pSE-125 (Table 6), indicating that the genetic defect in this strain is in the hydrogenase. Complementation of this mutation by the 2.8-kb *Sal*I fragment containing hydrogenase led to restoration of the parental phenotype, which was similar to SE-67(pSE-128). However, the production of FHL activity by this strain is inhibited by plasmids pSE-126, pSE-129, and pSE-130. In the presence of these plasmids, the total hydrogenase and FDH-2 levels were unaltered (as compared with the levels in SE-67 [pSE-125]), but the FHL activity was reduced by greater than 90%. These results suggest that the presence of the DNA fragments in their unique size and orientations in plasmids pSE-126, pSE-129, and pSE-130 (Fig. 3) lead to the inhibition of FHL activity in this genetic background (strain SE-67; *hydB107*).

An inhibitory effect of these plasmids on FHL induction was also observed with an *E. coli* K-12 prototroph, strain K-10, during an induction experiment (Fig. 5). The growth characteristics of strain K-10 and strain K-10(pSE-130) were similar except that the final cell yield in the strain with plasmid pSE-130 was lower. The rate of differential induction of FHL activity was also similar in both strain K-10 and K-10(pSE-130), but the strain with the plasmid failed to produce FHL activity during the early exponential phase of growth. In both cultures, the specific activity started to decline as the culture reached the stationary phase of growth.

These results (Table 6, Fig. 5) establish that plasmids pSE-130, pSE-126, and pSE-129 produce a product that is inhibiting the flow of electrons between FDH-2 and hydrogenase. This inhibitory effect of FHL activity by these plasmids was detected in other *hyd* mutant strains as well (Table 4), although the effect was not as pronounced as with strains SE-67 and K-10. In all these experiments, hydrogenase activity was always greater than 500 units and thus not rate limiting for FHL activity.

DISCUSSION

Hydrogenase, the primary enzyme responsible for H_2 metabolism in *E. coli*, requires the presence of gene products from at least two operons (Table 2, Fig. 2). The two *hyd* genes have been termed *hydA* and *hydB* (22). The *hyd* mutation described by Pascal et al. (24) is in the *hydA* gene, as determined by the complementation of the defect in strain LCB 850 (Table 2). The genetic location of the *hyd* mutations described by other investigators (12, 15, 20, 21, 25) among

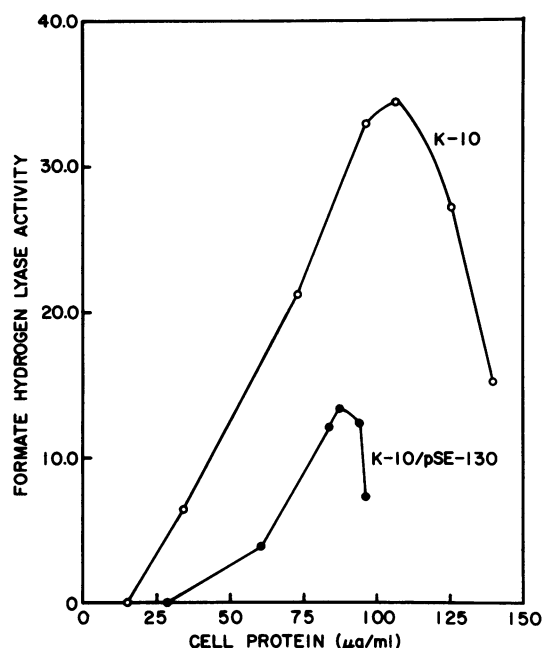

FIG. 5. Differential induction of FHL activity by strains K-10 and K-10(pSE-130), after a shift to anaerobic conditions. Aerobic cultures were used to inoculate LB medium. The culture vessel was sealed, and the gas phase was replaced by N_2. At different time periods, samples were removed and growth, cell protein, and FHL activity were determined as described previously (22).

the two *hyd* genes is unknown at present. The *hyd* mutant isolated by Glick et al. (strain H-61 [12]) is not complemented by any of the three plasmids used in this study (pSE-111, pSE-201, and pSE-290). The *hyd* plasmid pBL-101 constructed by Glick et al. (13) failed to complement the hydrogenase defect in strain SE-19 or SE-61. Strain H-61 was reported to produce hydrogenase activity with a defect in the ability to reduce viologen dyes effectively (12). Plasmid pBL-101 was isolated by these investigators from a gene bank as a plasmid capable of complementing an anaerobic growth defect in strain H-61 and thus may not carry the genes needed to complement mutant strains SE-19 or SE-61.

E. coli has two hydrogenase-dependent activities; production of H_2 from formate (FHL) and consumption of H_2 as a source of reductant and energy (HUP). The hydrogenase involved in these two activities may differ biochemically (3). Ackrell et al. (1) have described the presence of isoenzymes of hydrogenase in *E. coli*, based on polyacrylamide gel electrophoresis. Yamamoto and Ishimoto (28) also have reported differences in the R_f values, after polyacrylamide gel electrophoresis, for hydrogenase from cells grown in HF medium and glucose minimal medium. The reported differences in the molecular weight of hydrogenase is also in agreement with the possible existence of isoenzymes. Adams and Hall (2) have reported a molecular weight of 112,000, whereas Graham et al. (15) have found that the hydrogenase from *E. coli* has a molecular weight of 56,000 with no evidence of dimerization for the transmembrane protein (14). Although our results show the presence of two genes for hydrogenase activity, both genes are essential for hydrogenase. Mutation in either gene abolishes all the hydrogenase-dependent activities in the cell (22). It is probable that these *hyd* genes code for the hydrogenase structural gene(s) or regulatory proteins essential for hydrogenase synthesis or accessory proteins needed for activation of apoportein to active hydrogenase. Additional experiments are necessary to determine the gene-product relationships of these *hyd* genes.

Plasmid pSE-128 also carries a gene for FDH-2 (*fdv*) and an electron transport protein (*fhl*) besides the *hydB* gene (Table 5). Graham et al. (16) have isolated two groups of mutant strains defective in FDH and mapped the *fdhA* and *fdhB* mutations in the *E. coli* chromosome at 79 and 40 min, respectively. These mutant strains produced NO_3^- reductase activity, but lacked the ability to respire with NO_3^- as the terminal electron acceptor, indicating a defect in FDH-1 activity (18). Strain SE-68, found to be defective in the production of FDH-2 (Table 5), produced normal levels of FDH-1 (22) and also was capable of NO_3^- respiration. The mutation in strain SE-68 mapped in a region between *cys* and *srl* in the *E. coli* chromosome corresponding to about 59 min (4). Based on both biochemical and genetic evidence, this gene affecting FDH-2 is a gene that until now has been unreported. We would like to define this gene as *fdv* to represent the FDH activity that reduced BV and a part of the FHL system because *fdhA* and *fdhB* denote the FDH-1 (16) that is a component of the NO_3^- respiration.

Both FDH-1 and FDH-2 are selenoproteins (18) but differ in their molecular weights. Cox et al. have observed that FDH-1 and FDH-2 had molecular weights of 110,000 and 80,000 respectively (8). The two enzymes also failed to cross-react immunologically (11). It is possible that the *fdv* gene codes for the protein with a molecular weight of 80,000.

The *hydB fdv* cluster also carries a gene that is essential for the production of FHL activity (Table 4, Fig. 4). This gene product is not essential for either FDH-2 or hydrogenase activity but influences the level of FHL activity in the cell. This gene is defined as *fhl* to designate these characteristics. Yerkes et al. (29) have isolated mutant strains of *E. coli* with defects in the anaerobic electron transport (*ant*). These mutant strains produced active hydrogenase. The *ant* gene mapped between *srl* and *cysC* in the *E. coli* chromosome. The *ant* mutants were isolated as strains that reduced BV from formate at a lower rate. The genetic map location and the similarity of the *ant* phenotype to the *fhl* phenotype raise the possibility that the two genes may be the same or similar. However, differences in the restriction map of the *ant* gene region (λ *ant* [29]) and plasmid pSE-111 (Fig. 1) can be observed. Although the two genes map in the same region, it seems probable that *ant* and *fhl* are two different genes and both gene products are essential for anaerobic formate-dependent electron transport.

It is not known whether the three genes, *hydB*, *fhl*, and *fdv* constitute a single operon or two or three independent operons. However, interestingly, in this context strain SE-4 produces low levels of FDH-2 (16 units), and the FDH-2 synthesis is restored to the parental levels by reversion to Hup^+ (65 units). The reversion frequency for this *hyd* mutation (*hydB104*) is about 2×10^{-5}. This high reversion frequency for both defects (*hydB* and *fdv*) suggest that the mutation lies in the hydrogenase gene and the decline in the FDH-2 levels is a consequence of polar mutation affecting the *fdv* gene. An alternate hypothesis involving a physiological effect of the *hyd* mutation on FDH-2 production can be ruled out based on the results of cloning and complementation experiments (Table 5) because strain SE-64(pSE-125) produced only 13 units of FDH-2 activity. FDH, hydrogenase, as well as the unidentified electron transport protein(s) constitute the FHL complex (17, 30) and thus conceivably are a part of one operon. The lack of good biochemical

information on these proteins leads to ambiguity on the nature of the gene products.

The close interaction among the three genes is also evident from the inhibitory effect of some of the plasmids on FHL activity (Table 6). Plasmids pSE-126, pSE-129, and pSE-130 inhibited the FHL activity in strain SE-67 without affecting hydrogenase or FDH-2 levels. The difference between plasmids pSE-125 and pSE-126 is the orientation of the 2.8-kb insert with respect to the vector (pBR-322). The plasmid pSE-125 allowed the production of normal levels of FHL activity, whereas plasmid pSE-126 completely inhibited it. The 2.8-kb chromosomal DNA fragment is inserted in the restriction endonuclease *SalI* site in the vector which inactivates the tetracycline resistance gene. It is possible that a hybrid protein comprising a part of Tc-gene product and another part of *fdv* gene product is produced by the plasmid pSE-126. Because the Tc-gene product (7) and FHL (14, 18) are membrane proteins, the hybrid protein may lead to production of inactive FHL complex by inhibiting the flow of electrons between FDH-2 and hydrogenase. Yerkes et al. (29) also have observed a similar inhibition with cloned DNA fragments, presumably containing genes for anaerobic electron transport proteins in *E. coli*. The insert in this plasmid did not exhibit any structural homology with either pSE-111, pSE-201, or pSE-290. In this connection, it should be noted that plasmid pSE-111 inhibited the production of hydrogenase in some of the mutant strains that produced hydrogenase but lacked FHL activity (data not shown).

The inhibitory effect on FHL activity by the gene products produced from plasmid pSE-130 can be observed even in prototrophic strains but only during the early stages of induction after a shift to anaerobic conditions (Fig. 5). Once the induction of active FHL begins, the rate of induction was similar to the control without the plasmid. The total FHL levels in the cells never reached the maximum because of the cessation of growth, probably a secondary consequence of organic acid accumulation (formate) during fermentative growth. Because these proteins are membrane proteins (14), cellular growth is an essential requirement for maximum induction. Plasmid pSE-130 enhanced the production of FHL activity in strain SE-64 (Table 5). It is possible that the plasmid may not carry the complete *fhl* gene and that the truncated gene product is active in strain SE-64, whereas the same gene product inhibits the FHL activity in strains that produce normal *fhl* gene product from the chromosome (strain K-10; Fig. 5). Although this is one plausible interpretation of the available data, other alternative explanations are possible and must await additional information on the *fhl* gene and its role in FHL activity.

The FHL activity is also low whenever the fumarate-dependent H_2 uptake activity is high (Tables 4, 5, and 6). This indicates a metabolic (biochemical?) connection between the two pathways. Because H_2 is a common component, these two pathways could interact at the level of hydrogenase.

In summary, the results presented in this paper indicate that there are two genes for hydrogenase (*hydA* and *hydB*) in *E. coli*. *hydB* may be a part of an operon consisting of *hydB*, *fhl* (an electron transport protein), and *fdv*, which codes for FDH-2. All three genes are essential for maintaining active FHL complex in the cell.

ACKNOWLEDGMENTS

We thank M. C. Pascal and W. G. Martin for providing the bacterial strains and plasmid pBL-101. This work was supported by subcontract XK-2-02100-01 from Solar Energy Research Institute, Golden, Colo., and in part by grant PCM-8118350 from the National Science Foundation, contract AID/ta-C-1376 from the U.S. Agency for International Development, and contract MCS 2221 from the Gas Research Institute of the University of Florida Institute for Food and Agricultural Sciences regional biomass program.

LITERATURE CITED

1. **Ackrell, B. A. C., R. N. Asato, and H. F. Mower.** 1966. Multiple forms of bacterial hydrogenases. J. Bacteriol. **92**:828–838.
2. **Adams, M. W. W., and D. O. Hall.** 1979. Purification of the membrane-bound hydrogenase of *Escherichia coli*. Biochem. J. **183**:11–22.
3. **Adams, M. W. W., L. E. Mortenson, and J. Chen.** 1981. Hydrogenase. Biochim. Biophys. Acta **594**:105–176.
4. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12, edition 7. Microbiol. Rev. **47**:180–230.
5. **Bochner, B. R., H.-C. Huang, G. L. Schieven, and B. N. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143**:926–933.
6. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. L. Falkow.** 1977. Construction and characterization of new cloning vehicles II. A multipurpose cloning system. Gene **2**:95–113.
7. **Chopra, I., and T. G. B. Howe.** 1978. Bacterial resistance to the tetracyclines. Microbiol. Rev. **42**:704–724.
8. **Cox, J. C., E. S. Edwards, and J. A. DeMoss.** 1981. Resolution of distinct selenium-containing formate dehydrogenases from *Escherichia coli*. J. Bacteriol. **145**:1317–1324.
9. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. A manual for genetic engineering. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. **77**:7347–7351.
11. **Giordano, G., C. Medani, M. A. Mandrand-Berthelot, and D. H. Boxer.** 1983. Formate dehydrogenases from *Escherichia coli*. FEMS Lett. **17**:171–177.
12. **Glick, B. R., P. Y. Wang, H. Schneider, and W. G. Martin.** 1980. Identification and partial characterization of an *Escherichia coli* mutant with altered hydrogenase activity. Can. J. Biochem. **58**:361–367.
13. **Glick, B. R., J. Zeisler, A. M. Banaszuk, J. D. Friesen, and W. G. Martin.** 1981. The identification and partial characterization of a plasmid containing the gene for membrane associated hydrogenase from *E. coli*. Gene **15**:201–206.
14. **Graham, A.** 1981. The organization of hydrogenase in the cytoplasmic membrane of *Escherichia coli*. Biochem. J. **197**:283–291.
15. **Graham, A., D. H. Boxer, B. A. Haddock, M. A. Mandrand-Berthelot, and R. W. Jones.** 1980. Immunological analysis of the membrane-bound hydrogenase of *Escherichia coli*. FEBS Lett. **113**:167–172.
16. **Graham, A., H. E. Jenkins, N. H. Smith, M.A. Mandrand-Berthelot, B. A. Haddock, and D. H. Boxer.** 1980. The synthesis of formate dehydrogenase and nitrate reductase proteins in various *fdh* and *chl* mutants of *Escherichia coli*. FEMS Lett. **7**:145–151.
17. **Gray, C. T., and H. Gest.** 1965. Biological formation of molecular hydrogen. Science **148**:186–192.
18. **Haddock, B. A., and C. W. Jones.** 1977. Bacterial respiration. Bacteriol. Rev. **41**:47–99.
19. **Jones, R. W.** 1980. The role of the membrane-bound hydrogenase in the energy-conserving oxidation of molecular hydrogen by *Escherichia coli*. Biochem. J. **188**:345–350.
20. **Karube, I., N. Urano, T. Yamada, H. Hirochika, and K. Sakaguchi.** 1983. Cloning and expression of the hydrogenase gene from *Clostridium butyricum* in *Escherichia coli*. FEBS Lett. **158**:119–122.
21. **Krasna, A. I.** 1984. Mutants of *Escherichia coli* with altered hydrogenase activity. J. Gen. Microbiol. **130**:779–787.
22. **Lee, J. H., P. Patel, P. Sankar, and K. T. Shanmugam.** 1985. Isolation and characterization of mutant strains of *Escherichia*

coli altered in their H_2 metabolism. J. Bacteriol. **162**:344–352.
23. **Macy, J., H. Kulla, and G. Gottschalk.** 1976. H_2-dependent anaerobic growth of *Escherichia coli* on L-malate: succinate formation. J. Bacteriol. **125**:423–428.
24. **Pascal, M. C., F. Casse, M. Chippaux, and M. Lepelleiter.** 1975. Genetic analysis of mutants of *Escherichia coli* K12 and *Salmonella typhimurium* LT2 deficient in hydrogenase activity. Mol. Gen. Genet. **141**:173–179.
25. **Pecher, A., F. Zinoni, C. Jatisatienr, R. Wirth, H. Hennecke, and A. Böck.** 1983. On the redox control of synthesis of anaerobically induced enzymes in enterobacteriaceae. Arch. Microbiol. **136**:131–136.
26. **Peck, H. D., Jr., and H. Gest.** 1957. Formic dehydrogenase and the hydrogen lyase enzyme complex in coli-aerogenes bacteria. J. Bacteriol. **73**:706–721.
27. **Rodriguez, R. L., R. W. West, R. C. Tait, J. M. Jaynes, and K. T. Shanmugam.** 1981. Isolation and characterization of the *his*G and *his*D genes of *Klebsiella pneumoniae*. Gene **16**:317–320.
28. **Yamamoto, I., and M. Ishimoto.** 1978. Hydrogen-dependent growth of *Escherichia coli* in anaerobic respiration and the presence of hydrogenases with different functions. J. Biochem. **84**:673–679.
29. **Yerkes, J. H., L. P. Casson, A. K. Honkanen, and G. C. Walker.** 1984. Anaerobiosis induces expression of *ant*, a new *Escherichia coli* locus with a role in anaerobic electron transport. J. Bacteriol. **158**:180–186.
30. **Zajic, J. E., N. Kosaric, and J. D. Brosseau.** 1978. Microbial production of hydrogen. Adv. Biochem. Eng. **9**:57–109.