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Mutational Analysis and Characterization of the *Escherichia coli hya* Operon, Which Encodes [NiFe] Hydrogenase 1

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Deletion mutants of *Escherichia coli* specific for hydrogenase isoenzyme 1 (HYD1) have been constructed and characterized. The *hya* operon, which contains genes for the two HYD1 structural subunits and four additional genes, was mapped at 22 min on the *E. coli* chromosome. The total hydrogenase activities of the HYD1-negative mutant and wild-type strains were similar. However, the formate dehydrogenase activity associated with the formate hydrogen lyase pathway was lower in the mutant. The *hya* mutant (strain AP1), complemented with only the hydrogenase structural genes (*hyaAB*), produced antigenically identifiable but inactive HYD1 protein. The first five genes of *hya* (*hyaA* to *hyaE*) were required for the synthesis of active HYD1, but wild-type levels of HYD1 activity were restored only when mutant cells were transformed with all six genes of the operon. When AP1 was complemented with *hya* carried on a high-copy-number plasmid, the HYD1 structural subunits were overexpressed, but the excess protein was unprocessed and localized in the soluble fraction of the cell. The products of *hyaDEF* are postulated to be involved in the processing of nascent structural subunits (HYAA and HYAB). This processing takes place only after the subunits are inserted into the cell membrane. It is concluded that the biosynthesis of active HYD1 is a complex biochemical process involving the cellular localization and processing of nascent structural subunits, which are in turn dependent on the insertion of nickel into the nascent HYD1 large subunit.

Hydrogen metabolism in *Escherichia coli* is tightly regulated by parameters of growth (2, 27) and involves three discrete nickel-containing hydrogenases: two electrophoretically stable, membrane-bound heterodimeric enzymes, hydrogenase 1 (HYD1) (28) and hydrogenase 2 (HYD2) (3), and a labile hydrogen-evolving hydrogenase, hydrogenase 3, which is as yet uncharacterized (27). HYD1 has been purified from anaerobically grown cells and biochemically characterized (28). It has a molecular mass of 200 kDa, is composed of two large (60-kDa) and two small (32-kDa) subunits, and contains 11 nonheme iron atoms and 1 g-atom of nickel per mol of enzyme (28). The genes encoding the large and small subunits of HYD1 have been sequenced and shown to occur in a single operon (*hya*) which contains four additional open reading frames (ORFs) (22). The derived amino acid sequences of the HYD1 small and large subunits have 70% homology to those of the [NiFe] hydrogenases from *Azotobacter chroococcum* (7), *Azotobacter vinelandii* (20), *Bradyrhizobium japonicum* (29), and *Rhodobacter capsulatus* (15) and about 30% homology to those of the [NiFe] and [NiFeSe] hydrogenases from the sulfate-reducing bacteria (17, 21, 35).

Comparison of the nucleotide-derived amino acid sequence of the small subunit of HYD1 with the NH_2-terminal amino acid sequence of the mature small subunit, as determined by protein sequencing (8), indicates the presence of a long mitochondrionlike signal peptide (22). The large subunit lacks a recognizable NH_2-terminal signal peptide, since the NH_2-terminal protein sequence (8) and the nucleotide-derived protein sequence are colinear (22). Both peptides appear to be processed, however, and the possibility of other modifications of the large subunit has not been eliminated.

Many hydrogenase mutants of *E. coli* have been isolated and can be broadly classified into three types, as follows: (i) mutants lacking all hydrogenase activity and involving loci regulating nickel uptake and/or processing (5, 36–38) and other yet-to-be-characterized loci (16, 26) (many of these mutations, clustered at 58 min, have been shown to be lesions in the *hyp* operon, which regulated the activities of all three *E. coli* hydrogenase isoenzymes [18]); (ii) mutants defective in hydrogen evolution from formate, i.e., lacking one or more structural or regulatory components of the formate hydrogen lyase system (16, 24, 25, 30, 33); and (iii) mutants unable to utilize hydrogen in the presence of electron acceptors (16, 32). None of the mutants, however, have been shown to specifically impair HYD1 activity, indicating that the mutations are not in the *hya* operon. In this paper, we report the construction, biochemical characterization, and genetic analysis of HYD1-specific deletion mutants.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All bacterial strains used in this study are derivatives of *E. coli* K-12 and are listed in Table 1. Bacteria were cultured in Luria broth with 0.4% glucose as the carbon source (LBG). Antibiotics were added at final concentrations of 50 μg/ml (kanamycin), 100 μg/ml (ampicillin), and 20 μg/ml (chloramphenicol). For studying the incorporation of nickel into HYD1, cells were grown anaerobically in LBG in the presence of 1.2 μM $^{63}NiCl_2$ (1 mCi/ml for 5 h).

Anaerobic growth. Overnight cultures of different strains were inoculated at a final concentration of 1% (vol/vol) into LBG in 250-ml tissue culture bottles. The bottles were filled to the top, sealed tightly, and incubated for 4 to 5 h at 37°C without shaking.

Aerobic growth. One-liter flasks containing 250 ml of Luria broth were inoculated with overnight cultures at a final concentration of 1% (vol/vol) and incubated at 37°C with

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TABLE 1. *E. coli* strains and plasmids used

Strain, phage, plasmid, or plasmid construct	Description	Source or reference
Strains		
TG1	*hsdR supE thi* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q} Z(M15)]	10
AP1	TG1 Δ(*hyaU*)*1* Km	This study (6.4-kb *Bam*HI deletion)
AP2	TG1 Δ(*hyaAB*)*1* Km	This study (2-kb *Sal*I deletion)
BW545	Δ(*lacU*)*169 rpsL*	Laboratory collection
KL188	*galK2 pyrD34 trp-45 his-68 thyA25 rpsL118 malA1 xyl-7 mtl-2 thi-1*	CGSC 4211
KL282	HfrPO2A *tonA22 phoA4*(Am) (*serS14 serS16*) *serC13 ompF627 supD32* (*serU132*) *relA1 pit-10 spoT1*	CGSC 4297
AB2829	*aroA354 supE42* F$^+$ (?)	CGSC 2829
SE1000	*cysC43 thr-1 leu-6 thi-1 proA2 his-4 argE3 srl-300*::Tn*10 lacY1 galK2 ara-14 xyl-5 mtl-1 rpsL31 tsx-33 supE44*	Laboratory collection
3000X111	HfrPO1 Δ(*gpt-lac*)*5 relA1 spoT1 thi-1*	CGSC 5263
SE1301	BW545 *cys*	This study
SE1663	BW545 Δ(*hyaU*)1 Km	P1 transduction (BW545 × AP1)
SE1664	3000X111 Δ(*hyaU*)*1* Km	P1 transduction (3000X111 × AP1)
SE1669	SE1663 *hydE*	This study (H_2-BV selection; 4)
SE1713	SE1301 *hydE cys*$^+$	P1 transduction (SE1301 × SE1669)
Phages		
P1	Tn*9* Cm$^{rclr-100}$	Laboratory collection
HMS 273	Contains pGp-1	34
Plasmids		
pBR322		
pTZ19R		
pT7-5		34
pT7-6		34
pKK223		Pharmacia
pMAK7000		11
Plasmid constructs		
pCL1	7.5-kb *Eco*RI fragment containing *hya* operon in pBR322	
pCL2	Opposite orientation of pCL1 insert in pBR322	
pCL47	*hyaA* to *hyaF* in pBR322	
pCL30	*hyaA* to *hyaE* in pBR322	
pCL18	*hyaA* to *hyaD* in pBR322	
pCL5	*hyaA* to *hyaC* in pBR322	
pCL23	*hyaA* to *hyaB* in pBR322	
pCLA	*hya* operon in pT7-5	
pCLB	*hyaC* to *hyaF* in pT7-5	
pCLC	*hyaC* in pT7-5	
pCLD	*hyaE* to *hyaF* in pT7-6	
pCLE	*hyaF* in pT7-6	
pCLD$^-$	*hyaD* IFD (294-nucleotide deletion) in pT7-5	
PNKM1	pCL1 insert with 6.4-kb *Bam*HI deletion and Km cassette in *Bam*HI site in pMAK7000 (Fig. 1)	
pNKM2	pCL1 insert with a 2-kb *Sal*I deletion and Km cassette in *Sal*I site in pMAK7000 (Fig. 1)	
p*hyaA* IFD	888-bp deletion in *hyaA* in pCL47 (sites used: *Bam*HI to *Aat*2)	
p*hyaB* IFD	42-bp deletion in *hyaB* in pCL47 (sites used: *Rsa* to *Pml*1)	
p*hyaC* IFD	390-bp deletion in *hyaC* in pCL47 (sites used: *Cla*I to *Nco*I)	
p*hyaD* IFD	294-bp deletion in *hyaD* in pCL47 (sites used: *Xmn*I to *Xmn*I)	

shaking (350 rpm) for 3 to 4 h. Strains AP1 and AP2 were grown in the presence of kanamycin, while mutants transformed with plasmids were grown in the presence of kanamycin and ampicillin.

Preparation of crude extracts. Cells from 25-ml cultures were centrifuged at 10,000 × *g* for 15 min, and the pellets were resuspended in 250 μl of TE buffer (Tris-HCl [pH 8.0], 50 mM; EDTA, 10 mM) containing lysozyme at a concentration of 10 mg/ml. After 30 min of incubation at 37°C, spheroplasts were disrupted by sonication with a microtip probe (Heat Systems model W-385) three times for 40 s each time on ice. DNase (10 μg/ml) and Triton X-100 (final concentration, 2.5% [vol/vol]) were added, and the extraction was continued with gentle shaking at room temperature for 30 min. The extracts were centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatants (S10) were used for determining hydrogenase activity.

Preparation of membrane fractions. Cells from 250-ml batch cultures were harvested, washed, resuspended in 4 ml of Tris-HCl buffer (50 mM, pH 7.3), and sonicated at medium power with a macrotip probe three times for 45 s each time on ice. Unbroken cells were cleared by centrifu-

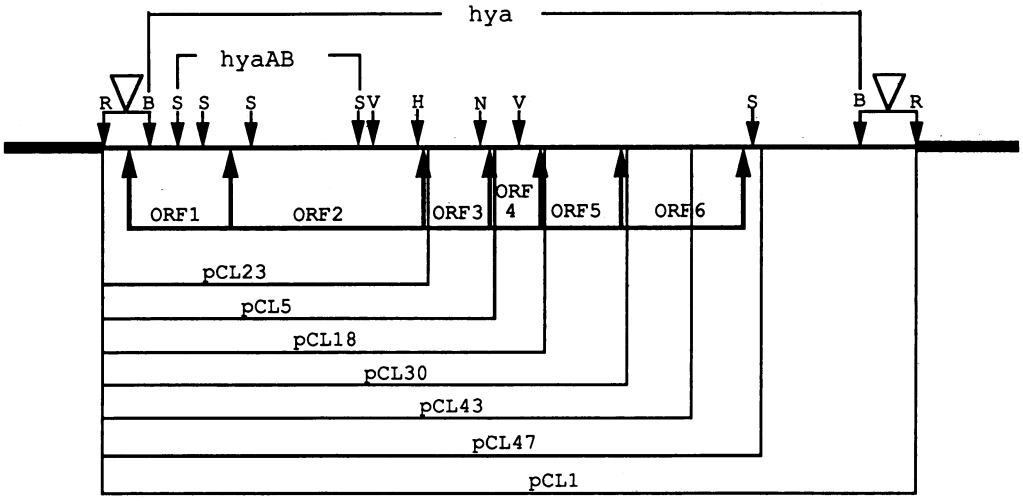


FIG. 1. Restriction map of the *hya* operon contained on a 7.5-kb *Eco*RI restriction fragment of *E. coli* DNA. Restriction sites: B, *Bam*HI; H, *Hpa*I; R, *Eco*RI; V, *Eco*RV; S, *Sal*I; N, *Nco*I. Deletions in strains AP1 and AP2 are indicated as hya and hyaAB, respectively. Probe 1 is the 6.4-kb *Bam*HI fragment designated hya, and probe 2 is a mixture of two *Eco*RI-*Bam*HI fragments designated ▽. The six putative ORFs of *hya* are designated ORF1 to ORF6, and the limits of the subclones used for complementation studies are indicated as pCL23, etc., and described in Table 1.

gation at 10,000 × *g* for 30 min. The supernatants were centrifuged at 110,000 × *g* for 60 min at 4°C, and pelleted membranes were washed twice with Tris-HCl buffer containing 10 mM EDTA. Membranes were resuspended in Tris-HCl buffer containing Triton X-100 (2.5% [vol/vol]) and stored at −70°C when not used immediately. Triton X-100-insoluble material was pelleted from thawed membranes by centrifugation at 125,000 × *g* for 45 min, and the supernatants (S100) constituted the membrane fractions.

Enzyme assays. The deletion of HYD1 did not affect the total hydrogenase activities of the mutants when assayed with viologen dyes as electron donors and/or acceptors. Therefore, all assays for HYD1 were performed after electrophoretical separation of HYD1 from other hydrogenase isoenzymes in a nondenaturing polyacrylamide gel electrophoresis (PAGE) system (7.5%). HYD1 was visualized with a benzyl viologen-linked hydrogen uptake assay (2). The activity bands were fixed by the addition of 1 mM triphenyl tetrazolium chloride. All other enzyme activities were determined as previously described (16).

Antibody screening. In a previous report, it was demonstrated that antibodies specific for the two structural subunits of *B. japonicum* [NiFe] hydrogenase cross-reacted with the denatured large and small subunits of one of the *E. coli* hydrogenases but not with the native enzyme (12). This result is not surprising, as a comparison of the primary amino acid sequences of the *B. japonicum* and *E. coli* HYD1 large and small subunits showed about 70% homology. Western blot (immunoblot) analysis in our laboratory also showed that affinity-purified antibodies raised against the small and large subunits of *B. japonicum* hydrogenase cross-reacted specifically with the corresponding subunits of purified *E. coli* HYD1. Therefore, these antibodies were used to analyze the plasmid-encoded hydrogenase subunits in mutant and wild-type strains. Western blotting was performed as previously described (21). The large and small subunits were resolved on 10 and 12% sodium dodecyl sulfate (SDS)-acrylamide gels, respectively.

Expression of polypeptides encoded by the *hya* operon. Constructs encompassing the different ORFs and the complete *hya* operon were made in the T7 polymerase-regulated pT7-5 and pT7-6 vectors (Table 1). The expression of proteins directed by each construct was analyzed by SDS-PAGE after the incorporation of [^{35}S]methionine (34).

Genomic hybridizations. All hybridizations were done in 50% formamide at 42°C for 14 to 16 h. Probes were labelled by the random primer technique with digoxin-labelled UTP, in accordance with manufacturer specifications (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Construction of mutants. To study the role of HYD1 in the anaerobic metabolism of *E. coli*, we constructed two specific deletions in the *hya* operon. In the first mutant, the *Bam*HI internal fragment in the *hya* operon (Fig. 1) was deleted, resulting in a mutant lacking the entire *hya* operon (strain AP1). The second mutant consisted of a deletion of the *Sal*I internal fragment within the two structural subunits (Fig. 1; strain AP2). Since the operon contained additional *Sal*I sites outside the structural genes, a clone containing only *hyaAB* (pCL23) was used to make this construct.

The mutants were constructed by a modification of a previously described technique (11), which utilizes the temperature-sensitive pSC101 replicon in plasmid pMAK7000 to enhance gene replacements. The deleted regions in the *hya* operon were replaced by a kanamycin cartridge which served as a selection marker. Since the deletions were much larger than those originally described, the selection of cointegrates as described previously (11) was not successful. Hence, the following modifications were introduced. Deletion constructs cloned into pMAK7000 (pNKM1 and pNKM2) were transformed into *E. coli* TG1, and the transformants were selected by their ability to grow on kanamycin and chloramphenicol at 30°C. The transformants were then grown in Luria broth containing kanamycin at the nonpermissive temperature of 44°C. The cultures were serially transferred (1:100 dilution) every 6 h for 48 h at 44°C. This procedure yielded 100% cointegrates, as judged by the ability of colonies to grow either in kanamycin or in chloramphenicol plus kanamycin at 44°C. Isolated colonies of cointegrates were next grown at 30°C in the absence of chloramphenicol for 48 h with six to eight serial transfers.

This procedure allowed the plasmids to replicate and be excised from the chromosome. The cultures were then returned to 44°C in the absence of chloramphenicol, facilitating the curing of the plasmids as a consequence of their inability to replicate at that temperature. The final step involved the isolation of plasmid-cured mutants as chloramphenicol-sensitive, kanamycin-resistant strains at 30 and 42°C. In the case of the 6.4-kb *Bam*HI deletion, 2 of 600 mutant clones tested carried the deletion. In the second case, however, over 40% of the tested colonies had the expected 2.2-kb *Sal*I deletion.

Construction of clones containing different *hya* ORFs. Deletion clones were generated in pTZ19 with the DNase I technique (9). Clones containing deletions in *hya* progressing in the 5′ to 3′ direction and different numbers of complete *hya* ORFs, as judged by sequencing, were used for complementation assays. Since initial experiments with the parental clone in pTZ19 gave inconsistent results on transformation into the mutant strains, the inserts were subcloned with *Eco*RI and *Hin*dIII into pBR322. The recombinant clones in pBR322 were stable and yielded reproducible results (Fig. 1).

Construction of IFDs of *hyaA* to *hyaD*. In-frame deletions (IFDs) of various ORFs were constructed with internal restriction sites (Table 1). After digestion, the sites were blunt end repaired with T4 DNA polymerase or the Klenow fragment and religated. To ensure that only nonpolar mutations resulted, we checked all constructs by sequencing, Western blot analysis of transformed cell extracts, activity staining of cell extracts, and expression of the constructs in the T7 vector system.

Genetic analysis. All experiments were performed as described previously (23).

Materials. Restriction enzymes were purchased from New England BioLabs. A nonradioactive DNA labelling kit was purchased from Boehringer. Reagents for Western blotting were purchased from Promega Corp., Madison, Wis. $^{63}NiCl_2$ and [^{35}S]methionine were purchased from Amersham Corp., Arlington Heights, Ill.

RESULTS AND DISCUSSION

Characterization of *hya* deletion mutants. Figure 1 presents a restriction map of a 7.5-kb fragment of *E. coli* chromosomal DNA which encompasses the *hya* operon. Two HYD1 deletions, *hya* (strain AP1 [*hyaU*]) and *hyaAB* (strain AP2 [*hyaAB*]), were constructed in the wild-type strain TG1 (Fig. 1) with plasmids pNKM1 and pNKM2, respectively. To establish the authenticity of the mutant constructs, we digested genomic DNAs from strains AP1, AP2, and TG1 with restriction enzymes and probed them with a 6.4-kb *Bam*HI fragment encompassing *hya* (probe 1) and a mixture of two *Eco*RI/*Bam*HI fragments which flank the 5′ and 3′ ends of probe 1 (probe 2) (Fig. 1). No signal was obtained when probe 1 was hybridized to digests of genomic DNA from AP1, whereas genomic DNA from TG1 showed the expected 6.4-kb band (data not shown). With probe 2, genomic DNA from TG1 showed two bands, since there were internal *Eco*RV sites in the operon (Fig. 2a, lane 1). In the case of AP1, a single band was obtained, since the internal *Eco*RV sites had been deleted (Fig. 2a, lane 2). Strain AP2 also showed the expected shifts in hybridization patterns (data not shown).

There were no differences in growth rates among strains AP1, AP2, and TG1 grown anaerobically in LBG or LBG plus formate. HYD1 activity was absent in both AP1 and AP2, and data for AP1 are presented (Fig. 2b). Western blot analysis of S10 extracts demonstrated the absence of the antigenically cross-reactive HYD1 large subunit in AP1 (Fig. 2c). The lower band in Fig. 2c is the HYD2 large subunit, as deduced from Western blot analysis of a HYD2-specific deletion mutant (data not shown), indicating that HYD2 expression was not affected in strain AP1. There were no changes in the total hydrogenase activities in deletion strains, as measured by tritium exchange or hydrogen-dependent reduction of benzyl viologen or fumarate (Table 2). However, strain AP1 consistently produced significantly lower levels of formate dehydrogenase activity (about 60% of the parental level). Complementation of strain AP1 with pCL2, which carries the *hya*$^+$ operon in a multicopy plasmid, restored HYD1 expression and formate dehydrogenase activity but did not influence the levels of total hydrogenase activity or hydrogen-dependent fumarate reduction. In the presence of the plasmid, however, BV reduction was reduced to about 40% of the parental level. These results showed that the levels of total hydrogenase and hydrogen uptake activities were not influenced by the absence of HYD1 and indicated that the deletion of the *hya* operon did

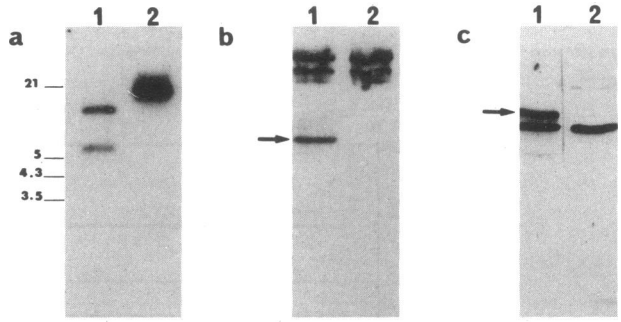


FIG. 2. Analysis of the AP1 deletion strain. (a) DNA analysis. Southern blots of wild-type (TG1, lane 1) and *hya* (AP1, lane 2) DNAs digested with *Eco*RV were hybridized to ^{32}P-labeled probe 2 (Fig. 1) and exposed to X-Omat AR X-ray film. *Hin*dIII-digested lambda standard markers are indicated. (b) Enzyme analysis. S10 extracts of TG1 (lane 1) and AP1 (lane 2) were fractionated in 7.5% native polyacrylamide gels and stained for hydrogenase activity with a BV-linked uptake assay (see the text). The arrow indicates HYD1. (c) Protein analysis. S100 fractions of TG1 (lane 1) and AP1 (lane 2) were blotted to nitrocellulose filters and probed with antibodies specific for the large subunit. The arrow indicates the HYD1 large subunit, and the lower band is the large subunit of HYD2.

TABLE 2. Biochemical properties of *hya* mutant strain SE1663 and its parent strain, BW545

Strain	Sp act of[a]:				
	HYD	HUP with:		FHL	FDH-H
		Benzylviologen	Fumarate		
BW545	503	2,203	178	235	748
SE1663	434	2,115	173	225	440
SE1663(pBRC12)	406	909	177	169	609

[a] HYD, hydrogenase activity (nanomoles of tritiated water produced per minute per milligram of cell protein; HUP, hydrogen uptake activity determined with either benzyl viologen or fumarate as the electron acceptor; FHL, formate hydrogen lyase; FDH-H, formate dehydrogenase associated with FHL. All enzyme activities, except for HYD, are expressed as nanomoles per minute per milligram of cell protein.

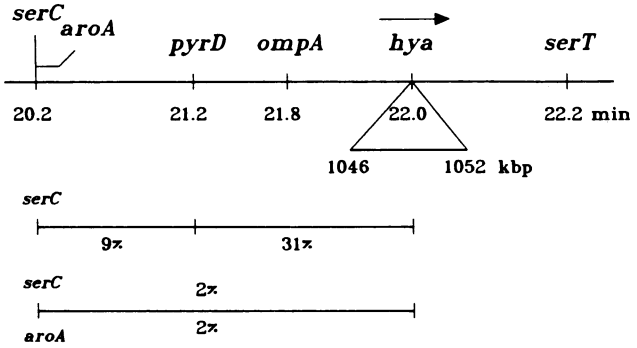


FIG. 3. Genetic mapping of *hya*. The minutes represent the *E. coli* chromosome in minutes as described by Bachmann (1). 1046 and 1052 kbp designate the physical location of the *hya* operon (14, 19). Other numbers are bacteriophage P1-mediated cotransduction frequencies between adjacent genes.

not exert pleiotropic effects on the other hydrogenase isoenzymes.

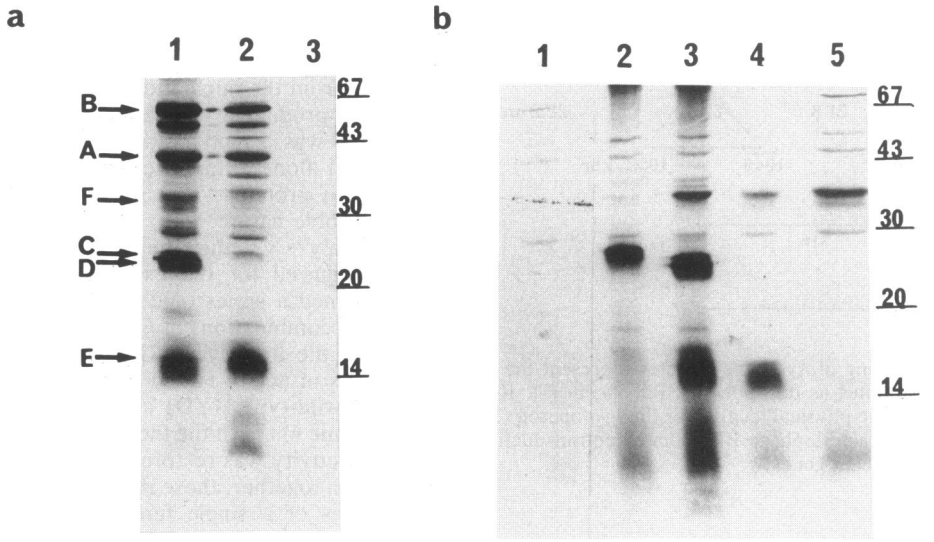
Genetic localization of *hya*. To determine the genetic location of the *hya* operon, we transduced the *hya* mutation from AP1 to an Hfr strain (3000X111). The resultant strain, SE1664, was used as a donor in a conjugation experiment with strain SE1000 as the recipient, and kanamycin-resistant exconjugants were selected. On the basis of the time of entry and expression of kanamycin resistance, the *hya* operon was located between 21 and 25 min on the *E. coli* chromosome (1). Bacteriophage P1-mediated transductional analysis revealed that the *hya* mutation was cotransducible with the *aroA*, *serC*, and *pyrD* genes. The *hya* operon was cotransducible with *pyrD* at a frequency of about 30%, and the cotransduction frequency with either *aroA* or *serC* was less than 5%. The cotransduction frequency between *pyrD* and *serC* was about 10%. These results suggest that the *hya* operon is located at about 22 min on the *E. coli* chromosome (Fig. 3). This map location was confirmed by comparing the restriction map of the *hya* operon and the restriction map of the *E. coli* chromosome (14, 19). On the basis of this analysis, the *hya* operon was located between 1,046 and 1,052 kbp on the physical map of the *E. coli* chromosomal DNA (Fig. 3). The direction of transcription of the *hya* operon is clockwise towards the *serT* gene.

Identification of *hyaA* to *hyaE* gene products. Our attempts to isolate a 6-kb mRNA to demonstrate that the six ORFs of *hya* were transcribed were unsuccessful. To determine whether the ORFs of *hya* were transcribed and translated, we made various constructs in the pT7-5 and pT7-6 vectors, which place recombinant DNAs under the regulation of T7 polymerase promoters. The translation products of a plasmid containing the entire *hya* operon (pCLA) (Fig. 4a) exhibited a number of minor radioactive bands, making it difficult to conclusively identify six *hya*-specific gene products. Since the products of *hyaA* and *hyaB* were identified by their cross-reaction with antibodies specific for the large and small subunits of *B. japonicum* hydrogenase, subclones containing *hyaC* to *hyaF* in various combinations (Fig. 4b) were constructed. The product of *hyaD* was identified by comparing the labelled protein patterns of pCLA and pCLD$^-$ (Fig. 4a). The latter contained the *hya* operon with an IFD of *hyaD* (Fig. 4a). The protein profile shown by pCLD$^-$ also indicated that the deletion was not polar, since both HYAE and HYAF were synthesized. The identities of *hyaE* and *hyaF* were also established (Fig. 4). The molecular masses of the resultant T7-expressed gene products of all *hya* ORFs agreed well with the calculated molecular masses derived from the nucleotide sequence (Fig. 4c). HYAC was the only product to exhibit anomalous behavior in that its mobility was slightly slower in SDS gels when it was produced alone as opposed to when it was produced with other *hya* proteins. This result may be due in part to its hydrophobic nature.

To analyze whether the protein products of ORFs 3 to 6 were required for the expression of HYD1 activity, we transformed a series of deletion clones (Fig. 1) containing different combinations of *hya* ORFs into AP1 and AP2. As is evident, the first five ORFs (pCL30) are essential for the synthesis of active HYD1 in both mutants, restoring about 30% of wild-type HYD1 activity (data for AP1 are shown; Fig. 5, lane 6). With the inclusion of the sixth ORF (pCL47), HYD1 activity was restored to wild-type levels (Fig. 5, lane 7). Taken together, these results indicated that the six ORFs are genes of a single functional operon required for the synthesis and activation of HYD1.

Localization and processing of the structural subunits. (i) Large subunit. Immunological analysis of S10 extracts of AP1 complemented with various deletion clones showed that the HYD1 large subunit was overexpressed in transformed mutant cells compared with wild-type cells (Fig. 6a). This result was expected, since the constructs were carried on multicopy plasmids. The levels of active HYD1, however, were equivalent in transformed AP1 and TG1 cells (Fig. 5, lanes 1 and 7). Thus, increased levels of protein synthesis did not translate to higher HYD1 activity. Analysis of S100 fractions of the same samples revealed that the levels of membrane-bound large subunit were only slightly higher in fully complemented mutant cells than in wild-type cells (Fig. 6b, lanes 6 and 7). This result implied that most of the excess protein expressed by the multicopy plasmids was inactive and soluble. Membrane fractions of AP1 complemented with only the structural genes (*hyaA* and *hybB*), on the other hand, contained significantly lower levels of antigenically cross-reactive protein than did wild-type cells (Fig. 6b, lane 2). This result suggested that the expression of one or all of the additional genes in *hya* was required for the localization of HYAA and HYAB to the cell membrane.

Two membrane-bound forms of the HYD1 large subunit (Fig. 6b, bands A and B) were apparent in S100 fractions of AP1 complemented with various clones. S100 fractions from wild-type cells or purified enzyme samples, on the other hand, only had the smaller subunit. It was also observed that the ratio of the smaller form to the larger form increased with the progressive inclusion of additional *hya* ORFs in complementation assays. The apparent decrease in size also appeared to occur concurrently with the appearance of active HYD1 in transformed cells. These findings are consistent with the occurrence of a processing step which converts the large form of HYD1 to the small form during activation. It was also evident, however, that even when the mutant was complemented with a high-copy-number plasmid carrying all six genes of *hya*, there was still unprocessed large subunit present on the membrane (Fig. 6b, lane 6, band A). This last result suggested that other factors, in addition to the proteins encoded by the *hya* operon, regulated the levels of processed HYD1. Indeed, we have observed the presence of the two forms of the large subunit in membrane extracts of an *hyb* (HYD2) deletion mutant which showed lower levels of HYD1 than did the wild type (unpublished data). Similar observations on the presence of the two forms of the large



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Protein	Nucleotide Derived Molecular weight	Experimental Molecular weight
HYAA	40.6	43.0
HYAB	66.2	67.0
HYAC	27.6	26.0
HYAD	21.5	23.0
HYAE	14.9	15.0
HYAF	31.5	33.0

FIG. 4. Analysis of *hya* gene products. (a and b) Autoradiograms of ^{35}S-labeled proteins directed by *hya* and subclones of *hya* in a T7 promoter-polymerase expression system (see the text). The plasmids used for these experiments are described in Table 1. (a) Lane 1, *hyaA* to *hyaF* (pCLA); lane 2, *hyaD* IFD (pCLD$^-$); lane 3, pT7-6. The gene products of *hyaA* to *hyaF* are indicated by the letters A to F, respectively. (b) Lane 1, pT7-5; lane 2, *hyaC* (pCLC); lane 3, *hyaC* to *hyaF* (pCLB); lane 4, *hyaE* to *hyaF* (pCLD); lane 5, *hyaF* (pCLE). Molecular mass markers are indicated in kilodaltons. (c) Nucleotide-derived and experimentally derived molecular weights (in thousands) of HYAA to HYAF.

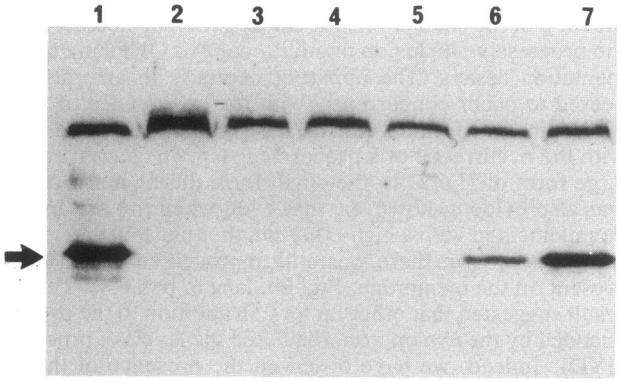

FIG. 5. Activity gel of S10 extracts of AP1 complemented with various plasmids. Lanes: 1, TG1; 2, AP1; 3, pCL23; 4, pCL5; 5, pCL18; 6, pCL30; 7, pCL47. The arrow indicates the HYD1 activity band.

subunit have been made in *Desulfovibrio gigas* [NiFe] hydrogenase (19a) and *D. baculatus* [NiFeSe] hydrogenase cloned into *E. coli* (21). N-terminal amino acid sequencing of the HYD1 large subunit (8) has shown that the first 15 residues are identical to those of the nucleotide-derived amino acid sequence; thus, the small form does not arise by the removal of a signal peptide from the large form. At present, we do not know whether the existence of two forms of the HYD1 large subunit represents processing of the peptide chain at the carboxy terminus or modification by another mechanism.

The failure to obtain higher levels of hydrogenase activity in cells complemented with high-copy-number plasmids could be explained in part by the following. (i) The amount of space for the insertion of HYD1 nascent polypeptides into the cell membrane is limiting. (ii) The processing enzymes required for the activation (metal insertion, etc.) of nascent HYAA and HYAB are limiting. (iii) Electron carriers associated with HYD1 are limiting. Our findings differ from those obtained by others working with *E. coli* fumarate reductase, another membrane-bound enzyme involved in anaerobic metabolism. A significant increase in active fumarate reductase occurred upon an increase in the copy number of the

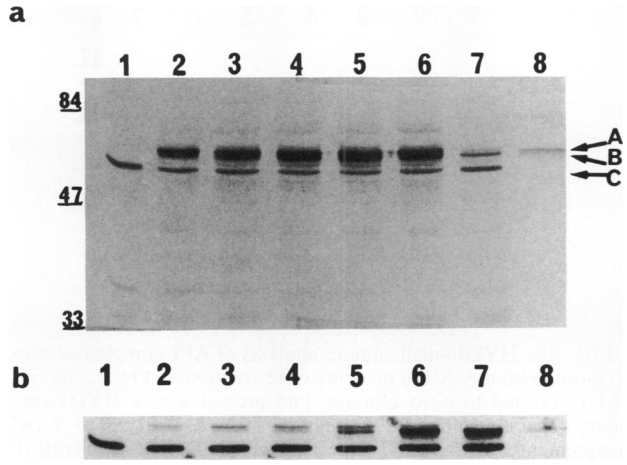

FIG. 6. Analysis of the HYD1 large-subunit protein from soluble extracts (S10) (a) and membrane fractions (S100) (b) of cells complemented with various plasmids. Soluble extracts and membrane fractions were probed with a hydrogenase large-subunit-specific antibody. Lanes: 1, AP1; 2 to 6, AP1 transformed with pCL23 (lane 2), pCL5 (lane 3), pCL18 (lane 4), pCL30 (lane 5), and pCL47 (lane 6); 7, control protein from wild-type cells (TG1); 8, purified HYD1 from *E. coli*. Molecular mass markers are indicated on the left in kilodaltons. A and B, two electrophoretic forms of the large subunit of HYD1; C, large subunit of HYD2. The two forms are readily distinguished in membrane preparations, i.e., lane 5 in panel b.

genes. In this case, even excess enzyme which remained in the cytoplasm was active (6).

(ii) Small subunit. Immunological screening with antibody specific for the HYD1 small subunit showed that the small subunit is present as membrane-bound 40- and 35-kDa proteins in S100 fractions of *hya*-complemented AP1 (Fig. 7, lane 3, bands A and B). The size of 40 kDa agrees well with the nucleotide-derived amino acid sequence of the small subunit inclusive of the putative signal peptide (22). The nascent 40-kDa small subunit could be processed to the 35-kDa protein, the size of the immunogenic band seen in wild-type S100 fractions (Fig. 7, lanes 2 and 3). The apparent decrease of 5 kDa is indicative of the removal of the putative leader peptide (46 amino acids). Even though detectable

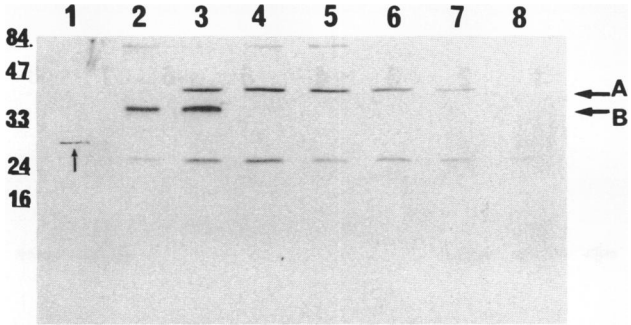

FIG. 7. Analysis of the HYD1 small-subunit protein from membrane fractions (S100) of AP1 complemented with various plasmids. Lanes: 1, purified HYD1; 2, TG1; 3 to 7, AP1 transformed with pCL47 (lane 3), pCL30 (lane 4), pCL18 (lane 5), pCL5 (lane 6), and pCL23 (lane 7); 8, AP1. Molecular mass markers are indicated on the left in kilodaltons. Two electrophoretic forms of the HYD1 small subunit are labelled A and B and are discussed in the text.

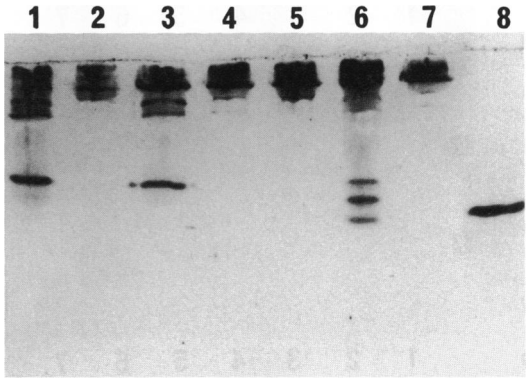

FIG. 8. Enzyme analysis of AP1 complementation. Lanes: 1, TG1; 2, AP1; 3 to 7, AP1 complemented with pCL47 (*hya* operon) (lane 3), p*hyaA* IFD (lane 4), p*hyaB* IFD (lane 5), p*hyaC* IFD (lane 6), and p*hyaD* IFD (lane 7); 8, purified HYD1.

levels of unprocessed small subunit were present, complementation of AP1 with *hya* restored wild-type levels of processed small subunit. When AP1 was transformed with the first five genes (*hyaA* to *hyaE*) of *hya*, low levels of activity (10% of wild type) were detected and, similarly, only low levels of processed small subunit were observed (Fig. 7, lane 4).

The 35-kDa processed small subunit is larger than the 29-kDa small band observed in purified hydrogenase (Fig. 7). This result is in agreement with previous results demonstrating that during purification, the 35-kDa small subunit gave rise to 31- and 29-kDa polypeptides (28).

Analysis of gene function. To further characterize the role of various genes of *hya* in the biosynthesis of active HYD1, we generated several deletion clones which consisted of the *hya* operon with a gene or part of a gene deleted. The deletions were termed IFDs, as they were tested to ensure that the resultant transcripts directed the synthesis of all proteins encoded both upstream and downstream of the deletions.

A clone containing an 888-nucleotide deletion of *hya* which encompassed 296 amino acids of the gene encoding the small subunit (p*hyaA* IFD) was constructed and transformed into AP1. This clone failed to restore HYD1 activity to AP1 (Fig. 8, lane 4). Since over 80% of the small subunit gene was deleted, it was not surprising that the small subunit was not detected immunologically. The large subunit (HYAB) was synthesized by *hyaA* IFD constructs, but in comparison with the results with AP1 complemented with the entire operon, only low levels of the protein were localized on the membrane, and HYD1 activity was undetectable. These results, along with data obtained from site-directed mutagenesis of conserved residues in the small subunit (20a), indicated that the expression of the small subunit was required for the biosynthesis of active hydrogenase. Other groups have reported the purification of active monomeric forms of HYD1 containing only the large subunit (8). Our findings suggest that the biosynthesis of active HYD1 requires the concurrent processing of nascent large and small polypeptides and that it is only subsequently that the two subunits can be separated, with the generation of the active monomeric large subunit.

A clone containing a 42-nucleotide deletion in *hya* encompassing the conserved cysteinyl residues at the carboxy terminus of the large subunit (p*hyaB* IFD) failed to restore

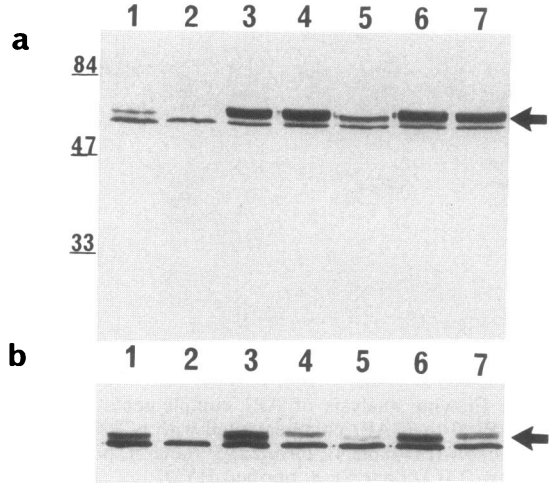

FIG. 9. HYD1 large subunit analysis of AP1 complementation. Shown is a Western blot analysis of soluble (a) and membrane-bound (b) extracts probed with a hydrogenase large-subunit-specific antibody. Lanes: 1, TG1; 2, AP1; 3 to 7, AP1 complemented with pCL47 (lane 3), p*hyaA* IFD (lane 4), p*hyaB* IFD (lane 5), p*hyaC* IFD (lane 6), and p*hyaD* IFD (lane 7). The arrow indicates the doublet containing two electrophoretic forms of the HYD1 large subunit. Molecular mass markers are indicated on the left in kilodaltons.

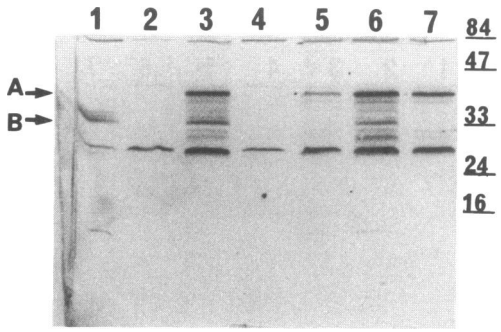

FIG. 10. HYD1 small subunit analysis of AP1 complementation. Membrane-bound (S100) proteins were fractionated by 12.5% SDS-PAGE, blotted to nitrocellulose, and probed with a HYD1 small-subunit-specific antibody. Lanes: 1, TG1; 2, AP1; 3 to 7, AP1 complemented with pCL47 (lane 3), p*hyaA* IFD (lane 4), p*hyaB* IFD (lane 5), p*hyaC* IFD (lane 6), and p*hyaD* IFD (lane 7). Two electrophoretic forms of the HYD1 small subunit are labeled A and B. Molecular mass markers are indicated on the right in kilodaltons.

HYD1 activity when transformed into AP1 (Fig. 8, lane 5). Although this deletion encompassed only 14 amino acids, S10 extracts of this cell line showed significantly lower levels of the HYD1 large subunit than did AP1 complemented with *hya* (Fig. 9a, lanes 4 and 6). Analysis of membrane fractions revealed negligible, if any, antigenically cross-reactive large subunit associated with the membrane. The small subunit, however, was localized on the membrane but was present as a 40-kDa unprocessed polypeptide. Presuming that the set of conserved cysteinyl residues is involved in nickel binding, these results seem to indicate that the binding of nickel to the large subunit may be a prerequisite to the localization of the large subunit on the membrane. Alternatively, it is possible that in the absence of incorporation of the active center, protein stability is affected, resulting in the lower levels of antigenically cross-reactive protein observed in the S10 extracts.

A clone containing a 390-nucleotide deletion in *hyaC* (p*hyaC* IFD) resulting in the deletion of three of four computer-predicted membrane-spanning regions of HYAC (22) was constructed. This construct was the only IFD which complemented AP1 to wild-type levels of HYD1 activity, clearly demonstrating that this protein is not required for the processing or activation of HYD1. Expression of the *hyaC* IFD construct, however, consistently resulted in the appearance of multiple forms of HYD1 in transformed AP1 cells (Fig. 8, lane 6). The large and small subunits of all three forms were membrane bound, indicating that HYAC was not solely responsible for anchoring the enzyme to the membrane. Multiple forms of HYD1 have also been observed during purification (28), but purified hydrogenase from wild-type cells comigrated with only the fastest-moving form (Fig. 8, lanes 6 and 8). This result seems to indicate that HYAC may be an integral part of the HYD1 complex. The three forms present in *hyaC* IFD-transformed cells may result from differential folding of HYD1 in the absence of HYAC or perhaps increased susceptibility of the enzyme to proteolysis in the absence of HYAC. At present, we do not know whether a deletion in HYAC results in the loss of the in vivo electron-transferring capacity of HYD1.

Complementation of AP1 with an *hya* clone encoding a 98-amino-acid IFD in HYAD did not restore HYD1 activity. This deletion resulted in the expression and localization of both large and small HYD1 subunits in the cell membrane, but neither subunit was processed (Fig. 9, lane 7; Fig. 10, lane 7). Thus, the gene product of *hyaD* is essential for the processing of nascent large and small subunits.

Active HYD1 was obtained when a clone containing *hyaA* to *hyaE* was introduced into AP1. Complemented cells had about 30% wild-type hydrogenase activity, a level which was slightly enhanced by the addition of 100 μM nickel to the medium for 6 h (Fig. 11). Cells grown in 100 μM nickel for 8 to 10 h, however, showed wild-type levels of HYD1 activity (data not shown). With the inclusion of *hyaF*, wild-type levels of HYD1 activity were obtained within 4 to 5 h of anaerobic growth in Luria broth.

Studies on the incorporation of ^{63}Ni into nascent HYD1 revealed that the complementation of strain AP1 with *hya* resulted in the incorporation of wild-type levels of nickel into gel-fractionated HYD1 (results not shown). No detectable band of ^{63}Ni was observed when the mutant was comple-

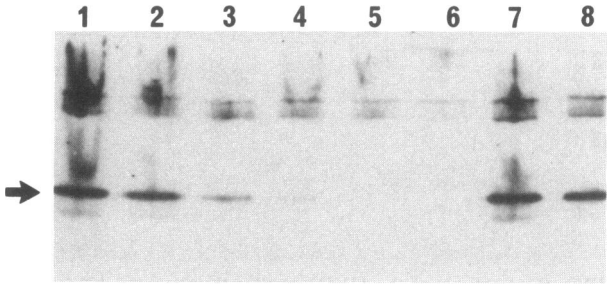

FIG. 11. Effect of nickel on HYD1 activity. Wild-type cells (TG1, lanes 7 and 8) and AP1 cells complemented with *hyaA* to *hyaF* (pCL47, lanes 1 and 2), *hyaA* to *hyaE* (pCL30, lanes 3 and 4), and *hyaA* to *hyaD* (pCL18, lanes 5 and 6) were grown in Luria broth (lanes 2, 4, 6, and 8) or in Luria broth supplemented with 100 μM $NiCl_2$ (lanes 1, 3, 5, and 7). The arrow indicates HYD1.

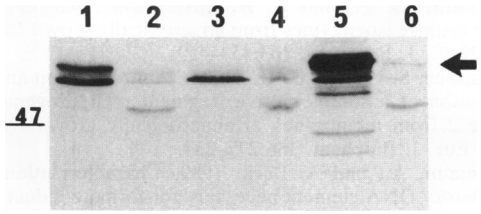

FIG. 12. Anaerobic induction of HYD1. Western analysis of extracts from wild-type cells (TG1, lanes 1 and 2), AP1 cells (lanes 3 and 4), and AP1 cells complemented with pCL47 (lanes 5 and 6), grown either anaerobically (lanes 1, 3, and 5) or aerobically (lanes 2, 4, and 6). The arrow indicates HYD1.

mented with *hyaA* to *hyaE*, probably because only low levels of HYD1 activity were restored with *hyaA* to *hyaE*. Labelling studies with ^{63}Ni did not detect nickel bound to nascent polypeptides when AP1 was complemented with *hyaA* to *hyaD*, *hyaB* IFD, or *hyaD* IFD. These results suggest one of the following. (i) Nickel is not bound to nascent structural polypeptides (HYAA or HYAB) unless the remaining gene products of *hya* are expressed. (ii) Nickel may be bound to nascent structural subunits but at undetectable levels when not all genes of *hya* are expressed. (iii) Nickel bound to nascent HYD1 polypeptides is dissociated from the protein on electrophoresis in native gels when processing has not occurred.

Factors affecting HYD1 induction and activation. (i) Anaerobic control. Anaerobic control of the induction and expression of the *hya* operon was monitored by the accumulation of hydrogenase-specific proteins (Western blot analysis) as well as enzymatic activity. Mutants complemented with the *hya* operon and grown aerobically did not produce enzyme or immunologically cross-reactive proteins (Fig. 12). These results were unlike those obtained when the [NiFe] hydrogenase genes from *D. gigas* and *D. baculatus* were expressed in *E. coli*. In this case, unprocessed structural subunits were synthesized under aerobic conditions (17, 21).

Many anaerobically regulated genes in *E. coli* have been shown to contain a consensus "anaerobox" which is induced by binding of the *fnr* gene product (31), and *fnr* has been implicated in the regulation of both HYD1 induction and HYD2 induction (13). Sequence analysis of 0.3 kb of DNA 5′ to *hyaA*, however, did not reveal homologies to the consensus sequence, suggesting that *hya* was not directly regulated by anaerobiosis. In an *fnr* background, however, in which HYD1 is not induced, the product of *hydC* (a nickel transport and/or processing protein) restores HYD1 activity (38). It has also been shown that *hypB*, which pleiotropically affects the expression of all three *E. coli* hydrogenases, is regulated directly by an *fnr* promoter (18). These last two observations and the data reported here suggest that the *fnr* gene product has an indirect effect on the anaerobic expression of HYD1 via the regulation of *hydC* and *hypB*.

(ii) Nickel regulation. Analysis of S10 extracts of strains SE1713 (HydE$^-$) and SE1669 (HydE$^-$ *hya*) complemented with *hya* showed that the HYD1 large subunit was present in both strains (Fig. 13). This result is in agreement with recent results for the *hyp* operon of *E. coli* (18), which encompasses the *hydE* locus (now designated *hypB*). Although HYD1 structural subunits were expressed, HYD1 activity was absent in both extracts. Analysis of S100 fractions from SE1713, SE1669, and SE1669 complemented with *hya* showed that the large subunit was expressed in the absence

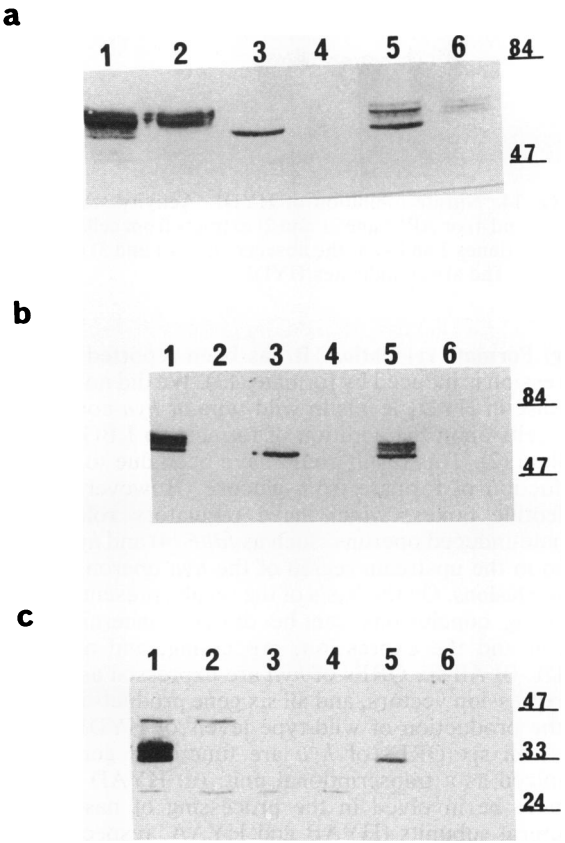

FIG. 13. Activation of HYD1 and HYD2 by nickel. Soluble (a) and membrane-bound (b and c) proteins from *hya*-complemented SE1669 (lanes 1 and 2), SE1669 (lanes 3 and 4), and SE1713 (lanes 5 and 6). Cells were grown in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of 800 μM $NiCl_2$, fractionated by 10% SDS-PAGE, blotted to nitrocellulose, and antigenically screened for the HYD1 large subunit (a and b) or small subunit (c). The arrows in panels a and b indicate the HYD1 large subunit. Molecular mass markers are indicated on the right in kilodaltons.

of exogenous nickel but was not localized on the membrane (Fig. 13). The small subunit, on the other hand, was present as an unprocessed 40-kDa membrane-bound polypeptide. The HYD1 structural subunits both became membrane bound and processed upon the addition of 800 μM nickel to the medium. Since it has been proposed that the product of *hypB* is involved in the activation or processing of nickel (18), these experiments suggest that the large subunit is bound to the membrane only after it binds nickel and that the processing of the small subunit requires its interaction with the precursor of the large subunit.

(iii) Nitrate regulation. In the presence of nitrate, wild-type cells do not express HYD1 activity or immunoreactive protein. However, *hya*-complemented AP1 grown in nitrate showed low levels of active HYD1 (Fig. 14). One possible explanation for this observation is that in wild-type cells, nitrate affects the induction of *hya*, directly or indirectly, via a transcriptional repressor, but the repressor is diluted out in *hya*-transformed AP1 cells, which contain multiple repressor binding sites on multiple copies of *hya*. The result would be variable expression of the operon in the presence of nitrate. The HYD2 large subunit was expressed in nitrate-grown cells, in agreement with previously published results (13), but it was inactive.

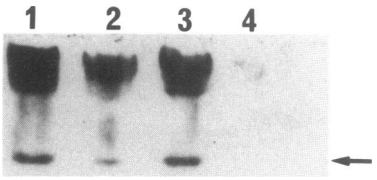

FIG. 14. Nitrate inhibition of HYD1. Activity staining of TG1 (lanes 3 and 4) or AP1 (lanes 1 and 2) extracts from cells grown in the presence (lanes 2 and 4) or the absence (lanes 1 and 3) of nitrate (see the text). The arrow indicates HYD1.

(iv) Formate regulation. It has been reported that HYD1 expression is induced by formate (13). We did not observe an increase in HYD1 levels in wild-type or *hya*-complemented AP1 cells upon the addition of formate to LBG or minimal medium (2). This result could have been due to the cellular production of formate from glucose. However, consensus nucleotide boxes, which have regulatory roles in other formate-induced operons, such as *fdhF* (4) and *hyc*, were not found in the upstream region of the *hya* operon.

Conclusions. On the basis of the results presented here, the following conclusions can be drawn concerning the *hya* operon and the expression, processing, and activation of HYD1. (i) All six ORFs of *hya* are expressed as proteins in T7 expression vectors, and all six gene products are required for the production of wild-type levels of HYD1 in *E. coli*. Thus, all six ORFs of *hya* are functional genes and are organized as a transcriptional unit. (ii) HYAD and HYAE seem to be involved in the processing of nascent HYD1 structural subunits (HYAB and HYAA, respectively), and data suggest that these proteins may act as a processing complex. (iii) Although *hyaA* to *hyaE* are sufficient to encode active HYD1, the inclusion of *hyaF* increases the levels of active HYD1 to wild-type levels. This effect is not observed in nickel-supplemented growth medium; thus, *hyaF* may enhance nickel incorporation into the nascent enzyme subunits. (iv) Even though the transformation of AP1 with *hya* on a multicopy plasmid resulted in at least a 10-fold enhancement of HYD1 precursor levels, levels of HYD1 activity were always equivalent to wild-type levels, indicating that factors other than the proteins encoded by *hya* are limiting for the processing and activation of nascent HYD1 structural proteins or that the levels of active HYD1 are posttranslationally regulated. (v) If the nascent large subunit (HYAB) fails to bind nickel, either it fails to localize to the cell membrane or the nickel is loosely bound and easily washed off during cell fractionation procedures. (vi) Membrane localization and processing of the HYD1 small subunit are essential for the biosynthesis of active HYD1. Therefore, active monomeric HYD1 consisting of the large subunit of HYD1 most probably arises only after the addition of metal centers and concurrent processing of HYD1 small- and large-subunit precursors.

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