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Molecular Cloning of Structural and Regulatory Hydrogenase (*hox*) Genes of *Alcaligenes eutrophus* H16

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A gene bank of the 450-kilobase (kb) megaplasmid pHG1 from the hydrogen-oxidizing bacterium *Alcaligenes eutrophus* H16 was constructed in the broad-host-range mobilizable vector pSUP202 and maintained in *Escherichia coli*. *hox* DNA was identified by screening the *E. coli* gene bank for restoration of hydrogenase activity in *A. eutrophus* Hox mutants. Hybrid plasmids that contained an 11.6-kb *Eco*RI fragment restored soluble NAD-dependent hydrogenase activity when transferred by conjugation into one class of Hos$^-$ mutants. An insertion mutant impaired in particulate hydrogenase was partially restored in Hop activity by an 11-kb *Eco*RI fragment. A contiguous sequence of two *Eco*RI fragments of 8.6 and 2.0 kb generated Hox$^+$ recombinants from mutants that were devoid of both hydrogenase proteins. *hox* DNA was subcloned into the vector pVK101. The resulting recombinant plasmids were used in complementation studies. The results indicate that we have cloned parts of the structural genes coding for Hos and Hop activity and a complete regulatory *hox* DNA sequence which encodes the thermosensitive, energy-dependent derepression signal of hydrogenase synthesis in *A. eutrophus* H16.

Hydrogenase plays a major role in lithoautotrophic metabolism of aerobic H_2-utilizing bacteria. It catalyzes the hydrogen uptake reaction $H_2 \rightleftarrows 2H^+ + 2e^-$. Species of *Alcaligenes* have evolved two distinct types of hydrogenase, a soluble, NAD-linked enzyme (31) and a membrane-bound, electron transport-coupled protein (26). The enzymes differ in catalytic and molecular properties. The soluble, NAD-dependent hydrogenase of *Alcaligenes eutrophus* H16 is a tetramer consisting of four nonidentical subunits of M_r 63,000, 56,000, 30,000, and 26,000 (32). The membrane-bound hydrogenase is a dimer composed of M_r 62,000 and 30,000 peptides (26). The soluble hydrogenase in its native configuration is immunologically distinct from the particulate protein (27). Recent immunofluorescence studies with antibodies raised against the isolated peptides indicate a relationship between the large subunit of the membrane-bound hydrogenase and the M_r 56,000 peptide of the NAD-linked enzyme (30).

Despite considerable knowledge of the physical and kinetic properties of hydrogenases from various sources (22), the molecular structure of these enzymes is unknown. Toward this goal, attempts were made to clone hydrogenase genes from *Rhizobium japonicum* (4, 14, 18), *Desulfovibrio vulgaris* (37), and *Escherichia coli* (25). Genetic transfer and the analysis of hydrogenase mutants indicated that there were plasmid-borne hydrogenase (*hox*) genes in *A. eutrophus* (1, 8). Structural and regulatory *hox* genes are located on the conjugative, 450-kilobase (kb) plasmid pHG1 of *A. eutrophus* H16 (5, 8, 13). This paper describes the construction of a gene bank with the *E. coli*-specific, Mob site-containing vector pSUP202 (34) and purified megaplasmid DNA (12) from *A. eutrophus* H16. *hox*-specific DNA was identified by restoration of the hydrogenase activity of hydrogenase-deficient mutants. Recombinant plasmids are characterized.

(Preliminary accounts of some of this work have been published [9].)

MATERIALS AND METHODS

Bacterial strains. Strains of *Alcaligenes* spp. and mutants derived therefrom are listed in Table 1. Transposon mutagenesis was carried out as described previously (36). Transposon Tn*5* was transferred from *E. coli* SM10 to *A. eutrophus* HF39 by the vector pSUP2011 (34) to yield mutant HF148 or by the vector pSUP5011 (33) to yield mutant HF221 (Table 1). *E. coli* S17-1, a derivative of *E. coli* K-12, was the host of plasmid pSUP202 (34). The broad-host-range vector pVK101 (17) was used for complementation studies.

Growth conditions. *A. eutrophus* strains were grown either in complex medium consisting of nutrient broth (NB) or in the mineral salts medium described by Schlegel et al. (28). The gas atmosphere for autotrophic growth contained a mixture of hydrogen, oxygen, and carbon dioxide in a ratio of 8:1:1 (vol/vol/vol). Organic carbon sources were supplied at a concentration of 0.4% (wt/vol) unless otherwise stated. *E. coli* strains were cultured in LB liquid medium or on solid NB agar. Antibiotics were added as indicated in the text.

Conjugation. Donor and recipient strains were grown to the late exponential phase of growth and concentrated 10-fold by centrifugation. Spot matings were performed on NB agar by plating 0.1 ml of donor and recipient at a cell ratio of 1:1. After 16 h of incubation at 30°C, transconjugants were selected by plating appropriate dilutions on selective medium. Patch matings were conducted by streaking cell material from single colonies on recipient cells spread onto the appropriate selective medium. The plates were incubated under lithoautotrophic growth conditions for 6 to 10 days.

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TABLE 1. Bacterial strains

Strain[a]	Relevant phenotype[b]	Reference or source
A. eutrophus		
H16	Hos Hop HoxTs Hoxd	Wild type (ATCC 17699)
HF18	Hos$^-$ Hop$^-$	10
HF39	Hos Hop Str^r	36
HF148	Hos$^-$ Hop$^-$ Kan^r	Tn*5*-induced mutant of HF39 (this study)
HF14	Hos$^-$ Hop	29
HF15	Hos$^-$ Hop	13
HF08	Hos Hop$^-$	13
HF221	Hos Hop$^-$ Kan^r	Tn*5*-induced mutant of HF39 (this study)
HF117	Hos Hop HoxTr Hoxd	11
A. hydrogenophilus M50	Hos Hop HoxTr Hoxi	Wild type (23)

[a] Strains with HF designations are derived from *A. eutrophus* H16.
[b] Hos, NAD-linked hydrogenase; Hop, particulate hydrogenase; Hoxd, hydrogenase derepression by energy limitation; Hoxi, H_2-dependent hydrogenase induction; HoxTs or HoxTr, thermosensitive or thermoresistant, respectively, hydrogenase synthesis.

Enzyme assays. Cells were grown in mineral medium with a substrate mixture of fructose and glycerol, which allows hydrogenase to be formed at a high derepressed level (6). The activity of the NAD-linked enzyme was measured spectrophotometrically (31) by a whole-cell assay (6). Membrane-bound hydrogenase was measured by H_2-dependent methylene blue reduction (26) with the particulate fraction of a crude extract (6). Soluble and particulate hydrogenase antigens were determined by immunodiffusion with antibodies raised against the purified proteins as described previously (27). Protein was assayed by the method of Lowry et al. (19).

DNA isolation, digestion, ligation, and transformation. Megaplasmid DNA was isolated as described previously (12). Large-scale vector and recombinant DNA preparations from *E. coli* were obtained by ethidium bromide-cesium chloride gradient centrifugation after alkaline lysis of the cells (3). Rapid, small-scale DNA isolation for clonal analysis was done by the method of Birnboim and Doly (3). Restriction endonuclease and T_4 DNA ligase were purchased from C. F. Boehringer & Soehne, Mannheim, Federal Republic of Germany, and used as recommended by the manufacturer. The *recA* strain of *E. coli* S17-1 was transformed with the ligation mixture by the method of Mandel and Higa (20). Tetracycline-resistant transformants were selected on NB plates supplemented with 10 μg of tetracycline per ml.

Construction of a pHG1 gene bank. Megaplasmid pHG1 was fractionated from the chromosomal DNA of *A. eutrophus* as described previously (12). This method enabled us to clone specifically plasmid-encoded genes and thus increased the probability of successfully identifying *hox* DNA. Megaplasmid pHG1 DNA was partially digested with *Eco*RI (2 h at 37°C with 0.3 to 0.8 U/μg of DNA) to generate a high proportion of relatively large fragments. This DNA was mixed with completely *Eco*RI-digested suicide vector pSUP202 (34) at a ratio of approximately 7:1 (wt/wt) and ligated with T4 DNA ligase (12 h at 4°C, 1.5 U/μg of DNA). The vector pSUP202 cannot replicate in *A. eutrophus*. The ligated DNA was introduced into *E. coli* S17-1 by transformation. From 10 to 15% of the Tc^r transformants proved to be Cm^s. In subsequent experiments with dephosphorylated vector DNA, insert-containing clones occurred at a frequency of 80 to 90%. A total of 150 individually isolated Tc^r Cm^s clones were chosen for plasmid analysis; 56% of these clones had inserts smaller than 10 kb, 42% were in the range of 10 to 20 kb, and 2% were larger than 30 kb.

Subcloning of *hox* DNA-containing hybrid plasmids. The originally cloned hybrid plasmids that gave rise to hydrogenase recombinants of various mutants contained several *Eco*RI restriction fragments of plasmid pHG1. To demonstrate that a specific fragment was necessary to restore Hox activity, one plasmid of each group was reestablished in *E. coli* HB101 by transformation, isolated, and subcloned into pSUP202 (34) and the broad-host-range vector pVK101 (17) after complete digestion with *Eco*RI. Subclones of pSUP202 that carried the described single fragment were identified after clonal analysis of Tc^r Cm^s *E. coli* S17-1 transformants and used in subsequent marker rescue experiments. The *Eco*RI restriction site in plasmid pVK101 is not located in an antibiotic resistance gene (17). Thus, hybrid plasmids derived from pVK101 were identified by analyzing lysates of Km^r transformants of *E. coli* S17-1 for the occurrence of size-enlarged vector DNA. These recombinant plasmids, designated pGE, were used in complementing *hox* mutations.

Restriction endonuclease mapping and hybridization. Stan-

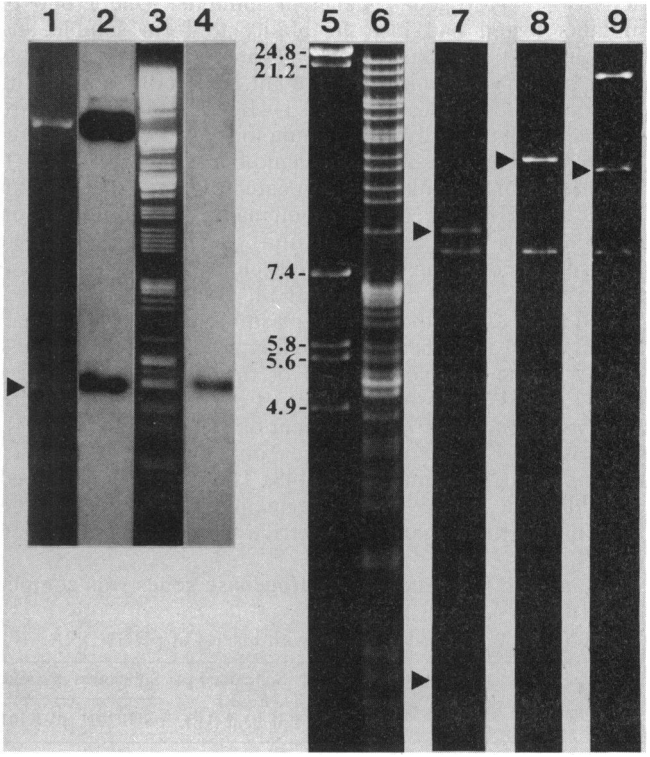

FIG. 1. Autoradiographs of Southern blots and *Eco*RI restriction digests of cloned *hox* DNA from plasmid pHG1 of *A. eutrophus*. *Eco*RI digests of plasmid pCH102 (lane 1); pCH102 probed with ^{32}P-labeled pCH102 (lane 2); megaplasmid pHG1 separated by electrophoresis in 2% agarose (lane 3); pHG1 probed with ^{32}P-labeled pCH102 (lane 4); digested λ DNA (with sizes indicated in kilobases) (lane 5); *Eco*RI digests of pHG1 (lane 6), pCH104 (lane 7), pCH128 (lane 8), and pCH122 (lane 9) run in 0.8% agarose. Arrows indicate *hox* DNA-containing bands.

dard procedures involving single and double digestions by the restriction endonucleases described in Results were used to identify the relative positions of cleavage sites within purified recombinant plasmid DNA. Agarose gel electrophoresis was performed as previously described (12). DNA fragments obtained from restriction endonuclease digestions were separated by agarose gel electrophoresis and then transferred to nylon GeneScreen Plus membranes (New England Nuclear Corp., Boston, Mass.) by the method of Southern (35). Recombinant DNA was labeled in vitro to a specific activity of 0.7 to 1 mCi/μg by incorporation of [^{32}P]dCTP (Amersham Buchler GmbH & Co. KG, Braunschweig, Federal Republic of Germany) by the nick translation procedure of Rigby et al. (24). The blotted fragments were hybridized with the ^{32}P-labeled probes at 65°C for 24 h and exposed to X-ray film.

Chemicals. Biochemicals were obtained from C. F. Boehringer & Soehne GmbH, Mannheim, Federal Republic of Germany. Antibiotics and agarose type V were purchased from Sigma Chemical Co., St. Louis, Mo. Agar was from Oxoid Ltd., London, England, and nutrient broth from Difco, Detroit, Mich. All other chemicals were purchased from E. Merck AG, Darmstadt, Federal Republic of Germany.

RESULTS

Identification of *hox* DNA. *E. coli* clones with inserts of pHG1 DNA were examined for the ability to restore Hox activity in hydrogenase-deficient mutants. One group of mutants tested had lost the ability to form soluble and particulate hydrogenase (Hox$^-$). Two representatives of this class are the revertible point mutant HF18 (10) and the Tn5 insertion mutant HG148 (Table 1). Both Hox$^-$ mutants are devoid of catalytically and immunologically active hydrogenase protein as a result of a plasmid-borne mutation (8, 24). The pHG1 DNA bank was conjugated by patch mating with HF18 and HF148. Hox$^+$ recombinants were selected on antibiotic-free mineral medium under H_2-dependent autotrophic conditions. Among 600 clones tested, 7 gave rise to Hox$^+$ recombinants with HF18. A clonal analysis revealed that all of the hybrid plasmids contained a small *Eco*RI insert, identified as a 2-kb fragment, that was scarcely visible in the complex pattern of the megaplasmid digest (Fig. 1, lanes 3 and 6). However, it clearly hybridized with the corresponding *Eco*RI fragment of pHG1 (Fig. 1, lane 4). Of the seven Hox-restoring clones, only five generated Hox$^+$ recombinants with mutant HF148. These clones contained an additional 8.6-kb *Eco*RI fragment that could easily be identified in the megaplasmid retriction map (Fig. 1, lanes 6 and 7).

The search for structural hydrogenase genes was complicated by the fact that direct selection for recombinants that had recovered either soluble hydrogenase (Hos) or particulate hydrogenase (Hop) activity was not possible. Mutants impaired in Hos activity are still able to grow with H_2 due to catalysis of the Hop enzyme. However, autotrophic growth of these mutants is significantly slower than that of the wild type, whereas mutants deficient in Hop activity but still active in Hos grow with H_2 as fast as the parent strain does (13).

In marker rescue experiments we had observed that approximately 80% of the Hox$^+$ recombinants were Tc^r. These heterogenote recombinants resulted from a single crossover that led to the incorporation of the whole hybrid DNA in homologous DNA sequences of the recipient genome. Plasmids from heterogenote recombinants exhibited the 8-kb vector DNA when analyzed by restriction endonuclease digestion (data not shown). We took advantage of heterogenote recombinants and identified genes for soluble hydrogenase by conjugating the pHG1 gene bank with Hos$^-$ recipients and primarily selecting heterotrophically for Tc^r recombinants. Two representative recipients used in this study were HF14 and HF15. Both strains are defective in catalytic activity of Hos but still contain Hos antigen and normal Hop activity (13, 29). Tc^r recombinants were replica plated onto mineral agar and examined for wild-type-specific autotrophic growth. Two independently isolated clones gave rise to Hos$^+$ recombinants with mutant HF15. They contained a common 11.6-kb *Eco*RI fragment (Fig. 1, lane 8). Not a single Tc^r recombinant of mutant HF14 proved to be restored in Hos activity.

In the course of analyzing Tn*5*-induced mutants we accidentally detected a mutant (HF221) with an insertion in an 11.0-kb *Eco*RI fragment of plasmid pHG1 that was associated with the loss of Hop activity (C. Kortlüke and B. Friedrich, manuscript in preparation). The corresponding 11.0-kb wild-type fragment was identified in the clone bank (Fig. 1, lane 9) by random screening for pHG1 inserts.

Characterization of plasmids with hydrogenase-restoring function. The subcloned hybrid plasmids (Table 2) were retested for the ability to generate Hox transconjugants with

TABLE 2. Hybrid plasmids with inserts of pHG1 DNA

Plasmid of the gene bank[a]	*Eco*RI fragments of insert (kb)[b]	Subcloned *Eco*RI fragment (kb)	Subcloned plasmid	
			pSUP202	pVK101
pCH101	8.6, 2.0, 1.0	8.6, 2.0	pCH104	pGE4
		8.6	pCH103	pGE6
		2.0	pCH102	pGE8
pCH113	11.6, 1.6	11.6	pCH128	pGE5
pCH122	20.0, 11.0, 3.7, 2.2	11.0	pCH129	pGE1
pCH116	2.0			

[a] pCH116 was constructed from a gene bank of megaplasmid pHG1-17 isolated from the HoxTr mutant HF117 (11).
[b] Determined on agarose gels.

TABLE 3. Transfer of *hox* genes necessary for the expression of both hydrogenases

Recipient	Donor plasmid	Occurrence of Hox transconjugants[a]
HF18	pCH104	+
	pGE4	+
HF148	pCH104	+
	pGE4	+
HF18	pCH103	−
	pGE6	−
HF148	pCH103	+
	pGE6	(+)
HF18	pCH102	+
	pGE8	−
HF148	pCH102	−
	pGE8	−
HF18	pCH116	+

[a] Transconjugants generated with pCH plasmid-harboring *E. coli* S17-1 donors were isolated on minimal agar with H_2-CO_2-O_2 after spot-mating with the given mutants. pGE plasmid-containing transconjugants were isolated heterotrophically on fructose-mineral medium supplemented with tetracycline. Symbols: +, wild-type growth with H_2; (+), slow growth with H_2; −, no growth with H_2.

TABLE 4. Restoration of hydrogenase activity by inserts that contain presumptive structural *hox* genes

Recipient	Phenotype	Donor plasmid	Phenotype of transconjugants[a]
HF15	Hos$^-$	pCH128	Hos$^+$
		pGE5	Hos$^+$
HF14	Hos$^-$	pCH128	Hos$^-$
		pGE5	Hos$^-$
HF08	Hop$^-$	pCH129	Hop$^-$
		pGE1	Hop$^-$
HF221	Hop$^-$	pCH129	Hop$^{(+)}$
		pGE1	Hop$^{(+)}$

[a] Hos$^+$, Wild-type soluble hydrogenase activity; Hop$^{(+)}$, low particulate hydrogenase activity.

different classes of hydrogenase mutants. The conjugal transfer of the hybrids was several orders of magnitude higher than the reversion to hydrogen prototrophy for these mutant strains. Since the mutants used were Rec$^+$, the possibility of a low level of recombination within the pVK101 hybrid-bearing transconjugants cannot be eliminated. However, the recombinant pGE plasmids were relatively stably maintained in *A. eutrophus* H16. In cultures that had been grown for 3 days to obtain cells for enzymatic analysis, 70 to 90% of the population still expressed the pVK101-encoded antibiotic resistances. This proportion was even higher under selective pressure when tetracycline-or kanamycin-supplemented medium was used. Hybrid plasmids, such as pGE4, were retransferred from *A. eutrophus* to *E. coli* without losing their *hox*-complementing function. Reisolation and restriction analysis of the plasmid DNA did not reveal any detectable physical changes within the hybrid.

Wild-type-specific growth with hydrogen in mutant HF18 was restored by marker exchange with the plasmid pCH102, which contains the 2.0-kb *Eco*RI fragment (Table 3). However, the mutation in HF18 was not complemented by the corresponding plasmid pGE8. Complementation to Hox$^+$ required an additional 8.6-kb *Eco*RI fragment, present in pGE4. Mutant HF148 was not restored in Hox by marker exchange with the 2.0-kb insert-bearing hybrid but with the 8.6-kb fragment of pCH103. Transconjugants that arose from transfer of pGE6 into HF148 did not exhibit wild-type-specific lithoautotrophic growth but grew extremely slowly with hydrogen. The *hox* mutation in HF148 was complemented by plasmid pGE4 (Table 3). Recombinants of HF18 that were obtained with the hybrid pCH116-harboring donors grew with H_2 at 37°C. pCH116 had been constructed with megaplasmid DNA of the temperature-resistant (HoxTr) mutant HF117, and proved to contain the 2.0-kb *Eco*RI fragment (Table 2). The transfer of plasmid pGE4 to *A. hydrogenophilus* M50 led to Tc^r transconjugants that contained soluble hydrogenase activity under heterotrophic energy-limited conditions in the absence of H_2 and thus expressed the regulatory Hox phenotype of *A. eutrophus* H16. However, particulate hydrogenase in these transconjugants was still repressed (data not shown).

Tc^r transconjugants of the Hos$^-$ mutant HF15 had recovered wild-type soluble hydrogenase activity after conjugation with the 11.6-kb insert-bearing *E. coli* clones (Table 4). However, no return of Hos activity was observed after transfer of the 11.6-kb *Eco*RI fragment into mutant HF14. Tc^r transconjugants isolated after mating the Hop$^-$ mutants HF08 and HF221 with the 11.0-kb *Eco*RI insert-containing clones did not show wild-type particulate hydrogenase activity. Nevertheless, transconjugants of mutant HF221 revealed up to 6% of the Hop activity that was determined in the parent strain, *A. eutrophus* H16 (Table 4).

Localization of *hox* within plasmid pCH104. A series of hybrid plasmids that genetically complemented the Hox$^-$ mutants HF18 and HF148 had *Eco*RI fragments of 8.6 and 2.0 kb in common. Three lines of evidence suggest that these two fragments are arranged contiguously on the megaplasmid genome. (i) They were observed in five independently isolated hybrid plasmids, three of which differed with respect to at least one other *Eco*RI fragment. The latter therefore cannot be from siblings. (ii) Both inserts were essential for a *trans*-acting Hox restoration function. (iii) Marker exchange revealed that the mutational sites associated with the Hox$^-$ phenotype occurred within the subcloned DNA fragments of 8.6 and 2.0 kb. The physical map of the 10.6-kb insert DNA of pCH104 based on restriction endonuclease digestion is presented in Fig. 2. There is evidence for at least one additional *Sal*I restriction site within the sequences of 2.1 and 3.6 kb.

To identify the precise location of the *hox* gene(s), we cloned various parts of plasmid pCH104 into the vector pVK101. The resulting hybrid plasmids are listed in Fig. 2. They were transferred to the Hox$^-$ mutants HF18 and HF148 by conjugation. Transconjugants were selected heterotrophically for Tc^r and Km^r, respectively, and subjected to enzymatic analysis. As expected from the growth studies (Table 3), the complete 10.6-kb region of plasmid pGE4 restored Hos and Hop activities in both mutants to the wild-type level (Table 5). The 4.3-kb *Eco*RI–*Bam*HI fragment of plasmid pGE2 only complemented the *hox* mutation in mutant HF18. The introduction of the 2.1-kb *Eco*RI-*Sal*I fragment cloned in pGE7 generated Km^r transconjugants of HF18 which grew extremely slowly with H_2, and neither contained detectable Hos or Hop activity when cultivated heterotrophically under hydrogenase-derepressing conditions.

The 8.6-kb *Eco*RI fragment in plasmid pCH103 generated Hox$^+$ recombinants with mutant HF148 which exhibited wild-type growth with H_2 (Table 3). However, when introduced in *trans* by plasmid pGE6, the same fragment only restored 5 to 10% of Hos and Hop activity (Table 5). This result is consistent with the slow lithoautotrophic growth of these transconjugants. Extremely low Hos and Hop activi-

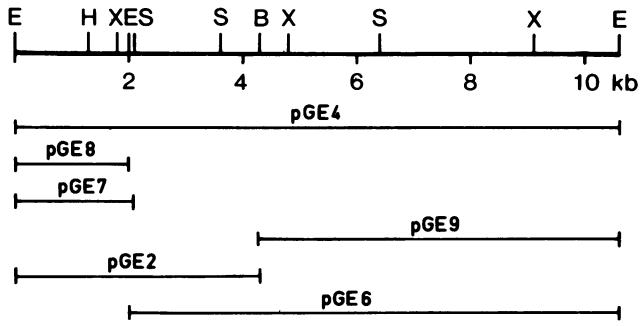

FIG. 2. Hybrid plasmids with *hox* DNA sequences. A map of the putative *hox* regulatory region of plasmid pHG1 cloned in plasmid pCH104 is shown, with distances given in kilobases. Pertinent restriction endonuclease recognition sites: E, *Eco*RI; H, *Hin*dIII; X, *Xho*I; S, *Sal*I; B, *Bam*HI. The lines indicate the location and size of individual restriction fragments which were cloned into vector pVK101. The resulting plasmid designations are given above the lines.

ties were found in derivatives of mutant HF148 bearing the 6.3-kb *Bam*HI-*Eco*RI fragment cloned in plasmid pGE9 (Table 5).

TABLE 5. Hydrogenase activities in mutants carrying subclones derived from plasmid pVK101

Recipient	Plasmid[a]	Enzyme activity (U/mg of protein)[b]	
		Hos	Hop
H16 (wild type)	None	3.10	1.10
HF18	None	0	0
	pVK101	0	0
	pGE4	2.53	0.54
	pGE8	0	0
	pGE7	0	0
	pGE6	0	0
	pGE2	2.38	0.96
HF148	pVK101	0	0
	pGE4	2.15	1.170
	pGE8	0	0
	pGE2	0.018	0
	pGE6	0.415	0.110
	pGE9	0.096	0.097

[a] See Fig. 2.
[b] Cells were grown in fructose-glycerol-mineral medium as described previously (6). Hos, Soluble hydrogenase activity; Hop, particulate hydrogenase activity.

DISCUSSION

We have constructed a megaplasmid pHG1 DNA library of *A. eutrophus* H16 to clone hydrogenase genes. pHG1-borne *hox* DNA was identified in hybrid plasmids by their ability to restore hydrogenase activity in three classes of hydrogenase mutants. A precise analysis of the cloned inserts revealed that the sizes of fragments previously deduced from the megaplasmid restriction map were generally overestimated. Thus, the numbers given in parentheses refer to fragment sizes reported in previous publications (7, 12).

One class of mutants difficient in soluble hydrogenase recovered Hos activity after transfer of the 11.6-kb (13.3-kb) *Eco*RI fragment. The mutants are impaired in H_2-dependent NAD reduction but still contain Hos antigen (13). Biochemical analysis revealed no changes in the structure or cofactor content of the Hos protein (S. Hornhardt, unpublished result). This indicates a mutation in one of the structural genes which may affect binding of the substrates H_2 and NAD or intramolecular electron transport. Thus, we assume that the 11.6-kb fragment bears parts of the Hos genes, presumably the start of the transcript, since the DNA was active in *trans* complementation. The fact that another class of Hos^- mutants was not restored in Hos activity by the 11.6-kb fragment suggests that the mutation maps at another locus of the megaplasmid DNA. Mutant HF14, a representative of the second group, is biochemically different from the Hos^- mutants discussed above. It still contains the two large Hos polypeptides but it does not exhibit cross-reactivity with antibodies raised against the two small subunits (15).

Preliminary evidence for the cloning of *hox* DNA associated with the particulate enzyme is based on a weak restoration of Hop activity in a mutant bearing an insertion in an 11.0-kb (12.4-kb) *Eco*RI fragment. The corresponding wild-type fragment did not generate Hop^+ transconjugants with another Hop^- mutant which contains a pHG1-borne point mutation (13). The insertion mutant is devoid of Hop protein (C. Kortlüke, unpublished result), whereas the point mutant exhibits wild-type Hop antigen in the soluble fraction of the extract (13). Thus, the latter strain may be impaired in a component which is necessary for the incorporation of the Hop enzyme into the membrane. If the 11.0-kb *Eco*RI fragment carries *hox* genes essential for the function of Hop activity, the results clearly show that this piece of DNA is not sufficient to allow full expression of the Hop enzyme. It is interesting that the 11.0-kb *Eco*RI fragment has recently been identified as the locus of megaplasmid-linked genes involved in autotrophic CO_2 fixation (16).

The third class of mutants that were shown to be complemented by pHG1 DNA are characterized by the loss of both Hos and Hop proteins and most probably represent regulatory hydrogenase mutants (Hox^-). The region necessary for generating Hox recombinants with mutant HF18 was shown to be 2.0 kb (2.4 kb). For *trans* complementation of this mutant, a larger sequence, located in the 4.3-kb *Eco*RI-*Bam*HI fragment, was necessary. Two lines of evidence suggest that this DNA region encodes a temperature-sensitive regulatory protein which is essential for the expression of both hydrogenases. (i) Hybrid plasmids containing the 2.0-kb fragment cloned from a temperature-resistant mutant generated HoxTr transconjugants. (ii) The introduction of the 4.3-kb hybrid plasmid to *A. hydrogenophilus* (5) affected the Hox regulation of this species. Hos activity of the transconjugants responded to the same energy-dependent derepression signal as found in *A. eutrophus* H16 (6).

The *hox* mutation in strain HF148 apparently maps apart from the 2.0-kb DNA region. Hos and Hop activity in this mutant were completely restored by marker exchange with the adjacent 8.6-kb (9.0-kb) *Eco*RI fragment. However, the same fragment led to only low hydrogenase activity when introduced in *trans*. For complementation the entire DNA sequence of 8.6 and 2.0 kb was necessary. We do not yet understand the nature of the *hox* mutation in mutant HF148. Its phenotype may result from a lesion in a second regulatory DNA sequence or a gene whose product is involved in the synthesis of active hydrogenase proteins. Although mutant HF148 is devoid of hydrogenase antigen, we discuss the possibility that this strain contains an extremely unstable protein. Attempts to restore hydrogenase activity in mutant HF148 by a high concentration of nickel in the growth medium were unsuccessful (B. Friedrich, unpublished result). Nickel-requiring hydrogenase mutants have recently been detected in *E. coli* (38).

The fact that only some types of mutants within each class are complemented by the cloned megaplasmid DNA indicates that there is more information to be discovered. Our previous results have shown that the system is even more complex, since the expression of a plasmid-encoded *hox* genes is dependent on the function of a chromosomal gene (13).

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