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A Hydrogen-Sensing System in Transcriptional Regulation of Hydrogenase Gene Expression in *Alcaligenes* Species

OLIVER LENZ,[1] ANGELIKA STRACK,[1] ANDREA TRAN-BETCKE,[2] AND BÄRBEL FRIEDRICH[1]*

Institut für Biologie der Humboldt-Universität zu Berlin[1] and Institut für Pflanzenphysiologie und Mikrobiologie der Freien Universität Berlin,[2] Berlin, Germany

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Heterologous complementation studies using *Alcaligenes eutrophus* H16 as a recipient identified a hydrogenase-specific regulatory DNA region on megaplasmid pHG21-a of the related species *Alcaligenes hydrogenophilus*. Nucleotide sequence analysis revealed four open reading frames on the subcloned DNA, designated *hoxA*, *hoxB*, *hoxC*, and *hoxJ*. The product of *hoxA* is homologous to a transcriptional activator of the family of two-component regulatory systems present in a number of H_2-oxidizing bacteria. *hoxB* and *hoxC* predict polypeptides of 34.5 and 52.5 kDa, respectively, which resemble the small and the large subunits of [NiFe] hydrogenases and correlate with putative regulatory proteins of *Bradyrhizobium japonicum* (HupU and HupV) and *Rhodobacter capsulatus* (HupU). *hoxJ* encodes a protein with typical consensus motifs of histidine protein kinases. Introduction of the complete set of genes on a broad-host-range plasmid into *A. eutrophus* H16 caused severe repression of soluble and membrane-bound hydrogenase (SH and MBH, respectively) synthesis in the absence of H_2. This repression was released by truncation of *hoxJ*. H_2-dependent hydrogenase gene transcription is a typical feature of *A. hydrogenophilus* and differs from the energy and carbon source-responding, H_2-independent mode of control characteristic of *A. eutrophus* H16. Disruption of the *A. hydrogenophilus hoxJ* gene by an in-frame deletion on megaplasmid pHG21-a led to conversion of the regulatory phenotype: SH and MBH of the mutant were expressed in the absence of H_2 in response to the availability of the carbon and energy source. RNA dot blot analysis showed that HoxJ functions on the transcriptional level. These results suggest that the putative histidine protein kinase HoxJ is involved in sensing molecular hydrogen, possibly in conjunction with the hydrogenase-like polypeptides HoxB and HoxC.

Alcaligenes hydrogenophilus is an aerobic, facultatively lithoautotrophic proteobacterium capable of obtaining energy from the oxidation of molecular hydrogen (5). Hydrogen oxidation in this strain is catalyzed by two hydrogenases (18). The soluble hydrogenase (SH) is closely related to the NAD-reducing hydrogenases present in *Alcaligenes eutrophus*, in the gram-positive *Rhodococcus* sp. strain 1b (5), and in the cyanobacterium *Anabaena variabilis* (42). The second hydrogenase of *A. hydrogenophilus* is a membrane-bound hydrogenase (MBH) coupled to the respiratory chain and involved in electron transport-dependent phosphorylation (18). The large and the small subunits of this enzyme show 75 and 90% identity, respectively, to the corresponding polypeptides of the *A. eutrophus* MBH (28, 53). Moreover, in both *Alcaligenes* species H_2-oxidizing ability is genetically linked to a megaplasmid (18) and dependent on the function of an RpoN-like sigma factor of RNA polymerase which is encoded on the chromosome (32, 38).

Despite the high degree of similarity, hydrogenase regulation in *A. hydrogenophilus* appears to be quite different from the mode of control observed in *A. eutrophus*. While the latter species produces both hydrogenases at a derepressed level when the growth rate is limited by the energy source, even in the absence of H_2 (20, 21), hydrogenase synthesis in *A. hydrogenophilus* strictly relies on the presence of molecular hydrogen (18) as described for a variety of aerobic H_2-oxidizing bacteria (17, 49). Although the molecular background for the more global energy-dependent hydrogenase gene expression on the one hand and the substrate-mediated regulation on the other hand is far from being understood, a principal transcription factor classified as a member of two-component regulatory proteins (reviewed in references 36 and 45) has been identified in several hydrogenase-containing bacteria. HoxA is essential for hydrogenase gene transcription in *A. eutrophus* (13, 54) and shares about 50% sequence identity with the *hoxA* gene product of *Bradyrhizobium japonicum* (48). A HoxA homolog has also been found in *Rhodobacter capsulatus* (HupR1) (37), which points to the existence of a common regulatory component in hydrogenase gene transcription of aerobic hydrogen oxidizers.

In a previous communication it was shown that the exchange of the hydrogenase-encoding megaplasmids between *A. hydrogenophilus* and *A. eutrophus* resulted in transconjugants which expressed the regulatory phenotype typical of the megaplasmid parent (18). To elucidate the molecular characteristics of H_2-responsive and energy source-dependent regulation on the genetic level, we conducted heterologous complementation studies with a megaplasmid-borne DNA library. In the course of these experiments we discovered a DNA region on the *A. hydrogenophilus*-specific megaplasmid pHG21-a consisting of four open reading frames, *hoxA*, *hoxB*, *hoxC*, and *hoxJ*. Sequence comparison revealed almost complete identity to the corresponding stretch of genes on megaplasmid pHG1 of *A. eutrophus* (13, 16) (accession no. U82564). We present evidence that the product of *hoxJ* in *A. hydrogenophilus* plays a crucial role in the regulation of SH and MBH gene expression by H_2 induction.

* Corresponding author. Mailing address: Institut für Biologie, Humboldt-Universität zu Berlin, Chausseestr. 117, D-10115 Berlin, Germany. Phone: 49-30-20938100. Fax: 49-30-20938102. E-mail: baerbel=friedrich@rz.hu-berlin.de.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Strains with names beginning with the letters HF were derived from the wild-type *A. eutrophus* H16. In *A. eutrophus* HF157 the indigenous megaplasmid

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Source or reference
Strains		
A. hydrogenophilus M50	Wild type, pHG21-a, pHG21-b	35
A. eutrophus		
H16	Wild type, pHG1	DSM 428; ATCC 17699
HF157	pHG1$^-$, pHG21-a	18
HF409	pHG1 *hoxA*Δ	This study
HF423	pHG21-a *hoxJ*Δ	This study
E. coli		
S17-1	Tra$^+$ *recA pro thi hsdR chr*::RP4-2	44
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac* (F′ *proAB lacI*^{q}ZΔM15 Tn*10*)	Stratagene Cloning Systems, Inc.
HB101	*supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13* Δ(*mcrC-mrr*) *hsdS20* (r$^-$ m$^-$)	6
Plasmids		
pBluescript SK+	Ap^r *lacZ′* T7 ϕ10 promoter f1 *ori*	Stratagene Cloning Systems, Inc.
pBluescript SK−	Ap^r *lacZ′* T7 ϕ10 promoter f1 *ori*	Stratagene Cloning Systems, Inc.
pBluescript KS+	Ap^r *lacZ′* T7 ϕ10 promoter f1 *ori*	Stratagene Cloning Systems, Inc.
pRK2013	Km^r Tra$^+$	12
pVK101	Km^r Tc^r Mob$^+$, RP4 *ori*	27
pVK102	Km^r Tc^r Mob$^+$, RP4 *ori*	27
pLO1	Km^r *sacB*, PR4, *oriT*, ColE1 *ori*	29
pCH220	3.6-kb *Xba*I-*Bam*HI *hoxA*-containing fragment of pGE97 in pTZ19R	13
pCH442	1.7-kb *Bam*HI-*Bgl*II *hoxJ*-containing fragment of pGE295 in pBluescript KS+	This study
pCH553	1.0-kb *Ssp*I-*Pst*I and 0.24-kb *Pst*I fragments of pCH220 inserted between *Eco*RV and *Pst*I of pLO1	This study
pCH554	Derivative of pCH442 with a deleted 1,026-bp *Nru*I fragment	This study
pCH555	0.64-kb *Nae*I-*Sma*I fragment of pCH554 in *Pme*I of pLO1	This study
pGE273	27-kb *Hin*dIII fragment of pHG21-a in pVK102	This study
pGE295	9.1-kb *Hin*dIII-*Bgl*II fragment of pGE273 in pVK101	This study
pGE296	7.4-kb *Eco*RI fragment of pGE273 in pVK101	This study
pGE303	1.7-kb *Bam*HI-*Bgl*II fragment of pGE273 in pVK101	This study

pHG1 was replaced by the *A. hydrogenophilus*-specific megaplasmid pHG21-a (18). *A. eutrophus* HF409 carries an in-frame deletion in *hoxA* of pHG1. Strain HF423, a derivative of HF157, carries a *hoxJ* deletion allele in pHG21-a. Plasmid pGE273 contains a 27-kb *Hin*dIII fragment of the *A. hydrogenophilus* megaplasmid pHG21-a cloned in plasmid pVK102 (27), and 9.1-kb *Hin*dIII-*Bgl*II, 7.4-kb *Eco*RI, and 1.7-kb *Bam*HI-*Bgl*II fragments of pGE273 were subcloned into pVK101 (27) to yield pGE295, pGE296, and pGE303, respectively.

Media and growth conditions. *Alcaligenes* strains were grown in nutrient broth, in a modified Luria-Bertani broth containing 0.25% sodium chloride (LSLB), or in mineral salts medium as described previously (14). Synthetic media for heterotrophic growth contained 0.4% (wt/vol) fructose (FN medium) or 0.2% (wt/vol) fructose and 0.2% (vol/vol) glycerol (FGN medium). Lithoautotrophic cultures were grown in mineral salts medium under an atmosphere of hydrogen, carbon dioxide, and oxygen (8:1:1, vol/vol/vol). Sucrose-resistant segregants of *sacB*-harboring strains were selected on LSLB plates containing 15% (wt/vol) sucrose (29). *Escherichia coli* strains were grown in Luria-Bertani medium (31). Solid media contained 1.2% (wt/vol) agar. Antibiotics were supplemented as follows: 400 μg of kanamycin per ml and 15 μg of tetracycline per ml for *A. eutrophus* and 25 μg of kanamycin per ml, 15 μg of tetracycline per ml, and 100 μg of ampicillin per ml for *E. coli.*

Construction of a cosmid library of the *A. hydrogenophilus* megaplasmid pHG21-a. Megaplasmid DNA from strain HF157 was isolated and purified as previously described (25). The DNA was partially digested with *Hin*dIII and ligated with the *Hin*dIII-treated pVK102 DNA. The resulting cosmids were introduced into λ phage particles by the aid of the DNA packaging kit (Boehringer Mannheim) following the instructions of the manufacturer. The packaging mixture was used for transduction of competent *E. coli* HB101 cells. The recombinant plasmids of the pHG21-a cosmid library were transferred to *A. eutrophus* via triparental mating using plasmid pRK2013 for mobilization (12).

Cloning and sequencing. Standard DNA techniques were applied in this study (2). DNA fragments were isolated from agarose gels with the GlassMax DNA Isolation Spin Cartridge System (GIBCO BRL). For sequence analysis, restriction fragments subcloned from pGE295 were inserted into pBluescript SK+, SK− and KS+. Serial deletions were generated by treatment with exonuclease III and S1 nuclease (24) with an Erase-a-Base kit (Promega Corp.), following the instructions of the manufacturer. Single-stranded templates were isolated from transformants of *E. coli* XL1-Blue after infection with helper phage R408 (Stratagene Cloning Systems, Inc.) and sequenced by the dideoxy chain termination method (39) using the Sequenase DNA Sequencing Kit (United States Biochemical) and ^{35}S-dATP (Amersham-Buchler). Nucleotide sequences were compiled and analyzed with the PC/GENE software package (IntelliGenetics, Inc.). Database searches were conducted with the National Biomedical Research Foundation BLAST facility (1).

Isolation of deletion mutants. An in-frame deletion in *hoxA* of *A. eutrophus* was constructed as follows. A 1.02-kb *Ssp*I-*Pst*I fragment of pCH220 (13) carrying the 5′ end of *hoxA* was cloned into *Eco*RV-*Pst*I-digested pLO1. The resulting plasmid was linearized by cutting with *Pst*I, and the construction was completed by inserting a pCH220-borne 0.24-kb *Pst*I fragment carrying the 3′ end of *hoxA*. The resulting plasmid pCH553 contained a 921-bp deletion in *hoxA*. An in-frame deletion of 1,026 bp was introduced into *hoxJ* of *A. hydrogenophilus* by religation of *Nru*I-treated plasmid pCH442. A 0.64-kb *Nae*I-*Sma*I fragment of the resulting plasmid, pCH554, carrying the *hoxJ* deletion allele, was subcloned into *Pme*I-digested pLO1 to yield pCH555. pCH553 and pCH555 were transferred to *A. eutrophus* by conjugation. Kanamycin-sensitive, sucrose-resistant survivors were isolated as previously described (29) and screened for the presence of the desired mutation as follows: an appropriate amount of colony material was resuspended in 0.5 ml of distilled water and boiled for 7 min. Then 10 μl of this lysate was used in a standard PCR (96°C for 1 min, 60°C for 30 s, and 72°C for 30 s for 25 cycles) with primers covering the deleted region. Deletion-carrying isolates were identified on the basis of altered electrophoretic mobility of the amplification products.

Southern hybridization. pHG21-a DNA (5 to 10 μg) was treated with appropriate restriction enzymes, separated by electrophoresis in 0.75% agarose gels, and transferred to Biodyne B nylon membranes (Pall Corp.) with a pressure blotting device (Stratagene Cloning Systems, Inc.). Following transfer the blots were UV cross-linked and baked for 2 h at 80°C. Hybridization was carried out at 68°C in QuickHyb Hybridization Solution (Stratagene Cloning Systems, Inc). Stringent washes were done in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–0.1% sodium dodecyl sulfate (SDS) at 68°C. A 1.24-kbp *Eco*RI-*Pvu*I digoxigenin (DIG)-labeled fragment of pCH220 was used as a probe. Labeling and detection were done with a DIG labeling and detection kit (Boehringer Mannheim) following the manufacturer's instructions.

Enzyme assays. Activities of SH (hydrogen:NAD$^+$ oxidoreductase; EC 1.12.1.2) and MBH (hydrogen:acceptor oxidoreductase; EC 1.18.99.1) were determined with cells grown lithoautotrophically or heterotrophically in FGN medium. SH activity was measured by spectrophotometric determination of H_2-dependent NAD reduction either with detergent-treated cells (19) or in soluble extracts

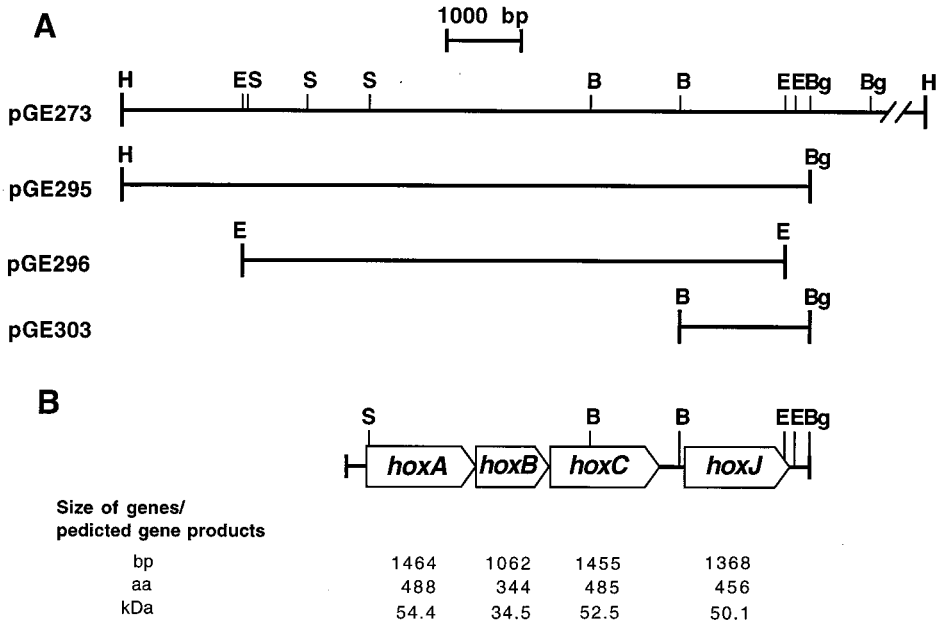


FIG. 1. (A) Schematic overview of the megaplasmid pHG21-a-borne subcloned DNA fragments which were used for heterologous complementation in this study. The designations of the respective plasmids are given on the left. (B) Genetic map of the sequenced hydrogenase regulatory region from *A. hydrogenophilus*. The arrows represent the predicted genes. A synopsis of the data deduced from the sequence is given in the lower part of the figure. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

(43). MBH activities were assayed with isolated membrane fractions by spectrophotometric measurement of H_2-dependent methylene blue reduction (40).

RNA dot blot analysis. Cells of *A. eutrophus* derivatives were grown in FGN medium to an optical density of 8 at 436 nm. Two milliliters of culture was rapidly collected by centrifugation. Isolation of total RNA was conducted as described previously (34). The RNA was denatured at 60°C for 15 min in a final volume of 20 μl containing 40 mM morpholinepropanesulfonic acid (MOPS; pH 7.0), 10 mM sodium acetate, 1 mM EDTA, 50% (vol/vol) formamide, and 6.5% (vol/vol) formaldehyde and subsequently transferred on Biodyne B nylon membranes by pipetting. *A. eutrophus* HF157 total DNA was isolated by the method of Chen and Kuo (8). A 715-bp fragment of the pHG21-a-borne *hupL* gene was amplified by standard PCR (96°C for 1 min, 60°C for 30 s, and 72°C for 30 s for 25 cycles) using the primer pair 5′-CGTCTACCTTCCGGACG-3′ and 5′-ACTTGTCGA CATTGGCC-3′ and pHG21-a DNA as a template. Both DNA preparations were labeled with the DIG DNA labeling kit (Boehringer Mannheim) following the instructions of the manufacturer. RNA-DNA hybridization was performed at 68°C for 2 h in QuickHyb hybridization solution (Stratagene Cloning Systems, Inc.). The membranes were washed twice for 10 min in 2× SSC–0.1% SDS at room temperature. The double washing steps were repeated at 60°C. The final step was conducted in 0.1× SSC–0.1% SDS for 15 min at room temperature. The DIG-labeled RNA-DNA hybrids were detected with the DIG DNA detection kit (Boehringer Mannheim) following the manufacturer's instructions.

Nucleotide sequence accession number. The sequence reported here was deposited in the EMBL/GenBank/DDBJ nucleotide sequence data banks under accession no. U82565.

RESULTS

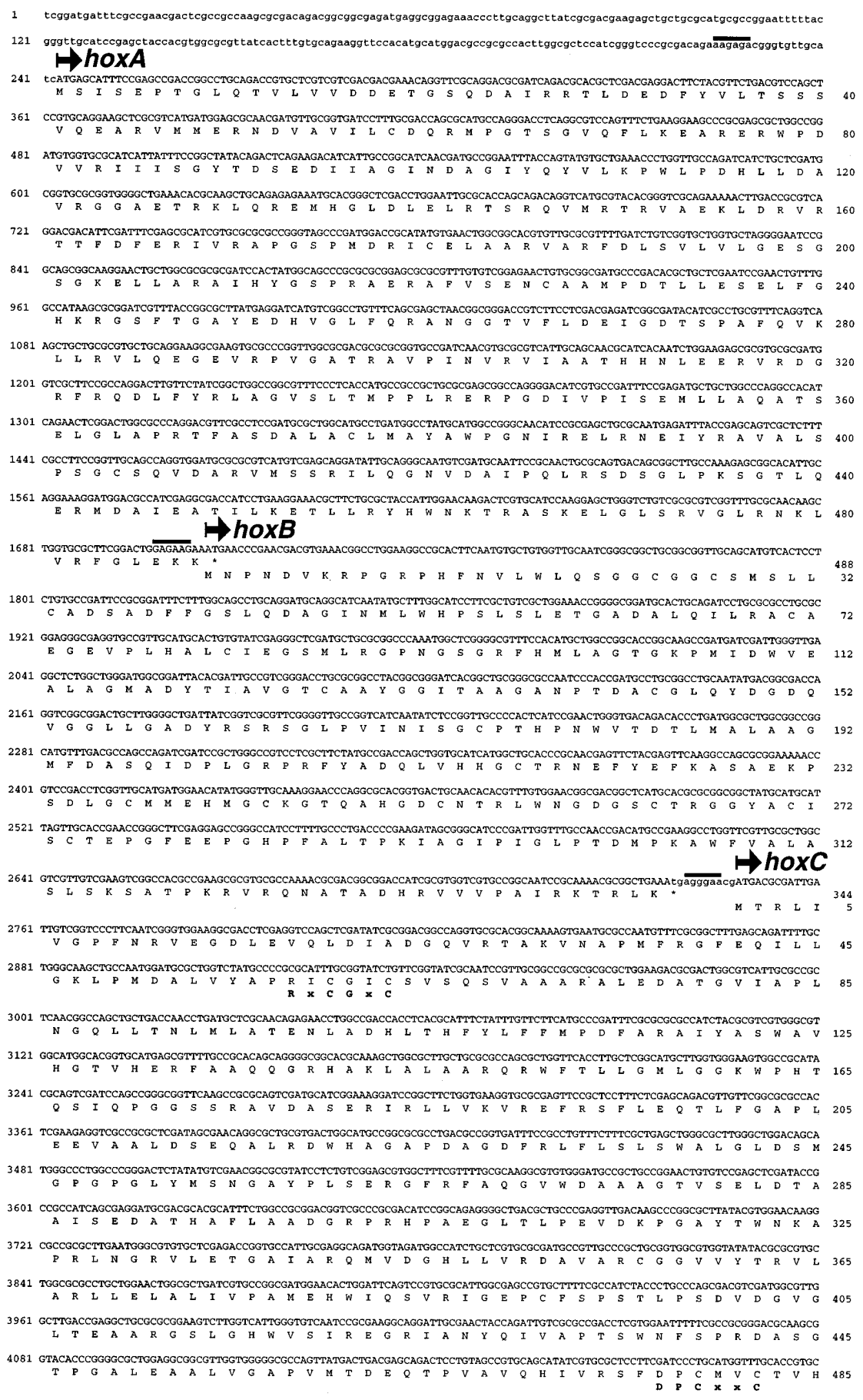
Identification of hydrogenase regulatory genes of *A. hydrogenophilus*. To identify regulatory determinants which participate in H_2-dependent hydrogenase gene expression in *A. hydrogenophilus*, we took advantage of its close phylogenetic relationship to *A. eutrophus* and selected this well-known strain as a recipient in heterologous gene transfer and expression. A cosmid library of megaplasmid pHG21-a DNA was constructed in the broad-host-range plasmid pVK102 (27) and was used for complementation of a hydrogenase-deficient mutant of *A. eutrophus*. This mutant (HF409) was unable to grow lithoautotrophically due to an in-frame deletion in the transcriptional activator gene *hoxA*. Of 313 tetracycline-resistant transconjugants tested, 16 isolates were restored in H_2-oxidizing ability. A representative cosmid (pGE273) containing a 27-kb *Hin*dIII insert of pHG21-a DNA (Fig. 1A) was chosen for further experiments.

An *A. hydrogenophilus* gene(s) alters hydrogenase gene expression in *A. eutrophus*. To identify the precise location of the *hoxA*-complementing determinant, we constructed a series of subclones of cosmid pGE273. One derivative (pGE295), bearing a 9.1-kb DNA insert (Fig. 1A), maintained its growth-restoring function in *A. eutrophus* HF409. Enzymatic analysis revealed that the pGE295-harboring cells contained a wild-type level of SH activity while MBH activity was absent (Table 2). Even more surprisingly, neither SH nor MBH was expressed in pGE295-containing transconjugant cells grown heterotrophically on fructose-glycerol. Under these conditions wild-type cells normally produce high levels of SH and MBH activity (Table 2). Moreover, the introduction of pGE295 into *A. eutrophus* H16 resulted in merodiploid transconjugants which almost failed completely to express the hydrogenase activities under heterotrophic derepression conditions, indicating that the *A. hydrogenophilus* DNA dominantly affects the

TABLE 2. Hydrogenase activities of *A. eutrophus* transconjugants

A. eutrophus strain	Relevant characteristic	Plasmid	Hydrogenase activity (U/mg of protein)[a] in:			
			H_2-CO_2-O_2		FGN medium	
			SH	MBH	SH	MBH
H16	Wild type	pVK101	1.67	2.06	2.81	0.54
HF409	*hoxA*Δ	pVK101	ND	ND	0.00	0.00
HF409	*hoxA*Δ	pGE295 (*hoxABCJ*)	1.52	0.00	0.00	0.00
H16	Wild type	pGE295 (*hoxABCJ*)	1.89	2.39	0.06	0.00
H16	Wild type	pGE296 (*hoxABCJ*Δ)	1.57	2.29	2.92	0.42

[a] Values give the average from at least two independent experiments. FGN medium, heterotrophic growth in mineral medium with fructose-glycerol; ND, not determined (strain HF409 does not grow under these conditions).


hoxA
hoxB
hoxC

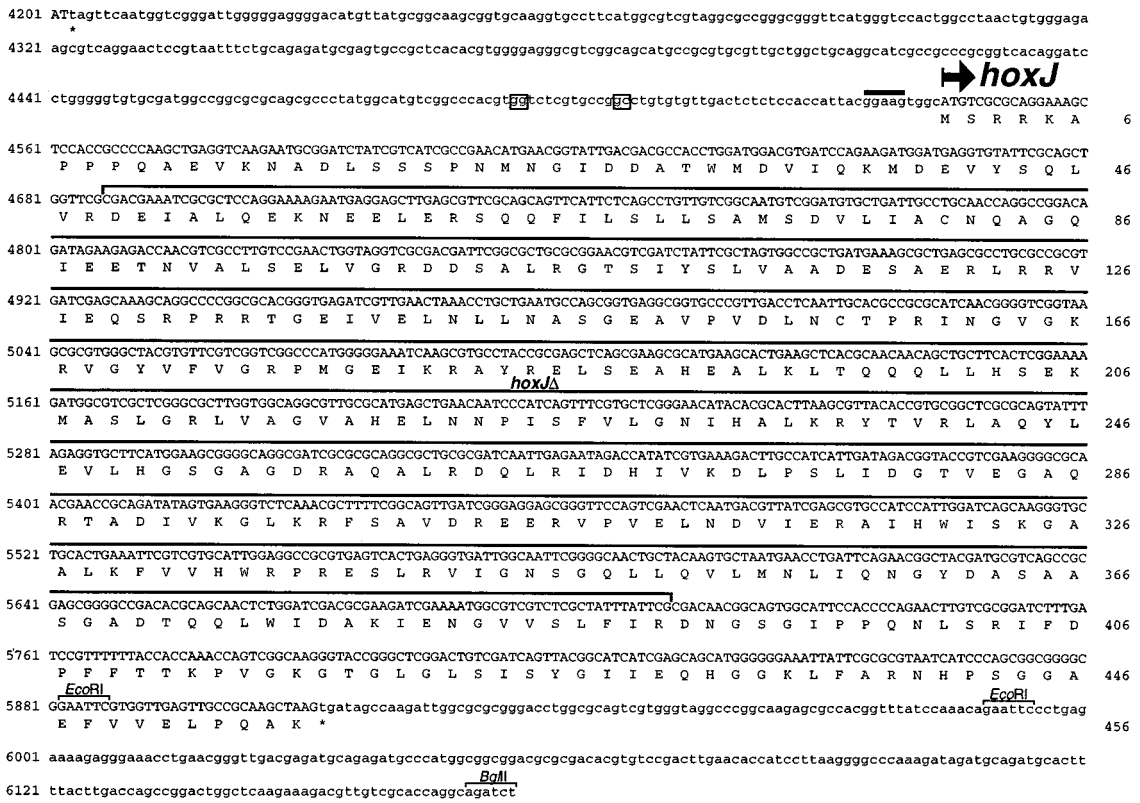


FIG. 2. Nucleotide sequence of a 6,174-bp segment of the *A. hydrogenophilus* regulatory region located on megaplasmid pHG21-a. Nucleotide coordinates are given on the right. The deduced amino acid sequences are given in standard single-letter code. Stop codons are marked by asterisks. Arrows indicate the start points of the predicted genes. Putative ribosome binding sites are emphasized by black bars. A possible −12/−24 promoter motif preceeding *hoxJ* is boxed. A bracket above the sequence marks the extent of the deletion in the *hoxJ* mutant allele. Two motifs in the deduced HoxC sequence which are conserved in [NiFe] hydrogenases (51) and participate in metal coordination in the active site of these enzymes are shown in boldface letters.

regulatory phenotype of *A. eutrophus* H16. The removal of relatively small pieces of DNA from both ends of the 9.1-kb insert, yielding plasmid pGE296 (Fig. 1A), released SH and MBH repression in the absence of H_2 (Table 2). This observation focused our attention on a careful analysis of the terminal sites of the 9.1-kb insert.

Structure of a hydrogenase regulatory DNA region on megaplasmid pHG21-a. DNA-DNA hybridization using an *A. eutrophus*-derived *hoxA*-containing fragment of plasmid pCH220 as a probe identified a *hoxA* homologous gene in *A. hydrogenophilus. hoxA* was localized on an internal *Sal*I-*Bam*HI fragment of plasmid pGE295 (Fig. 1A and data not shown). Correlating this observation with the complementation data described above led to the conclusion that the repression of SH and MBH in heterotrophically cultivated cells of pGE295-harboring transconjugants was probably triggered by a locus distant from *hoxA*. To search for this potential regulatory component, a 6,174-bp fragment of plasmid pGE295 was subjected to nucleotide sequence determination (Fig. 1B). Sequence analysis uncovered four contiguous open reading frames, designated *hoxA*, *hoxB*, *hoxC*, and *hoxJ*, each of which is preceded by a tentative ribosome binding site (Fig. 2). Stop and start codons of *hoxA* and *hoxB* overlap, while *hoxB* and *hoxC* are separated by 10 bp. This arrangement predicts the organization of an operon. The spacing between *hoxC* and *hoxJ* is 341 bp, and a possible RpoN-specific promoter sequence with typical −24 and −12 consensus elements (47) lies upstream of the *hoxJ* start codon (Fig. 2); procaryotic −35 and −10 consensus sequences appear to be absent in this region. A similar arrangement of open reading frames (*hoxA*, *B*, *C*, and *J*) has been identified on megaplasmid pHG1 of *A. eutrophus* H16 (16) (accession no. U82564).

The deduced amino acid sequence of the *A. hydrogenophilus*-borne *hoxA* revealed a product of 54.4 kDa which showed the expected similarity to response regulators of bacterial two-component systems (36, 45). A high degree of overall identity was found between the *A. hydrogenophilus* HoxA and the corresponding proteins from *A. eutrophus* (69.3%) (13), *B. japonicum* (47.9%) (48), *R. capsulatus* (42.8%) (37), *Salmonella typhimurium* (37.4%) (9), and *E. coli* (36.2%) (46) (Fig. 3). The products of *hoxB* and *hoxC* show homology to the small hydrogenase subunit VhoG of *Methanosarcina mazei* (10) and the large hydrogenase subunit HupL of an *Anabaena* sp. (7), respectively, and contain sequence elements which are typical for dimeric [NiFe] hydrogenases (Fig. 2) (51). Moreover, HoxB and HoxC are closely related to putative regulatory proteins encoded in the hydrogenase gene complex of *A. eutrophus* (16) (accession no. U82564), *B. japonicum* (4), and *R. capsulatus* (15). The predicted amino acid sequence of the fourth open reading frame, HoxJ, revealed typical signatures of histidine protein kinases which function as sensors in two-component regulatory systems (45). HoxJ appears to be a soluble protein. Its closest relatives (62.1 and 37.5% overall identity, respectively) were found to be HoxJ of *A. eutrophus* (16) (accession no. U82564) and HupT of *R. capsulatus* (15), as shown in Fig. 4.

Properties of a *hoxJ* mutant. The data of the sequence analysis and the transconjugant studies were consistent with the

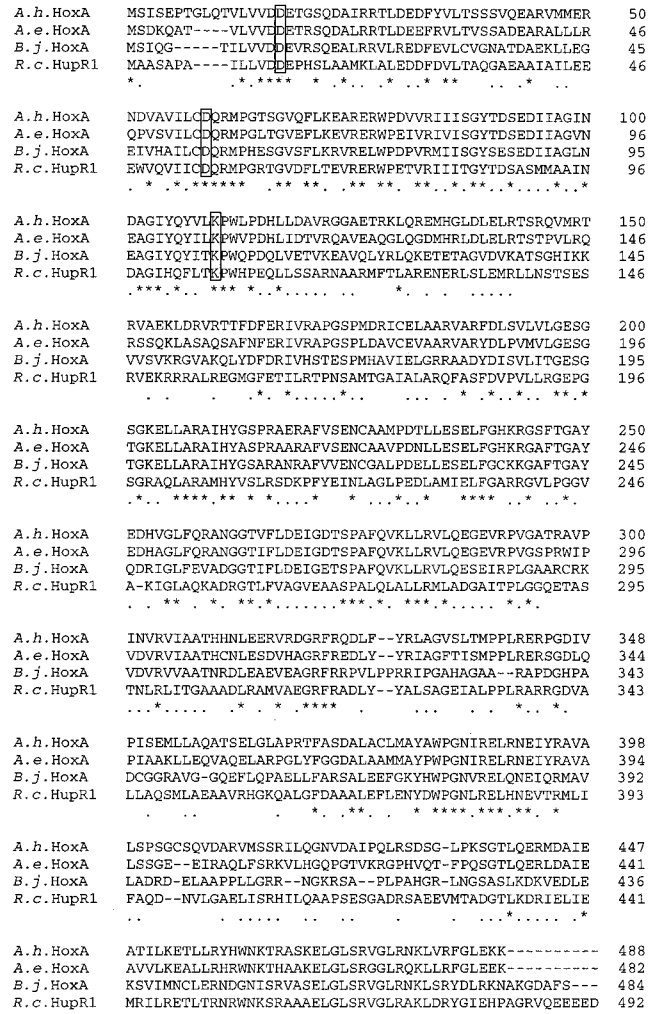


FIG. 3. Amino acid sequence comparison between *A. hydrogenophilus* HoxA and the hydrogenase-specific response regulators of other aerobic hydrogen oxidizers. Identical amino acid residues found in all sequences are marked by asterisks. Dots indicate similar residues. Highly conserved amino acid residues characteristic of response regulator N-terminal phosphorylation domains (36, 45) are boxed. Abbreviations: *A.h.*, *A. hydrogenophilus*; *A.e.*, *A. eutrophus*; *B.j.*, *B. japonicum*; *R.c.*, *R. capsulatus.*

assumption that the release of hydrogenase repression during heterotrophic growth of pGE296-harboring transconjugants may be due to inactivation of the putative sensor kinase, since subcloning of pGE296 had caused truncation of 7 amino acids from the C-terminal end of HoxJ (Fig. 1 and 2). To examine this hypothesis in more detail, the *A. hydrogenophilus*-borne *hoxJ* gene was disrupted in vitro by an in-frame deletion which eliminated 342 of the total 456 amino acid residues. The wild-type copy of *hoxJ* was replaced by the deleted allele via recombination as previously described (29). The mutation was introduced on plasmid pHG21-a in the host *A. eutrophus* HF157 (Table 1). Of the resulting isolates, mutant HF423 was further characterized. Autotrophic growth on H_2 was not affected in the *hoxJ* deletion mutant. However, enzymatic analysis clearly showed that SH and MBH expression differed remarkably from that in the parent strain (Table 3). While HF157 showed hydrogenase repression in the absence of H_2, which is characteristic of *A. hydrogenophilus* M50 (18), mutant HF423 expressed high levels of both hydrogenases on fructose-glycerol. Hydrogenase synthesis of HF423 was not constitutive, since both SH and MBH were almost completely repressed during growth on a rich carbon and energy source like succinate. Thus, the regulatory phenotype of strain HF423 resembled that of *A. eutrophus* H16 (Table 3). Finally, the introduction of *hoxJ* on plasmid pGE303 (Fig. 1A) reassigned the H_2-dependent mode of regulation to strain HF423 (Table 3), which confirms the significance of HoxJ in H_2-mediated control.

The *hoxJ* gene product functions on the transcriptional level. To show that HoxJ affects the formation of SH and MBH on the transcriptional level and to exclude its involvement in the complex maturation of catalytically active hydrogenase synthesis (3, 11), hydrogenase-specific mRNA was identified by dot blot analysis. Total RNA was isolated from cells of the *hoxJ*Δ mutant *A. eutrophus* HF423 grown on fructose-glycerol minimal medium and hybridized to an amplified DNA fragment of the large MBH subunit gene (*hupL*) (53) from *A. hydrogenophilus*. Total DNA of the parent strain *A. eutrophus* HF157 was used as a control. Figure 5A clearly demonstrates that disruption of *hoxJ* led to heterotrophic derepression of *hupL* transcript formation (lane 2), while only traces of hydrogenase-specific mRNA were detected in cells of the parent HF157 (lane 1) and the transconjugant HF423(pGE303) (lane 3). These observations are consistent with the enzymatic SH and MBH activities determined in cells cultivated under the

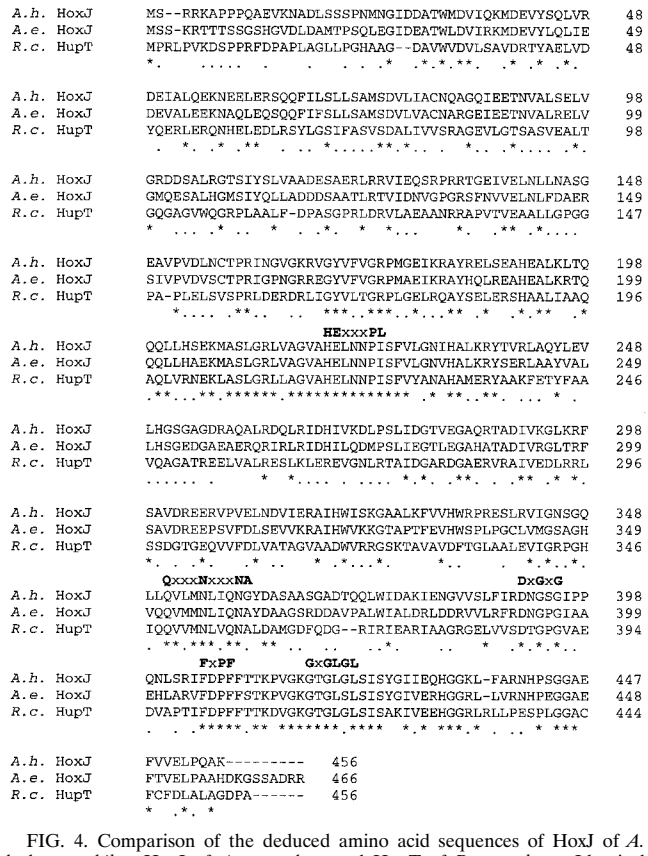


FIG. 4. Comparison of the deduced amino acid sequences of HoxJ of *A. hydrogenophilus*, HoxJ of *A. eutrophus*, and HupT of *R. capsulatus*. Identical residues are marked by asterisks. Dots indicate similar residues. Highly conserved motifs characteristic of histidine protein kinases (36) are emphasized above the sequences. Abbreviations: *A.h.*, *A. hydrogenophilus*; *A.e.*, *A. eutrophus*; *R.c.*, *R. capsulatus.*

TABLE 3. Hydrogenase activities of *A. hydrogenophilus* and *A. eutrophus* strains and mutants

Strain	Relevant characteristic	Hydrogenase activity (U/mg of protein)[a] in:					
		H_2-CO_2-O_2		FGN medium		SN	
		SH	MBH	SH	MBH	SH	MBH
A. eutrophus H16	Wild type (pHG1)	0.51	1.13	3.79	0.68	0.00	0.00
A. hydrogenophilus M50	Wild type (pHG21-a)	0.51	0.80	0.00	0.00	0.00	0.00
A. eutrophus HF157	pHG21-a	0.64	2.39	0.00	0.00	0.00	0.00
A. eutrophus HF423	pHG21-a *hoxJ*Δ	0.62	2.14	5.48	2.08	0.07	0.07
A. eutrophus HF423(pGE303) (*hoxJ*)	pHG21-a *hoxJ*Δ	0.80	2.79	0.17	0.00	ND	ND

[a] Values give the average from at least two independent experiments. FGN medium, heterotrophic growth in mineral medium with fructose-glycerol; SN, heterotrophic growth in minimal medium with succinate; ND, not determined.

same conditions (Table 2). Finally, the specificity of the dot blot analysis is evident from the control illustrated in Fig. 5B.

DISCUSSION

Hydrogenase synthesis in a number of facultatively H_2-oxidizing bacteria, including *A. hydrogenophilus* (18), *Azotobacter vinelandii* (26), *B. japonicum* (23), *Paracoccus denitrificans* (33), and *R. capsulatus* (50), depends on the availability of H_2. The H_2 concentration in the habitat of aerobic hydrogenotrophs is fairly low, since only traces of the anaerobically produced H_2 escape to oxic environments (41). Hence, the development of a regulatory system in which hydrogenase synthesis depends on the availability of consumable carbon and energy sources, as found in *A. eutrophus* (21) and *Acidovorax facilis* (52), may have a selective advantage for those organisms. These bacteria are well prepared to utilize H_2 instantaneously as a supplementary source of energy and reductant under conditions of general nutrient limitation, a situation they likely meet in their natural environments.

To learn more about the molecular signals which drive hydrogenase gene expression in response to either H_2 or the energy status of the cell, we selected two phylogenetically closely related species as representatives of the two divergent regulatory regimens. Synthesis of both hydrogenases of *A. eutrophus* H16 is derepressed coordinately during growth on slow-growth-supporting substrates like glycerol. On preferentially utilized carbon sources such as succinate, synthesis of SH and MBH is almost completely repressed (21). On the other hand, expression of the two hydrogenases in *A. hydrogenophilus* strictly correlates with the presence of external H_2 (18).

In this communication we report on a DNA region of the *A. hydrogenophilus*-borne plasmid pHG21-a, which shows remarkable similarity with respect to the arrangement and number of open reading frames within a regulatory DNA region on plasmid pHG1 of *A. eutrophus* H16 (16) (accession no. U82564). A *hoxA*-like gene was identified in *A. hydrogenophilus* by heterologous complementation of an *A. eutrophus hoxA* deletion mutant. The transconjugants were restored in autotrophic growth on H_2 and contained a normal level of SH activity. The lack of MBH activity may be explained by a low-transcription-stimulating function of the recombinant HoxA at the MBH promoter. Despite this partial deficiency, sequence analysis confirmed the expected overall homology of the *A. hydrogenophilus*-derived *hoxA* gene product and the corresponding regulatory proteins of *A. eutrophus* (13), *B. japonicum* (48), *E. coli* (46), *R. capsulatus* (37), and *S. typhimurium* (9). The typical N-terminal phosphorylation domain of HoxA predicts interaction with a cognate histidine protein kinase (36, 45). Based on previous studies with *A. eutrophus* (29) and *B. japonicum* (48), the product of *hoxX*, a gene located immediately upstream of *hoxA*, was discussed as a possible modulator of HoxA activity. However, it was emphasized that no convincing overall or local homology to histidine protein kinases was found (29). Extended sequence analysis of a region 3′ of *hoxA* revealed three additional open reading frames, *hoxB*, *hoxC*, and *hoxJ*, present in both *A. eutrophus* (16) (accession no. U82564) and *A. hydrogenophilus.*

Unlike HoxX, the *hoxJ* products of *A. hydrogenophilus* and *A. eutrophus* showed almost perfectly the consensus elements of histidine protein kinases. Thus, HoxJ appeared to be an excellent candidate for a sensor protein which might interact with HoxA via phosphorylation-dephosphorylation. Although autophosphorylation of HoxJ needs to be experimentally proven, the results obtained in this study showed for the first time that the introduction of a complete set of *A. hydrogenophilus*-derived *hoxA*, *B*, *C*, and *J* genes caused a severe repression of SH and MBH formation in *A. eutrophus* when H_2 was absent. This epistatic effect was dependent on a functional *hoxJ* product of *A. hydrogenophilus*. HoxJ homologs have so far been reported only for *R. capsulatus* (HupT) (15), *Rhodobacter sphaeroides* (HupT) (22), *E. coli* (HydH) (46), and *S. typhimurium* (HydH) (9). Insertional inactivation of the *hupT* gene in *R. capsulatus* led to overexpression of the structural gene operon, and the authors discussed an involvement of HupT in repression of hydrogenase synthesis (15). The behavior of the merodiploid transconjugants also pointed to a negative effect of the *A. hydrogenophilus*-derived HoxJ on hydrogenase gene

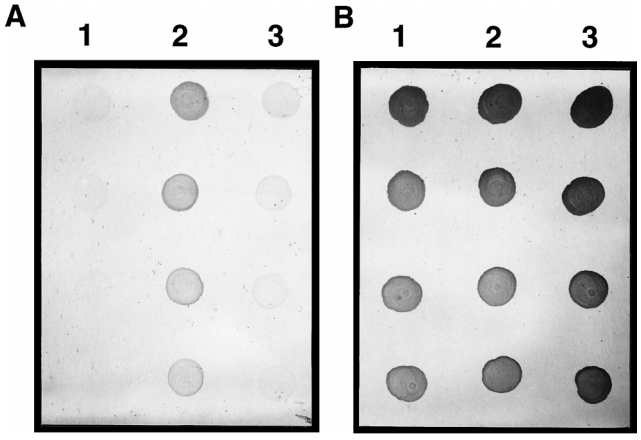


FIG. 5. Regulatory phenotype of *A. hydrogenophilus hoxJ* demonstrated in an RNA dot blot analysis. Total RNA isolated from *A. eutrophus* derivatives grown in FGN medium was hybridized with a *hupL* fragment of *A. hydrogenophilus* (A) and with HF157 total DNA as a control (B). The amount of RNA spotted on the membranes was, from top to bottom, 2, 1, 0.5, and 0.25 μg. Lanes 1, HF157; lanes 2, HF423; lanes 3, HF423(pGE303).

expression in *A. eutrophus*, although the results were difficult to interpret in view of the merodiploid background.

To avoid possible cross talk between the heterologous regulatory components, we investigated the function of HoxJ in a derivative of *A. eutrophus* H16 cured of its indigenous plasmid pHG1 but containing instead the *A. hydrogenophilus*-borne megaplasmid pHG21-a. Inactivation of *hoxJ* by an in-frame deletion released hydrogenase gene repression in the absence of H_2. This result was in agreement with the conclusions drawn from the merodiploid analysis. Moreover, RNA dot blot analysis showed that HoxJ exerts its regulatory function on the transcriptional level. Disruption of *hoxJ* did not generate a constitutive phenotype, since the formation of both hydrogenases was tightly regulated in mutant HF423. This regulation, however, was directed by the availability of the carbon and energy source. Thus, a mutation in *hoxJ* converted the H_2-specific control to a more global, substrate-responding phenotype.

In both regulatory systems HoxA and the RpoN-like sigma factor of RNA polymerase are essential for hydrogenase gene expression (13, 32, 38). This indicates that there may exist a second, HoxJ-independent form of HoxA modulation. In fact, we have preliminary evidence that *A. eutrophus* escaped H_2-dependent control by a mutation in *hoxJ* while HoxA maintains its transcription-stimulating activity (28a).

It is unlikely that the newly discovered *hoxBC* products directly interfere with HoxA. The predicted proteins show typical sequence motifs of dimeric [NiFe] hydrogenases (51). Cysteine residues of the small electron-transferring subunit which are supposed to coordinate Fe-S clusters are well conserved in HoxB. The HoxC polypeptide contains the consensus elements (R-X-C-G-X-C and D-P-C-X-X-C) (Fig. 2) which are considered to be important in the formation of the [NiFe]-active site buried inside the large subunit (51). Nevertheless, two major structural differences are apparent: HoxB is devoid of an N-terminal leader sequence which normally directs translocation of the dimeric hydrogenase, and HoxC is lacking the C-terminal extension which is essential for stable metal center assembly and maturation of [NiFe] hydrogenases (30, 49). Although more experimentation is required to elucidate the function of HoxB/C, it is an attractive hypothesis that these polypeptides act as the primary receptor of H_2 and are instrumental in relaying this environmental stimulus to HoxA via the putative protein kinase HoxJ. HoxB/C homologs have been found in *B. japonicum* (HupU/V) (4) and *R. capsulatus* (HupU) (15). *hupV* was shown to be necessary for transcriptional activation of hydrogenase gene expression in *B. japonicum*, and the authors suggested a sensing function for HoxU/V, although a HoxJ-like protein kinase remains to be reported for this bacterium.

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