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Randomly Induced *Escherichia coli* K-12 Tn*5* Insertion Mutants Defective in Hydrogenase Activity

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Systematic screening of 6.10^4 independent Tn*5* insertion mutants of *Escherichia coli* yielded one new hydrogenase locus, *hydF*, mapping near 64.8 min, i.e., close to the *hydL* locus (K. Stoker, L. F. Oltmann, and A. H. Stouthamer, J. Bacteriol. 170:1220–1226, 1988). It regulated specifically the activity of the hydrogenase isoenzymes, formate dehydrogenase and lyase activities being unaffected. In *hydF* mutants, hydrogenase 1 and 2 activities were reduced to 1% of the parental level, whereas the electrophoretically labile part was present at about 20% of the parental level. H_2 uptake was also reduced to about 20%, which suggested a relationship between these two activities. Experiments with ^{63}Ni indicated that hydrogenase isoenzymes 1 and 2 might be present in these strains but in an inactive form. The *hydF* product might therefore be a posttranslational activator. At least three other mutant classes were isolated. Additional data were obtained on coisolated, nickel-restorable *hydC* mutants (L. F. Wu and M.-A. Mandrand-Berthelot, Biochimie 68:167–179, 1986). These strains were found to suffer a general impairment of nickel uptake. Restoration of hydrogenase activities was specific for $NiCl_2$ and inhibited by chloramphenicol, which indicated an effect either on the transcription of hydrogenase(-associated) genes or by cotranslational incorporation in nickel-containing enzymes (e.g., in hydrogenases). The *hydC* mutation could not be complemented in *trans*, evidence that the *hydC* product is not a nickel transport protein but rather a *cis*-acting regulatory gene. Parent HB101, *hydF* mutants, and the other mutants were further analyzed by monitoring the induction of hydrogenase and hydrogenase-associated activities upon transition of cells from aerobic to anaerobic growth. These experiments also revealed a correlation between the early-induced H_2 uptake route and labile hydrogenase activity. The formate hydrogenlyase induction patterns followed quite well the slower induction patterns of hydrogenases 1 and 2.

Escherichia coli is able to evolve H_2 (formate hydrogenlyase [FHL] activity) as well as oxidize H_2 in an energy-conserving manner (H_2 uptake [Hup] activity) by means of hydrogenases (1, 21). Thus far, at least three isoenzyme activities have been demonstrated: activities of the two electrophoretically stable hydrogenases 1 and 2 (3, 4, 26) and a third activity, which is inactivated upon gel electrophoresis and may consist of one or more isoenzymes (25, 28). All of these isoenzymes probably contain nickel as a cofactor. In addition to hydrogenases, a formate dehydrogenase (FDH-H) is linked to the FHL pathway (23), whereas fumarate reductase and cytochromes are part of the second pathway, constituting an anaerobic respiratory chain.

Expression of most of these components is inducible, depending on the redox state of the environment, the presence of substrates (e.g., formate), and the source of energy (27). The underlying genetic regulation mechanisms, however, are still poorly understood. Although more than 10 genetic loci have been described thus far, mapping near 58 min (11, 15, 22, 24, 30), 65 min (15, 28), and 77 min (31), none of these searches was exhaustive, and it is still unknown whether these studies revealed all loci involved in H_2 metabolism. We therefore undertook a systematic screening of a large number of Tn*5* insertion mutants; this search yielded one new locus, which was further characterized.

Another problem is how the products expressed by these loci carry out their functions. This too is largely unknown, although some suggestions have been made. For example, *hydC* (31) and the loci affected in mutants FD-12 (11) and AK23 (6) might be involved in nickel metabolism, the *hyd-17* locus might make up the structural gene for the labile hydrogenase 2 (5), and *hydL* might regulate expression of the labile hydrogenase L and a second component, both required for Hup activity (28). In this paper, additional data are provided on *hydC* and a hypothetical function is assigned.

A third question dealt with here is which hydrogenase isoenzyme catalyzes the FHL reaction and which catalyzes the Hup pathway. Although several reports suggest a respiratory role for hydrogenase 2, our previous results (28) suggested that a labile hydrogenase species was involved in H_2 uptake. In this study, further evidence is provided in favor of the latter model.

MATERIALS AND METHODS

Bacterial strains. For isolation of mutants, *E. coli* RR1 (F$^-$ *pro leu thi lacZ gal xyl ara mtl hsdS phx supE rpsL*) or its *recA* derivative HB101 was used. Growth of λNK421 was on strain JMsu6 [F$^-$ Δ(*lac pro*) *ara argE*(Am) *nalA rif thi supB*(Am)] (20).

Donor strains for coconjugation experiments were PK191 [Hfr *thi* Δ(*lac pro*) *phx sup*(Am) *colV*], a noncolicinogenic derivative of PK19 (*oriT* at 43 min, clockwise transfer) (13) and KL14 (Hfr *phx thi rel*) (*oriT* at 68 min, clockwise transfer) (16). PK191-II, PK191-III, and KL14-IV were constructed by P1 *vir*-mediated transduction from prototype strains of classes II (BO5), III (BO32), and IV (BO26), respectively.

Recipient strains were CSH57B (F$^-$ *leu purE trp his argG ilvA met thi lac gal xyl ara mtl tonA tsx phx sup rpsL*) (19), KMBL1418 (F$^-$ *thyA his phx rpsL*), LBE1930 (*thyA serA his metG galE phx drm rpsL*), and KN126 [F$^-$ *trpE*(Am) *tyr*(Am) *ilv malT lam phx supD*(Ts) *rpoB ompC*] (29). These

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strains were obtained from the Phabagen Collection, University of Utrecht, Utrecht, The Netherlands.

For cotransduction experiments, we also used AT2699 (F⁻ *hisG thyA metC argG lacY gal-6 malT tsx-1 supE rpsL*), JM2071 (F⁻ *his leu ilvA* Δ*lac mglP galP*::Tn*10*), and JGC127 [F⁻ *leuB tonA lacY supE gal-6 hisG thyA argG rpsL malA dnaM*(Ts) *zhg-922*::Tn*10 xyl mtl deoC*] (7, 9), all supplied by the *Escherichia coli* Genetic Stock Center, Yale University, New Haven, Conn. For Tn*5* insertion mutagenesis, we used λNK421 (*c*I857 *b*221 *O*am*P*am *rex*::Tn*5*), a gift from G. E. De Vries.

Media and growth conditions. For anaerobic batch growth to obtain induction curves, freshly grown overnight cultures of parent strain HB101 and the four prototype strains, each representing a mutant class, were subcultured 1:100 to the same initial density (A_{600} = 0.05) in prewarmed brain heart infusion broth (38 g/liter, pH 7.4; GIBCO Laboratories, Grand Island, N.Y.) and distributed over a series of 250- or 100-ml flasks. The flasks were completely filled, tightly stoppered, and left at 37°C without shaking for 1 to 6 h. When used, chloramphenicol was added at a concentration of 170 μg/ml. Growth in the presence of $^{63}NiCl_2$ (specific activity, 0.6 mCi/μmol of Ni) was carried out at a concentration of 1.2 μM. All other media and growth conditions were as described previously (28).

Tn*5* insertion mutagenesis. Tn*5* insertion mutagenesis was performed as described previously (8, 14, 28).

MV-filter assay. Colonies were screened for hydrogenase activity by transfer to filter paper drenched in 20 mM methylviologen (MV) (E'_0 = 440 mV)–10 mM Tris chloride (pH 7.4). The filter paper was placed in an atmosphere of 5% H_2–95% N_2 as described by Glick et al. (10). Positive colonies turned blue within a few minutes.

Sample preparations. All steps were carried out at ice bath temperatures. Whole-cell samples were prepared by harvesting cells by centrifugation at 6,000 × *g* for 10 min and suspending the pellet in 50 mM phosphate buffer (pH 7). Densities were measured at 600 nm in cuvettes of 1-cm path length. Densities of the suspensions applied ranged from 25 to 150. Solubilized membranes were prepared by sonicating the whole-cell preparations described above on ice with five pulses of 20 s each (Sonifier, amplitude 16 at medium power; Branson Sonic Power Co., Danbury, Conn.). Triton X-100 was added after sonication at a final concentration of 0.5% (vol/vol). The S100 extract was the supernatant obtained after centrifugation of the solubilized membranes at 100,000 × *g* at 4°C for 1 h. Solubilized membranes and S100 extracts were prepared aerobically. Samples were used fresh or after storage at −20°C. Loss of enzymatic activity due to the latter treatment was limited, reproducible, and equal for all samples prepared and stored in the same manner.

Assays. Hydrogenase, FDH-H, and the pathways FHL, formate to NO_3^-, and H_2 to fumarate (Hup) were assayed manometrically in Warburg experiments at 35°C as described previously (28). In addition, relative (total) hydrogenase activities were determined colorimetrically by titration in 0.35-ml microdilution wells (28). Relative activities of the electrophoresable hydrogenases were read from an activity-stained neutral, nondenaturating polyacrylamide gel by comparing the intensity of the coloration (directly proportional to the activity) with that of a standard dilution series of a parental extract, applied on the same gel and prepared under identical conditions (28). Fumarate reductase was determined spectrophotometrically in a Thunberg cuvette (25). Protein concentrations were determined by the method of Lowry et al. (17). Cell suspensions having an optical density of 1 at 600 nm typically contained 2.5 × 10^8 cells and 0.2 mg of protein per ml.

PAGE. For polyacrylamide gel electrophoresis (PAGE), S100 extracts were electrophoresed on neutral, nondenaturating 7.5% (wt/vol) polyacrylamide gels (3) either as vertical slabs (0.2 by 12 by 15 cm) at 50 mA or as cylindrical gels (90 by 5 mm) at 5 mA per gel, both for 2 to 4 h at 15 to 20°C. Activity staining was for 1 to 16 h at room temperature as described previously (3).

Genetic experiments. Coconjugation experiments and P1 *vir*-mediated transduction were carried out according to the method of Miller (19).

Scintillation counting. Cylindrical gels loaded with ^{63}Ni-labeled material were fractionated into 60 1.5-mm slices and put in separate glass vials filled with 10 ml of scintillation fluid (Insta-Gel; Packard Instrument Co., Inc., Rockville, Md.). Counting was for 10 min per sample in an LKB Rack Beta (LKB/Wallac) in a 0 to 70 kEV window.

RESULTS

Isolation and physiological characterization of hydrogenase mutants. By means of Tn*5*-mediated insertion mutagenesis, 32 strains negative in the MV-filter assay were isolated. Six of them (class I) have been described recently (28). The remaining 26 were physiologically characterized (Table 1). In addition to being tested for properties related to hydrogen metabolism, the strains were analyzed for complementation by $NiCl_2$ and F-prime factors. As a result, three classes in addition to the class I mutants mentioned above could be discerned. Class II strains behaved as typical *hyd* mutants (hydrogenase⁻, FDH-H⁻, FHL⁻, Hup⁻), complementable by factor F'143 but not by nickel salts (11, 15, 22, 30). Class III strains were similar to class I mutants, since they were specifically impaired in hydrogenase activity and displayed normal levels of formate dehydrogenase or FHL activity. Both were complementable by factor F'116 and not by $NiCl_2$. In contrast to *hydL* mutants, however, class III mutants were also defective in hydrogenases 1 and 2. The activity of these isoenzymes, as determined by neutral, nondenaturing PAGE and activity staining, amounted to less than 1% of the parental level. The approximately 20% hydrogenase residual activity (Table 1) of these mutants was therefore concluded to be of the electrophoretically labile kind. The fact that Hup activity was also reduced to 15 to 20% of the wild-type level was considered another indication that the latter two activities might be related, as has been proposed previously (28).

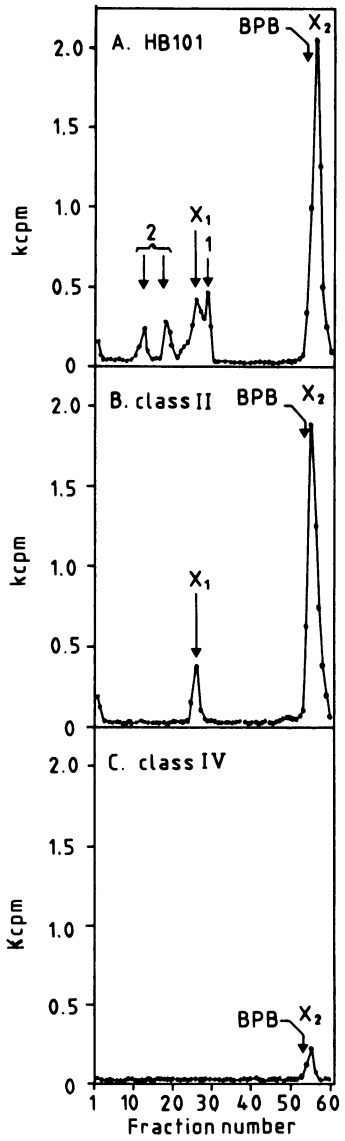
An intriguing observation with respect to the class III strains was that when a representative mutant (BO32) was grown in the presence of ^{63}Ni and hydrogenase content was analyzed by neutral, nondenaturing PAGE followed by scintillation counting, the hydrogenase isoenzymes could be demonstrated at normal levels (Fig. 1A). Apparently, the *hydF* product catalyzes a posttranslational activating step of hydrogenase synthesis.

Class IV mutants showed a phenotype very similar to that of the *hyd* mutants of class II, but their defects were completely restorable by nickel supplementation and not by the introduction of F'143 or F'116. When the FHL activity of class IV mutants was plotted against nickel concentration, an asymptotic curve was obtained which showed 50% restoration at 50 μM and reached a plateau at 250 μM $NiCl_2$ (not shown). The effect of nickel ions was highly specific. No effect of other divalent cations, such as Mg^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, Fe^{2+}, and Ca^{2+} could be observed. Palladium

TABLE 1. Physiological activities related to hydrogenase metabolism and other properties of parent strain *E. coli* HB101 and four classes of hydrogenase mutants[a]

Strain or mutant class (no. of strains)	Activity (nmol/mg per min)						PAGE activity (%)	Reaction or property				
	Hydrogenase[b]		FDH-H	FDH-N[c]	FHL[d]	Hup[e]		Complementation by:		Locus		
	Whole cells	Solubilized membranes						0.5 mM $NiCl_2$	F′ factor	Designation	Map position (min)	Reference(s)
Parent	5.6	12.5	3.2	94	6.6	13	100	−				
Mutant class												
I (7)	0.9	2.3	3.1	87	6.6	0.01	100	−	116	*hydL*	64.8	28
II (12)	0.01	0.02	0.01	92	0.01	0.01	0.1	−	143	*hyd*	~58	11, 15, 22, 24, 30
III (5)	0.9	2.1	3.2	90	6.5	2.1	0.1	−	116	*hydF*	64.8	This study
IV (5)	0.01	0.02	0.16	91	0.30	0.35	0.1	+	None	*hydC*	76.5	31, this study

[a] Cells were plate grown (under low-inducing conditions) (see also Fig. 1). Procedures were as described in Materials and Methods. Data are averages of all strains belonging to one class. The mutant strains tested grew normally in batch culture with or without O_2 and anaerobically on SV supplemented with 40 mM glycerol and 40 mM fumarate. Fumarate reductase activity was unimpaired. Three strains gave inconsistent results and were excluded from consideration.
[b] H_2: MV oxidoreductase activity was determined manometrically and by microdilution.
[c] Cells were grown in the presence of 0.35% KNO_3. We measured the formate-to-NO_3^- reaction.
[d] Formate to CO_2 plus H_2.
[e] H_2 to fumarate.



had a slight inhibiting effect on FHL residual activity (not shown).

Aerobic growth, anaerobic growth, or growth on glycerol and fumarate was unimpaired for all mutant strains. Also, FDH-N and fumarate reductase showed wild-type activity levels.

Genetic analysis. Conjugation experiments with Hfr strain PK191-II, carrying the class II mutation, showed a high coconjugation frequency between *hydII*::Tn*5* (Km^r) and *cysC* at 59.3 min, further confirmation that the locus interrupted was probably one of the *hyd* loci described previously (11, 15, 22, 24, 30). It was not further mapped.

Coconjugation with PK191-III yielded results that were almost identical to those obtained for class I (*hydL*) mutants described previously (28). This held true for cotransduction experiments as well (Table 2). Therefore, a second locus, referred to as *hydF*, was assigned near 64.8 min. The method used did not allow us to determine the orientation of *hydF* to *hydL*.

For mapping of class IV mutants, the interrupted locus (*hydIV*::Tn*5*) was transduced to a series of Hfr strains. It was found that Hfr KL14-IV could transfer the Km^r phenotype within 15 min. Subsequently, cotransduction frequencies were determined with *malT* (75.2 min) and *zhg* (75.8 min) (Table 2). From these data, it could be calculated (32) that the *hydIV* locus was localized at about 1.4 min from *malT* and 0.7 min from *zhg*, i.e., near 76.5 min. This finding, in conjunction with the physiological data on this mutant (Table 1), led to the conclusion that the *hydIV* locus is probably identical to the *hydC* locus previously described by Wu and Mandrand-Berthelot (31). To supplement the work

FIG. 1. ^{63}Ni-labeled components analyzed by neutral, nondenaturing PAGE. (A) HB101. An identical pattern was obtained when a *hydF* mutant was analyzed. (B) *hyd* strain BO5 (class II). (C) *hydC* strain BO26 (class IV). Cells were anaerobically grown in 50-ml batch cultures on brain heart infusion broth containing 40 μl of $^{63}NiCl_2$ to a density of 2.5×10^8 per ml. The cells were washed in phosphate buffer, pH 7.0, and converted into 1 ml of S100 extract. A 100-μl amount of sample was applied per cylindrical gel. Other details are as described in Materials and Methods. BPB, Bromophenol blue marker.

TABLE 2. Cotransduction frequencies of the Tn*5*-interrupted loci of *hydF* and *hydC* mutants with several markers[a]

Donor lysate[b] (relevant genotype)	Recipient strain (relevant genotype)	Selected phenotype (locus)	No. tested	Unselected	
				Phenotype locus	%
JM2071-III (*galP*::Tn*10* *hydL*::Tn*5*)	AT2699 (*metC*)	Tet^r (*galP*)	400	Km^r (*hydL*)	5
				Met$^+$ (*metC*)	1
		Met$^+$ (*metC*)	400	Km^r (*hydL*)	47
				Tet^r (*galP*)	1
KL-14 (*hydC*::Tn*5*)	KN126 (*malT*)	Mal$^+$ (*malT*)	200	Km^r (*hydC*)	2.5
JGC127-IV (*zhg*::Tn*10* *hydC*::Tn*5*)	RR1	Tet^r (*zhg*)	250	Km^r (*hydC*)	26

[a] Transduction, colony purification, and retesting for unselected markers were as described by Miller (9) except that 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was used instead of citrate. See Materials and Methods for complete genotypes.
[b] P1 *vir* host.

described by these researchers, we studied some other aspects of this mutant in more detail.

F-prime complementation of *hydC* mutants. On the basis of the mapping data, one would expect that *hydC* mutants could be complemented by factor F′140, carrying a chromosomal segment from 68 to 83 min. To rule out imperfect transfer or segregation, we first crossed *hydC*::Tn*5* into KN126 (*malT*) so that we could select for *malT* complementation (Table 3), a locus (75.2 min) very close to *hydC*::Tn*5* (76.5 min). As a result, we found that *hydC* mutants could be complemented in *trans* orientation with respect to the Mal$^-$ phenotype but not with respect to the hydrogenase-negative character of the mutants.

Chloramphenicol inhibition of nickel complementation. When class IV mutants were grown in the presence of 0.5 mM $NiCl_2$, the induction profile for FHL activity was identical to that of parent strain HB101 (data not shown). When $NiCl_2$ was added after 3.5 h of growth, within 30 min the strain had resumed maximum, wild-type activity. However, when the latter experiment was preceded by the addition of chloramphenicol after 2.5 h of growth, reactivation of the FHL pathway was completely abolished. As a consequence, we concluded that reactivation was not brought about by posttranslational nickel incorporation in one or more inactive core proteins. If nickel does exert its effect by incorporation, it can apparently do so only cotranslationally. Alternatively, nickel may act on the transcriptional level, as has been observed for the *hydC* locus (31).

Nickel processing in *hydC* mutants. Since at least two hydrogenase isoenzymes in *E. coli* have nickel as the catalytic center (3, 12), the proper import, transport, and incorporation of this atom is obligatory for the functioning of hydrogen metabolism. Therefore, the fact that the phenotypic defects in *hydC* mutants could be restored by growth in an excess of $NiCl_2$ strongly suggested that the gene inactivated might be involved in nickel processing (31). That this is the case was demonstrated by growing HB101 and mutant strains in the presence of β-emitting $^{63}NiCl_2$, preparing S100 extracts, and analyzing the extracts on gel (Fig. 1). The wild-type extract (Fig. 1A) displayed five distinct peaks. Three of them could be attributed to hydrogenases 1 and 2. Two peaks, X_1 and X_2, were unidentified and were assumed to be hydrogenase independent. This assumption was supported by the analysis of class II extracts (Fig. 1B). Class II mutants are hydrogenase negative, not only catalytically (Table 1) but probably also, as are *hyd* mutants, immunologically (11). The hydrogenase-associated peaks were absent, whereas the X_1 and X_2 peaks were present at wild-type levels. Whatever the nature of these labeled components might be, they served as useful internal markers for nickel utilization. It seems reasonable, therefore, to conclude from the low-intensity profile of *hydC* extracts (Fig. 1C) that these mutants were defective in nickel processing, possibly at the step of nickel uptake.

TABLE 3. Effect of factor F′140 on *hydC* mutants

Strain (relevant genotype)	Phenotype	Hydrogenase activity[a]
KN126 (*malT rpoB*)	Mal$^-$ Rif^r	+
KN126-IV (*malT rpoB hydC*::Tn*5*)	Mal$^-$ Rif^r Km^r	−
KN126-IVF1 (*malT rpoB hydC*::Tn*5* F′140)[b]	Mal$^+$ Rif^r Km^r	−

[a] Determined by the MV-filter assay as described in Materials and Methods.
[b] Plate conjugation of CGSC4289 (F′140 Km^s Rif^s) × KN126-IV, followed by transfer on grid to minimal agar plates for KN126 with kanamycin and rifampin and with maltose instead of glucose. This transfer procedure was repeated several times, also to rich agar plates. Each transfer plate was tested for hydrogenase activity. The parent strains did not grow on selective plates.

Growth under various FHL-inducing conditions. Thus far, we had been analyzing strains cultured under one physiological condition, plate grown, in order to test them under exactly the same conditions as were used for isolation. Plate growth might be considered a low-inducing condition with respect to FHL activity, since this activity could be increased 50-fold by anaerobic batch growth during several hours. Therefore, to extend our physiological characterization and to determine whether induction patterns could reveal something of the relationship between the individual hydrogenases and their physiological functions, we grew HB101 and a prototype *hydF* strain, as well as representatives of the other mutant classes, in batch cultures in closed vessels. The cultures were inoculated with aerobically grown cells in air-saturated broth. Samples were drawn, and several physiological parameters were determined (Fig. 2).

With respect to parent strain HB101, we found that hydrogenase isoenzymes 1 and 2 developed rather slowly, possibly under the influence of an increasing formate concentration, after an incubation period of 3 to 4 h (Fig. 2E), whereas total hydrogenase activity reached the maximum value after only 2 h of growth (Fig. 2C). This early development of hydrogenase activity, therefore, was brought about almost exclusively by the labile species and was induced by anaerobiosis rather than formate. Again, we observed a correlation between labile hydrogenase activity and the Hup pathway (Fig. 2C and D). This relationship was also found between isoenzymes 1 and 2 and FHL activity (Fig. 2E and F).

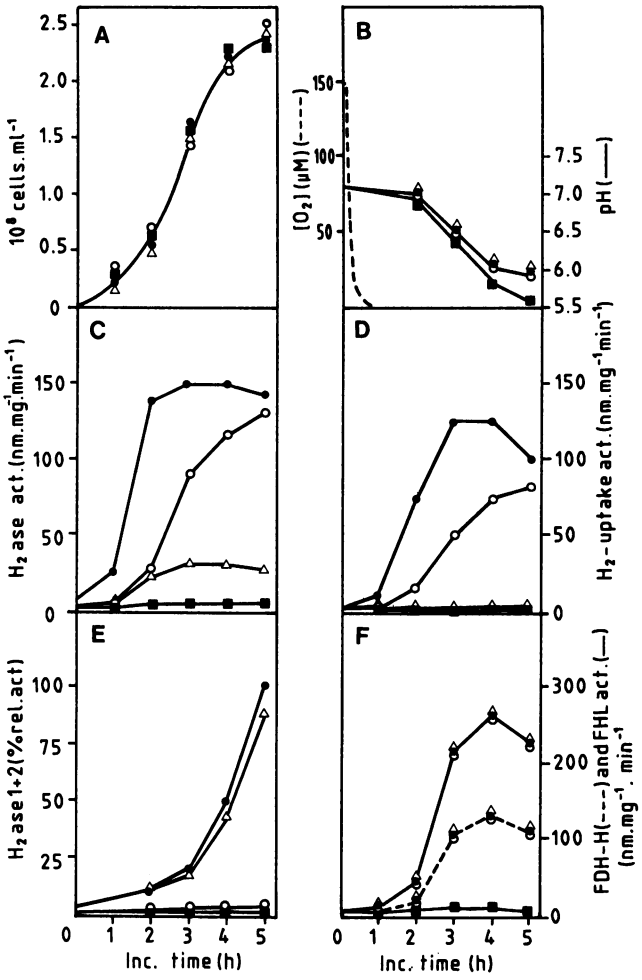


FIG. 2. Induction curves. Parent strain HB101 (●) and representative *hydL* (△), *hydF* (○), and *hyd hydC* (■) strains were grown in air-tight batch cultures. Samples were drawn and analyzed for several physiological parameters as described in Materials and Methods. (A) Cell density. (B) pH and O_2 concentration. (C) Total hydrogenase activity. (D) H_2-dependent fumarate reduction. (E) Hydrogenase isoenzymes 1 and 2 (considered here as one electrophoretically stable activity, since the intensity ratio among the individual bands did not vary significantly). Activity of HB101 at 5 h was set at 100%. (F) FDH-H (- - - - - -) and FHL (——————) activities. Fumarate reductase activity for all strains was approximately 90 μmol of benzyl viologen (reduced) oxidized per mg per min and remained constant during the culture period (not shown).

We observed for the *hyd*, *hydL*, and *hydC* mutant strains induction patterns which were normal in shape, although all values remained proportional to those given in Table 1. An exception was *hydF*. After 5 h of growth, total hydrogenase activity became almost equal to that of the parent strain (Fig. 2C). Hydrogenases 1 and 2, however, always remained at levels of less than 1%. From this result, one could deduce that it was only the labile activity that escaped repression due to the mutation in this strain. This result was accompanied by a parallel increase in Hup activity from 20 to 70%, an observation that was consistent with our speculation about a Hup function for at least part of this labile activity.

DISCUSSION

Missing hydrogenase loci. More than 6×10^4 Tn*5* insertion mutants were screened for defects in hydrogenase activity; use of this number of mutants ensured that each gene would have been hit once at least. Nevertheless, not all loci described in the literature were found. This discrepancy might be explained in part by the fact that we did not extensively analyze our set of *hyd*-like mutants (22). Several mutants mapping in this region, between 58 and 59 min, display more or less the same phenotype (hydrogenase$^-$, FDH-H$^-$, complementable by F'143). Therefore, our set of 12 class I mutants might be genetically heterogeneous and contain *hyd*, *hydA*, and *hydB* strains. However, none of the mutants was complementable by nickel supplementation, as has been reported for FD-12 (11) and *hydE* (6) mutants. Also, none had retained significant levels of hydrogenase 1 or 2, as was the case for the *hyd-17* strain (5, 24). Screening by means of the MV-filter assay, we apparently missed these types of mutants. It is not unlikely, however, that a *hyd-17* strain in particular will have a rather normal phenotype under these test conditions. Alternatively, the type of mutagen might determine whether a certain mutation will be found. When point mutations are induced, different hits within one gene may result in different phenotypes; also, the existence of double mutations cannot be excluded. These effects are not encountered when transposons are used, and therefore apparently fewer mutated loci are obtained. On the other hand, transposons may have polar effects that mask the presence of downstream genes.

The *hydF* locus. Despite the problems discussed above, one new hydrogenase locus, *hydF*, was characterized. The *hydF* product behaves as a specific regulator of hydrogenase activity. It may induce hydrogenase activity on a transcriptional or posttranslational level. Lee et al. (15) also reported mutations in this area. These *hup* mutations were roughly mapped at around 65 min and were suggested to affect the expression not of hydrogenases, but of a transmembranous electron transport protein coupling hydrogenase to viologens. In our *hydF* mutants, however, the amount of solubilized hydrogenase isoenzymes was also drastically reduced, as shown by neutral PAGE and activity staining, under which conditions the enzymes reacted directly with the dye. Furthermore, *hup* mutants were partially impaired in FDH-H and FHL activities, which was not found for *hydF* strains. Apparently, the 65-min region constitutes a complex gene cluster involved in hydrogen metabolism.

The *hydC* locus. The product of the *hydC* gene was suggested by Wu and Mandrand-Berthelot (31) to be involved in nickel metabolism. Our result showed that this mutation could not be complemented by introducing the homologous DNA segment as an episome. If the *hydC* product were a nickel uptake protein, this defect would be restored in this partially diploid situation. Since this was not found, we hypothesize that, because of the inactivation of *hydC*, a gene that somehow leads to a blockade of nickel uptake (2) becomes constitutively expressed. In that speculative model, the *hydC* product might be the *cis*-acting repressor of the latter gene. (An elegant proof would be the demonstration that a second mutation, in the latter gene, restores hydrogenase activity in *hydC* strains.)

Isoenzyme function. Thus far, our studies have provided three sources of evidence that the respiratory Hup pathway is catalyzed by a labile hydrogenase (hydrogenase L): (i) the simultaneous disappearance of labile hydrogenase activity and Hup activity in *hydL* mutants (28), (ii) the correlation between the rapid induction of labile hydrogenase activity and Hup function when measured in *E. coli* HB101 during the transition from aerobic growth to anaerobiosis (Fig. 2C and D), and (iii) the same correlation observed in *hydF*

mutants upon prolonged fermentative growth (Fig. 2C and D). In contrast, Sawers et al. found that under conditions that are assumed to stimulate anaerobic respiration, hydrogenase 2 in particular was induced (25). Also, Birkmann et al. reported the analysis of mutant *hyd-17*, which lacked specifically the labile hydrogenase activity (hydrogenase 3), similar to our *hydL* strains, but this characteristic was accompanied by a defect in FHL function (5). Unfortunately, the Hup activity in this strain was not reported. In short, it is still unclear whether hydrogenase 3 is identical to hydrogenase L and what the function of the enzymes is. Undoubtedly, more data will become available. In particular, isolation of unambiguously characterized structural mutants for one of the isoenzymes will be of great help.

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