Positive Transcriptional Feedback Controls Hydrogenase Expression in *Alcaligenes eutrophus* H16

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The protein HoxA is the central regulator of the *Alcaligenes eutrophus* H16 *hox* regulon, which encodes two hydrogenases, a nickel permease and several accessory proteins required for hydrogenase biosynthesis. Expression of the regulatory gene *hoxA* was analyzed. Screening of an 8-kb region upstream of *hoxA* with a promoter probe vector localized four promoter activities. One of these was found in the region immediately 5′ of *hoxA*; the others were correlated with the nickel metabolism genes *hypA1*, *hypB1*, and *hypX*. All four activities were independent of HoxA and of the minor transcription factor σ^{54}. Translational fusions revealed that *hoxA* is expressed constitutively at low levels. In contrast to these findings, immunoblotting studies revealed a clear fluctuation in the HoxA pool in response to conditions which induce the *hox* regulon. Quantitative transcript assays indicated elevated levels of *hyp* mRNA under hydrogenase-derepressing conditions. Using interposon mutagenesis, we showed that the activity of a remote promoter is required for hydrogenase expression and autotrophic growth. Site-directed mutagenesis revealed that P_{MBH}, which directs transcription of the structural genes of the membrane-bound hydrogenase, contributes to the expression of *hoxA* under hydrogenase-derepressing conditions. Thus, expression of the *hox* regulon is governed by a positive feedback loop mediating amplification of the regulator HoxA. These results imply the existence of an unusually large (ca. 17,000-nucleotide) transcript.

Alcaligenes eutrophus H16 is a facultative lithoautotroph that can utilize hydrogen as its sole source of energy. Two biochemically and physiologically distinct [NiFe] metalloenzymes catalyze the oxidation of H_2: a membrane-bound hydrogenase (MBH), which couples H_2 oxidation to electron transport phosphorylation, and a cytoplasmic hydrogenase (SH), which catalyzes H_2-dependent reduction of NAD^+ (reviewed in references 14 and 15). The hydrogenases supply the organism with energy during lithoautotrophic growth on CO_2. They are also synthesized in the presence of poor organic substrates, permitting the organism to utilize H_2 as a supplemental energy source (16).

Both *A. eutrophus* hydrogenases are nickel metalloenzymes. *A. eutrophus* possesses a high-affinity Ni permease which ensures a supply of Ni for their synthesis (12). Both enzymes undergo a complex maturation process, which converts the inactive precursor forms to catalytically active enzymes. The quintessential steps in the two maturation pathways lead to the assembly of the nickel-containing active sites. These sites contain a special coordination structure containing one nickel atom, one iron atom, and three diatomic ligands. The architecture of this metallocenter appears to be conserved in the [NiFe] hydrogenases (1, 13). The assembly of the hydrogenase [NiFe] metallocenter in *A. eutrophus* and in other bacteria is mediated by a set of specialized proteins encoded by the *hyp* genes. One of the functions of the Hyp proteins is to donate Ni to the hydrogenase apoprotein (7, 8). HypX may be instrumental in inserting the diatomic ligands into the nascent metallocenter (6). The underlying mechanism and the specific contributions of the Hyp proteins are fascinating but so far have

thwarted analysis (28). Metallocenter assembly seems to be intimately connected to C-terminal proteolytic processing of the Ni-containing large subunit of the hydrogenase enzyme. Both the MBH and the SH undergo C-terminal proteolytic processing, and each enzyme has its own specific protease (4, 22, 29, 43).

Detailed studies on the expression of the hydrogenase structural genes have been carried out in our laboratory (39). Both the MBH and SH genes are transcribed from σ^{54}-dependent promoters. Expression of these genes is controlled at the transcriptional level. The central regulatory agent governing the hydrogenase regulon is a transcriptional activator encoded by the gene *hoxA* (10). HoxA triggers the activation of the promoters in response to energy limitation, e.g., during growth on poor organic substrates such as glycerol or during autotrophic growth. The actual physiological cue is unknown. The deduced amino acid sequence of HoxA reveals several features typical of response regulators of the NtrC family. The N-terminal part of the protein is homologous to the receiver domains of the response regulators and has an aspartate residue at the usual position. The mode of action of this protein is, however, unconventional. HoxA mediates activation of the cognate promoters in the absence of a sensory kinase. Furthermore, mutations altering the conserved phosphoryl acceptor residue do not abolish transcriptional activation by HoxA (24). Recently, a hydrogen-sensing system which mediates H_2-dependent control of hydrogenase expression was discovered in strains of *Alcaligenes* (24). Remarkably, this system, consisting of a dimeric H_2 receptor and a histidine protein kinase, is cryptic in the wild-type strain but can be activated by a mutation leading to a single-amino-acid exchange. This mutation seems to occur at a high frequency and may represent a genetic switch allowing the organism to shift between two modes of regulation.

The main goal of the study reported here was to investigate the expression of the hydrogenase regulator, HoxA, itself. We used a promoter probe vector to search for and quantify promoters directing transcription of *hoxA*. Additional information

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on the transcription of the *hoxA* region was obtained from RNase protection experiments. Plasmid-borne translational fusions were used to monitor expression of *hoxA* under different growth conditions and to compare the expression of *hoxA* and other hydrogenase genes. Finally, the cellular levels of regulator were assayed via Western immunoblotting.

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *A. eutrophus* H16 is the wild-type strain harboring the endogenous megaplasmid pHG1. Strains HF09 (37) and HF18 (17) are derivatives of H16. *Escherichia coli* S17-1 (41) served as a donor in conjugative transfers. Strains of *A. eutrophus* were grown in a modified Luria broth containing 0.25% sodium chloride and 0.4% fructose (LBF medium) or in mineral salts medium as described previously (39). Synthetic media for heterotrophic growth contained 0.4% fructose (FN medium), 0.4% succinate (SN medium), or 0.2% fructose and 0.2% glycerol (FGN medium). Lithoautotrophic cultures were grown in mineral salts medium under an atmosphere of hydrogen, carbon dioxide, and oxygen of 8:1:1 (vol/vol/vol). For assays of hydrogenase activity, cells were cultivated in the above media containing 1 μM NiCl$_2$ in place of the standard trace elements mixture SL6. Strains of *E. coli* were grown in LB medium or in M9 medium containing glycerol (30). Solid media contained 1.5% agar. Antibiotics were added where appropriate (for *A. eutrophus*, kanamycin [350 μg/ml] and tetracycline [15 μg/ml]; for *E. coli*, kanamycin [25 μg/ml], tetracycline [15 μg/ml], and ampicillin [50 μg/ml]).

Plasmid and strain construction. For the generation of an interposon mutant, an Ω cassette was excised from plasmid pGMΩ1 (kindly supplied by H. P. Schweitzer, Calgary, Alberta, Canada) by digestion with *Sma*I and inserted into the *Eco*RV site of pCH427. The resulting plasmid (pCH679) was used to transfer the allele *hoxTΩR-B2* into *A. eutrophus* H16 via an allelic exchange procedure (25), yielding strain HF457. A deletion derivative with a lesion in the MBH promoter region was isolated by a similar strategy. Plasmid pCH128 was cut with *Bam*HI and *Bgl*II and religated. A 686-bp *Pst*I-*Xho*I fragment spanning the site of the deletion was transferred to the suicide vector pLO1, resulting in plasmid pCH680. The latter plasmid was used to generate the mutant HF491. Promoter test constructs were obtained by inserting the fragments listed in Table 1 directly into pEDY305. Oligonucleotides BF213 and BF214 were used to amplify a 233-bp segment of the *acoR* promoter region on plasmid pRZ10. The PCR product was cut with *Bam*HI and *Sma*I and inserted into pEDY305. Similarly, amplified segments of the *hypD* and *hypA1* upstream regions were obtained using the primer pairs BF250-BF251 and BF360-BF361, respectively. After cutting with *Nhe*I and *Bss*HIII (*hypD* fragment) and *Bgl*II and *Pvu*II (*hypA1* fragment), the products were likewise inserted into pEDY305.

Conjugative plasmid transfer. Mobilizable plasmids were transferred from *E. coli* S17-1 to *A. eutrophus* by a spot mating technique (41). Donor and recipient strains were grown on LB and LBF media, respectively. Transconjugants were selected on FN plates containing the appropriate antibiotics.

Recombinant DNA techniques. Standard DNA techniques were used in this study (2). Large-scale isolation of plasmid DNA was carried out by the alkaline lysis procedure followed by ethidium bromide-cesium chloride gradient centrifugation. Smaller amounts of pure plasmid DNA were isolated by using Qiagen Tip-20 columns (Qiagen GmbH) according to the manufacturer's instructions. DNA fragments used in plasmid constructions were isolated from agarose gels by using the Qiaex II system (Qiagen GmbH).

Western immunoblot analysis. Strains of *A. eutrophus* were grown under standard conditions as described above. Mid-log-phase cells were harvested, resuspended in 50 mM potassium phosphate buffer (pH 7.0), and homogenized by three passages through a French pressure cell. Soluble and membrane fractions were separated by spinning for 30 min at 140,000 $\times$ *g*; 20-μg samples were separated by polyacrylamide gel electrophoresis (PAGE) through sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. BenchMark Prestained Protein Ladder (Life Technologies Inc.) was used as a molecular mass standard. Following SDS-PAGE, proteins were transferred to Protran BA85 membranes (Schleicher & Schuell) (45). Blots were treated with rabbit polyclonal antisera (diluted 1:1,000) raised against HypD, HypX, or HoxA and developed with alkaline phosphatase conjugate (Dianova).

RNase protection assays. Riboprobes were synthesized by using a MAXIscript kit (Ambion, Inc.) and ^{32}P-labeled UTP (800 Ci/mmol; Dupont NEN). *Xmn*I-linearized plasmid pCH300 and *Nar*I-linearized plasmid pCH304 served as templates for the generation of the riboprobes E and F1C, respectively. The riboprobes were 354 and 91 nucleotides (nt) long, respectively. In vitro transcripts were purified by two rounds of ethanol precipitation. Total RNA was prepared by a hot-phenol procedure (19). Total RNA (5 to 20 μg) was added to 30 μl of hybridization buffer (40 mM PIPES [pH 6.4], 0.4 M NaCl, and 1 mM EDTA in a 1:4 (vol/vol) mixture of water-deionized formamide) containing 10^5 to 10^6 cpm of the appropriate riboprobe. After an initial denaturation step (5 min at 85°C), hybridization proceeded for at least 8 h at 45°C. Then 350 μl of RNase digestion cocktail (10 mM Tris-HCl, [pH 7.5], 300 mM NaCl, 5 mM EDTA, 40 μg of RNase A per ml, 2 μg of RNase T$_1$ per ml) was added, and the mixture was incubated for 30 min at 30°C. Treatment with proteinase K (10 μl of 20% [wt/vol]

SDS, 2.5 μl of proteinase K [20 mg/ml]; 37°C for 15 min) was followed by phenol extraction and precipitation in the presence of 10 μg of yeast tRNA. The pellet was dissolved in 3 to 5 μl of formamide loading buffer and applied to a 6% sequencing gel. In vitro transcripts of known length served as size standards. Quantitation of the protected fragments was done by analysis of scanned images obtained in a Molecular Dynamics PhosphorImager 445 SI, using IP-Labgel software (Scanalytics, Inc.).

Enzyme assays. Independent single colonies of the strains to be tested were picked from plates and inoculated in liquid media. Precultures were incubated for 15 to 20 h at 35°C. Since the hydrogenase system is repressed at temperatures above 33°C, this step ensures that the cells are uniformly devoid of hydrogenase at the beginning of an experiment. SH (hydrogen:NAD$^+$ oxidoreductase; EC 1.12.1.2) activity was assayed by spectrophotometric determination of H$_2$-dependent NAD reduction in detergent-treated cells. MBH (ferredoxin:H$^+$ oxidoreductase; EC 1.18.99.1) activity was determined by measuring H$_2$-dependent methylene blue reduction in isolated membranes. One unit of hydrogenase activity is the amount of enzyme which catalyzes the formation of 1 μmol of product per min. β-Galactosidase was assayed by the standard method (30), and the activity (in units) was calculated according to Miller except that cell density was measured at 436 nm. Unless otherwise indicated, enzyme activities were assayed in mid-log-phase cells, i.e., cells grown to optical densities of 3 in SN medium, 5 in FN medium, 8 in FGN medium, and 4 under lithoautotrophic conditions. Protein determinations were done according to the method of Lowry et al. (26).

RESULTS

Mapping promoters upstream of *hoxA*. As a first step in the analysis of the expression of the hydrogenase regulator HoxA, we screened the region upstream of *hoxA* (Fig. 1) by using a promoter assay vector. Plasmid pEDY305, a broad-host-range vector designed in our laboratory for promoter assays in gram-negative bacteria (39), was chosen for this study. DNA fragments from the 8-kb region containing genes *hypA1* through *hoxA* were inserted into the multiple cloning site of pEDY305. The resulting recombinant plasmids were introduced into *A. eutrophus* H16 via conjugal transfer from the *E. coli* donor strain S17-1. Transconjugants were streaked onto FN plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and scored for β-galactosidase activity. Four of the transconjugants tested gave a positive reaction in the plate test. These strains contained the plasmids pGE420, pGE322, pGE324, and pGE325, harboring DNA segments upstream of the genes *hypA1*, *hypB1*, *hypX*, and *hoxA*, respectively (Fig. 1). This indicated that the inserted DNA contained functional promoters which directed transcription of the vector-borne reporter gene. The four plasmid-harboring strains were cultivated in liquid media for quantitative β-galactosidase assays. Cultures were grown under hydrogenase-derepressing conditions (FGN medium). All four strains produced low but significant levels of β-galactosidase activity indicating moderate promoter activities (Table 2). The strongest activity (829 U for pGE420) resided in the *hypA1* upstream region; the weakest (173 U for pGE324) was in the *hypX* upstream region. For comparison, plasmids pGE319 and pGE320, containing the structural gene promoters P$_{MBH}$ and P$_{SH}$, respectively, were included in the experiment. These constructs produced 14,445 and 12,961 U, respectively (Table 2). We also measured the β-galactosidase activity in liquid cultures of selected transconjugants testing negative in the plate test. The activity of these strains was not more than double the background activity, i.e., the level of activity in the wild-type strain harboring vector plasmid pEDY305.

Cultures of the four transconjugants grown under hydrogenase-repressing conditions contained similar levels of β-galactosidase activity as under derepressing conditions (data not shown). In contrast, the activity of the structural gene promoters is negligible in repressed cells (39). This suggested that the promoters present on the four test plasmids are not subject to the same regulation as P$_{MBH}$ and P$_{SH}$. The latter promoters are controlled by the transcriptional activator HoxA (39). To

TABLE 1. Strains, plasmids, and synthetic oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics[a] or sequence	Source or reference
Strains		
A. eutrophus		
H16	MBH$^+$ SH$^+$	DSM428, ATCC 17699
HF18	H16 *hoxA18*, MBH$^-$ SH$^-$	17
HF09	H16 *rpoN09*, MBH$^-$ SH$^-$	37
HF457	H16 *hoxT*Ω*R-B2*	This study
HF491	H16 *hoxK*Δ*171-R4*	This study
E. coli		
S17-1	*recA pro thi hsdR* chr::RP4-2 Tra$^+$	41
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) e14$^-$ (F′ *lacI*^{q} *lacZ*Δ*M15 proAB traD36*)	48
Plasmids:		
pEDY305	Tc^r RK2 *ori* Mob$^+$, promoterless *lacZ* gene	39
pPHU234	Tc^r RK2 *ori* Mob$^+$, ′*lacZ* translational fusion vector	20
pPHU278	Φ(*aph-lacZ*), P$_{aph}$ deleted	20
pGMΩ1	Derivative of pUC18 carrying a polar Ω cassette	40
pLO2	*sacB oriT*$_{RP4}$ ColE1 *ori*, Km^r Mob$^+$, counterselectable suicide vector	25
pRZ10	Φ(*acoR-lacZ*)	23
pCH103	Derivative of pSUP202 carrying the *hyp* locus	11
pCH104	Derivative of pSUP202 carrying the *hyp* locus	11
pCH300	2.2-kb *Sal*I-*Bam*HI fragment of pGE43 in pBluescript KS+	This study
pCH301	2.2-kb *Pst*I-*Kpn*I fragment of pGE43 in pBluescript KS+	This study
pCH303	2.6-kb *Xho*I-*Sal*I fragment (Klenow enzyme treated) of pGE43 in pBluescript SK+	This study
pCH304	2.7-kb *Sal*I-*Xho*I fragment of pGE43 in pBluescript KS+	This study
pCH427	*hoxT* subcloned in pLO1, Km^r *sacB oriT*$_{RP4}$ ColE1 *ori*	4
pCH679	Ω cassette of pGMΩ1 inserted as a *Sma*I fragment into the *Eco*RV site of pCH427	This study
pCH680	Deletion derivative lacking 171 bp of the *hoxK* upstream region introduced into pLO2 as 686-bp *Pst*I-*Xho*I fragment	This study
pGE43	Derivative of pVK101 carrying the *hyp* region	10
pGE281	8.5-kb *Eco*RI (Klenow enzyme-treated)-*Hpa*I fragment of pCH104 in pPHU234, Φ(*hoxA-lacZ*)	This study
pGE282	6.1-kb *Bst*EII (Klenow enzyme-treated)-*Hpa*I fragment of pCH104 in pPHU234, Φ(*hoxA-lacZ*)	This study
pGE283	1.9-kb *Bsp*EI (Klenow enzyme-treated)-*Hpa*I fragment of pCH104 in pPHU234, Φ(*hoxA-lacZ*)	This study
pGE319	Derivative of pEDY305 with P$_{MBH}$ upstream of *lacZ*	39
pGE320	Derivative of pEDY305 with P$_{SH}$ upstream of *lacZ*	39
pGE322	667-bp *Xho*I-*Pvu*II fragment of pCH104 in pEDY305	O. Lenz and B. Friedrich
pGE323	516-bp *Pvu*II fragment of pCH303 in pEDY305	O. Lenz and B. Friedrich
pGE324	711-bp *Bam*HI-*Nhe*I fragment pCH104 in pEDY305	This study
pGE325	1739-bp *Nhe*I-*Hpa*I fragment of pCH104 in pEDY305	O. Lenz and B. Friedrich
pGE326	463-bp *Sna*BI fragment of pCH301 in pEDY305	This study
pGE327	721-bp *Fsp*I-*Eco*47III fragment of pCH303 in pEDY305	This study
pGE328	233-bp segment of pRZ10 subcloned as a PCR product in pEDY305	This study
pGE413	2.1-kb *Eco*RI-*Pvu*II fragment of pCH103 in pPHU234, Φ(*hypB1-lacZ*)	This study
pGE417	1-kb *Asc*I-*Nhe*I fragment of pCH301 in pEDY305	This study
pGE418	350-bp segment of pCH301 subcloned as PCR product in pEDY305	This study
pGE420	393-bp segment of pCH103 subcloned as a PCR product in pEDY305	This study
Oligonucleotides		
BF213	5′-gcgGATCCATGACCACCAGG-3′ (M90471, 150–166)	
BF214	5′-tggccCGGGCGCGTGCTGCGG-3′ (M90471, 383–368)	
BF250	5′-ccagCTAGCGATTCCCGCACG-3′ (X70183, 2466–2482)	
BF251	5′-ccgCGCGCCTCAGCGCGAACG-3′ (X70183, 2802–2785)	
BF360	5′-acagagatcTCGCGGAGTTGGTGTTGC-3′ (M96433, 8579–8596)	
BF361	5′-gcaacaGCTGAGCTCATGCATGGACAG-3′ (M96433, 8971–8951)	

[a] MBH, membrane-bound hydrogenase activity; SH, NAD-reducing hydrogenase activity; Tra, transfer of mobilizable plasmids; *ori*, origin of replication; chr::RP4-2, chromosomally integrated copy of RP4-2; Mob, mobilizability. For oligonucleotides, foreign or altered nucleotides are shown in lowercase, and accession numbers of DDBJ/EMBL/GenBank entries and coordinates are given in parentheses.

test the dependence of the promoters contained in pGE420, pGE322, pGE324, and pGE325 on HoxA, we introduced these plasmids into the *hoxA* mutant HF18 and assayed the β-galactosidase activity in cultures grown in FGN medium (Table 2).

All four plasmids produced significant levels of β-galactosidase in the HoxA$^-$ background. In the case of pGE325, the activity was even higher in the mutant than in the wild type. In contrast, the activity of the structural gene promoters was dramat-
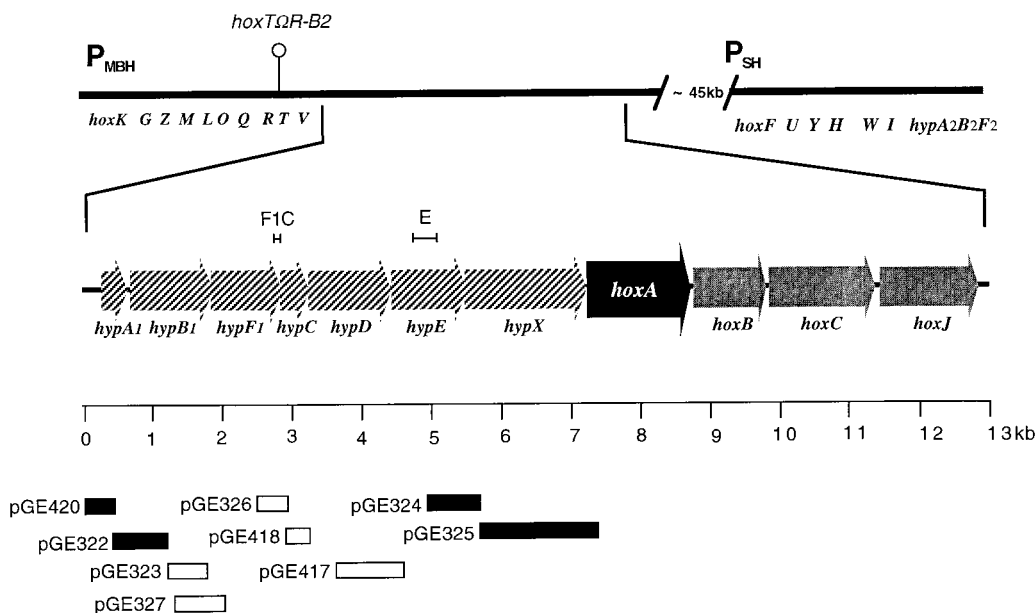
FIG. 1. The *A. eutrophus* hydrogenase genes. The upper part of the diagram represents the two hydrogenase gene clusters on the *A. eutrophus* megaplasmid pHG1. The genes of the MBH region (*hoxKGZMLOQRTV*) and SH region (*hoxFUYHWIhypA2B2F2*) are labeled below the bar. The promoters P_{MBH} and P_{SH} are indicated. The site of an interposon in the polar mutation in strain H16 *hoxTΩR-B2* is marked by a hairpin. The segment containing the regulatory genes *hoxA*, *hoxB*, *hoxC*, and *hoxJ* and the upstream *hyp* genes (hatched arrows) is enlarged. The scale bar pertains to the enlarged part of the drawing. The inserts of the various promoter test constructs are represented by bars. Open bars represent plasmids testing negative on X-Gal indicator plates; solid bars denote positive constructs. The brackets above the genetic map indicate the sequences used to generate the riboprobes F1C and E.

TABLE 2. Activities of promoter-test plasmids in wild-type and mutant strains of *A. eutrophus*

Strain	β-Galactosidase activity (U)[a]							
	pGE420	pGE322	pGE324	pGE325	pGE328 (P_{acoR})	pGE320 (P_{SH})	pGE319 (P_{MBH})	pEDY305
H16	829 ± 13	219 ± 3	173 ± 4	319 ± 13	450 ± 20	12,961 ± 960	14,445 ± 773	23 ± 1
HF09 (H16 *rpoN09*)	499 ± 54	227 ± 5	194 ± 3	1,052 ± 22	184 ± 22	146 ± 3	89 ± 5	18 ± 1
HF18 (H16 *hoxA18*)	579 ± 66	232 ± 7	233 ± 10	1,711 ± 56	346 ± 64	78 ± 2	62 ± 1	22 ± 0

[a] FGN cultures; mean values ± standard errors; $n = 5$.

ically higher in the HoxA[+] background. We also tested the four new constructs in a σ⁵⁴-deficient strain (HF09). Again the activities produced by the four plasmids were comparable to or higher than the wild-type background (Table 2). The σ⁵⁴-dependent promoters P_{MBH} and P_{SH} gave only basal levels of β-galactosidase in the mutant. Thus, the promoters cloned in the four test plasmids are dependent neither on HoxA nor on the alternative transcription factor σ⁵⁴. For comparison, we included a control plasmid with a constitutive promoter in the experiment. We chose the promoter of the *A. eutrophus acoR* gene, which controls the acetoin catabolism operon, since expression of this gene has been shown to be constant under different growth conditions (23). The test plasmid containing the *acoR* promoter (pGE328) showed significant promoter activity in both the RpoN⁻ and HoxA⁻ backgrounds, albeit at somewhat lower levels.

Expression of *hoxA* is independent of hydrogenase expression. One or more of the promoters identified in our screening could contribute to the expression of *hoxA*. To monitor the expression of this regulatory gene under different physiological conditions, we constructed in-frame fusions to the *lacZ* gene in a mobilizable, broad-host-range vector. Three Φ(*hoxA-lacZ*) fusions with identical fusion joints (codon 95 of *hoxA*) but different amounts of upstream sequence were assembled. A derivative of the same vector containing a promoterless kana-

mycin resistance gene fused in frame to *lacZ* (20) served as a control. An *A. eutrophus* transconjugant harboring the smallest fusion plasmid (pGE283) produced low levels of β-galactosidase activity (Fig. 2). The activities were, nevertheless, significantly higher than the background level assayed in the negative control, indicating that *hoxA* is in fact expressed under the control of a proximal promoter. Similar amounts of activity were assayed in both repressed (SN medium) and derepressed cells (FGN medium and lithoautotrophic cultures). Thus, HoxA is expressed constitutively at a low level. The second construct (pGE282), which carried additional upstream sequences and the promoter activity associated with the *hypX* upstream region, gave similar β-galactosidase values. Apparently the distal promoter does not contribute significantly to the expression of *hoxA*. The third fusion plasmid (pGE413) carried 8.5 kb of the region upstream of *hoxA* and, thus, a contiguous sequence spanning the segments contained in the promoter test plasmids pGE420, pGE322, pGE324, and pGE325. The β-galactosidase activities associated with this plasmid were higher than those determined for the smaller Φ(*hoxA-lacZ*) fusions. This indicates that distal promoters contribute to the transcription of *hoxA*, but the effect is not simply additive.

To compare the expression of *hoxA* and the upstream *hyp* genes, we constructed a comparable plasmid-borne fusion for
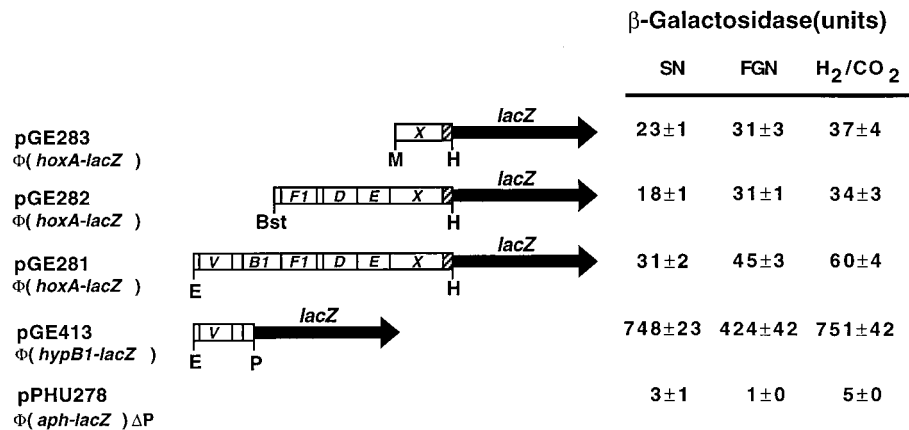
FIG. 2. Expression of *hoxA* and *hyp* genes. Translational fusions for *hoxA* and *hypB1* were generated by inserting restriction fragments into vector plasmid pPHU234. The resulting recombinants were mobilized into *A. eutrophus* H16, and reporter gene activity was monitored under hydrogenase-repressing (SN medium) and -derepressing (FGN medium and lithoautotrophic cultures) conditions. The relevant segment of each plasmid is shown schematically at the left; the corresponding β-galactosidase activities are shown at the right. The solid arrows denote the *lacZ* gene of the vector (not to scale). Bars represent the inserted *A. eutrophus* sequences. The shaded segment indicates the 5′ part of *hoxA*. Some of the genes are labeled for clarity (see Fig. 1). The restriction enzymes used to generate the insert are given below the bar. Bst, *Bst*EII; E, *Eco*RI; H, *Hpa*I; M, *Mro*I; P, *Pvu*II. pPHU278 is a control plasmid containing a Φ(*aph-lacZ*) fusion deleted for the *aph* promoter (20). Values are means of five independent measurements ± standard errors.

the *hypB1* gene. The Φ(*hypB1-lacZ*) also expressed β-galactosidase independent of the growth regimen (Fig. 2). The activities assayed were 10- to 20-fold higher than those produced by the Φ(*hoxA-lacZ*) fusions.

Immunoreactive HoxA is present only in derepressed cells. The results described above show that expression of the *hoxA* gene is constitutive within the scope of the experimental conditions tested. This finding agrees with the promoter activity data, which indicate that transcription from the promoters in the *hyp* region is not significantly modulated under the relevant growth conditions. Together the two sets of data suggest that the concentration of regulator in the cell is more or less constant. To assay the cellular levels of HoxA directly, we carried out immunoblotting experiments using anti-HoxA serum. Soluble extracts from cells of *A. eutrophus* grown under hydrogenase-repressing and -derepressing conditions were separated by SDS-PAGE and immobilized on nitrocellulose membranes. Surprisingly, significant amounts of immunoreactive HoxA were present in cells grown in FGN medium but not in succinate-grown cells (Fig. 3). Cells grown on other carbon sources known to mediate repression of the hydrogenase system were likewise devoid of immunologically detectable HoxA (data not shown). In some cases, an additional band of cross-reacting material was present in the immunoblots. However, control experiments with *hoxA* mutants and overproducing strains left no doubt that the 57-kDa species was HoxA (data not shown).

The paradoxical finding described above might be due to differential stability of protein HoxA. However, assays of the same extracts using anti-HypD and anti-HypX sera revealed a similar pattern for the two Hyp proteins: antigenic material was detectable in the glycerol-grown cells but not in the succinate-grown cells (Fig. 3). The appearance of immunologically detectable amounts of the three unrelated proteins following derepression on glycerol argues against differential stability as the basis of the variations in cellular HoxA concentration. Western analysis of soluble proteins from two mutant strains provided additional clues on the fluctuations of the cellular HoxA pool. *A. eutrophus* HF09 (RpoN⁻) and HF18 (HoxA⁻) were cultivated in FGN medium, and soluble extracts were tested along with extracts from the wild-type cells. Mutant and

wild-type extracts were screened on the same membrane to permit direct comparison (Fig. 3). Staining with anti-HoxA gave a scarcely visible band for the RpoN⁻ extract. Thus, the amount of HoxA in the RpoN⁻ background was significantly reduced. Staining with anti-HypD or anti-HypX gave scarcely visible bands, indicating radically lower levels of antigenic material in both cases (Fig. 3). Comparable findings were obtained for the HoxA⁻ extracts. Only traces of HypD and HypX
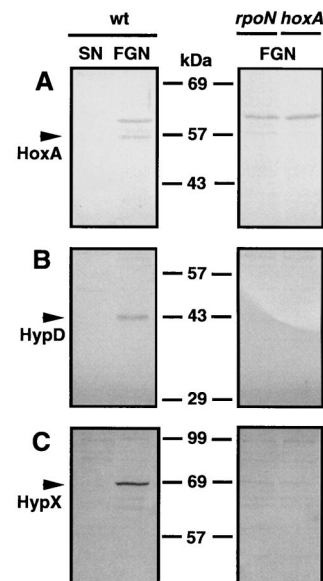

FIG. 3. Cellular levels of HoxA, HypD, and HypX in strains of *A. eutrophus* under different growth conditions. Cultures of *A. eutrophus* H16 (wild type [wt]) and of the mutant strains HF09 (*rpoN*) and HF18 (*hoxA*) were grown to mid-log phase under hydrogenase-repressing (SN) and -derepressing (FGN) conditions. Cells were homogenized, and 20-μg samples of the soluble proteins were separated by SDS-PAGE on 10% gels. Proteins were subsequently transferred to nitrocellulose membranes and stained with antisera directed against HoxA (A), HypD (B), and HypX (C). Arrows indicate the immunoreactive species. Positions of the molecular mass standards are given between the panels.
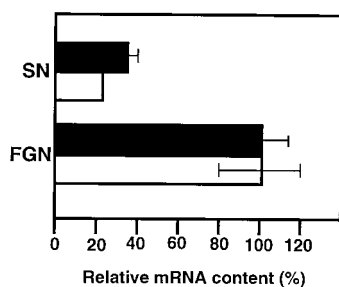
FIG. 4. Relative abundance of *A. eutrophus* *hyp* mRNA in repressed and derepressed cells. Total RNA was isolated from *A. eutrophus* cells grown to mid-log phase on SN or FGN medium; 5 to 20 μg of each sample was hybridized to ^{32}P-labeled riboprobe (10^5 to 10^6 cpm) for 8 h at 45°C. Following RNase treatment, the protected hybrids were separated in 6% sequencing gels. Labeled species were quantitated by densitometric analysis of scanned images. The bars (solid, probe E; open, probe F1C) represent radioactivities of protected fragments, taking the value for the FGN culture as 100% in each case. Each bar represents the average of determinations done on three separate cultures. Standard errors are indicated by brackets (in one case the standard error was too small for graphic representation).

were visible. As expected, a band with an apparent molecular mass of 57 kDa corresponding to HoxA was absent. These data show that the presence of immunologically detectable quantities of HoxA, HypD, and HypX in glycerol-grown cells requires σ^{54}-dependent transcription. Furthermore, the elevated levels of HypD and HypX require the hydrogenase regulator HoxA.

***hyp* mRNA is more abundant in derepressed cells.** The data described above led us to conclude that the fluctuation in the HoxA pool is not a protein-stability-related phenomenon. We hypothesized that an additional promoter or promoters direct transcription of the *hoxA* gene under hydrogenase-derepressing conditions. This promoter might have escaped detection in our screening procedure, or it might be located outside of the region screened. To investigate this, we used an RNase protection assay to quantitate transcripts from the *hyp* region in cells growing under repressing (SN medium) and derepressing (FGN medium) conditions. Since the immunological data showed that the expression of the genes *hypD*, *hypX*, and *hoxA* was affected in a similar fashion, we chose two different segments within this stretch of DNA to use as templates for generating riboprobes. The riboprobes were complementary to the *hypF1*-*hypC* border (probe F1C) and to an internal segment of *hypE* (probe E) (Fig. 1). The two riboprobes consistently yielded similar results: approximately three- to fourfold more *hyp* mRNA was present in derepressed cells (Fig. 4). This increase in transcript concentration, although not dramatic, could account for the observed fluctuation in the cellular levels of the three gene products.

The MBH promoter mediates high-level expression of *hoxA*. The increase in *hyp* mRNA levels in derepressed cells prompted us to search for an additional promoter. The data obtained with the translational fusion constructs suggested that the hypothetical promoter was located upstream of the *hyp* region. The region adjacent to *hypA1* contains the genes for the MBH enzyme and its auxiliary functions. The entire 9-kb segment has been sequenced, and the genetic determinants have been mapped to the nucleotide sequence (4, 21). We inserted the Ω cassette from plasmid pGMΩ1, which carries a strong transcriptional terminator, into a plasmid-borne copy of the MBH accessory gene *hoxT*. The resulting mutation was substituted for the wild-type gene via an allelic exchange procedure. The site of the insertion in the resulting homogenote is located more than 8.5 kb upstream of the initiator codon of *hoxA*. In-frame deletions in *hoxT* cause only a slight reduction in MBH activity (4). The biological function of the gene product is not known. The downstream accessory gene *hoxV* plays a more important role in MBH biosynthesis, and polarity on *hoxV* should reduce MBH activity to low levels. Nonpolar *hoxV* mutants and even MBH null mutants are viable, and lithoautotrophic growth is only slightly slower than growth of wild-type strains, since the synthesis of the SH enzyme is not affected (4). The newly isolated *hoxTΩR-B2* mutant (strain HF457) was first tested for lithoautotrophic growth on H$_2$. After 3 days of incubation under lithoautotrophic conditions, the control strains formed zones of confluent growth, whereas no growth was apparent in the zone where the mutant had been streaked. Assays of hydrogenase activity revealed that the cells were devoid of MBH activity (Table 3). This was not unexpected, assuming a polar effect of the interposon on *hoxV*. Remarkably, however, SH activity was significantly lower in the mutant strain. The low levels of SH activity represented a 10-fold decrease compared to the wild type. This drastic effect on SH activity cannot be attributed to the defect in *hoxT* or to a polarity on *hoxV*, since the synthesis of SH is not dependent on MBH-related functions, and must therefore be due to polarity on one or more genes further downstream. Polarity on *hypA1*, *hypB1*, or *hypF1* should have no effect on SH synthesis, since two copies of these genes are present and the second copy fully complements a knockout mutation in each case (7). A polar effect on either *hypC*, *hypD*, *hypE*, *hypX*, or *hoxA* should, however, have a marked effect on SH activity, since lesions in these genes either abolish or reduce both hydrogenase activities. Disrupting expression of these genes would, however, affect SH synthesis at different levels. Defects in the gene for the transcriptional activator HoxA block transcription of the SH operon, whereas lesions in any of the four *hyp* genes curtail maturation of the SH enzyme. To test for an effect of the polar insertion in HF457 on transcription of *hoxA*, which would in turn perturb transcription of the SH operon, we

TABLE 3. Lithoautotrophic growth, hydrogenase activities, and activities of the SH and MBH promoters in wild-type and mutant strains of *A. eutrophus*

Strain	Growth on H$_2$[a]	Activity (U)[b]			
		Hydrogenase		β-Galactosidase	
		SH	MBH	P$_{SH}$ (pGE320)	P$_{MBH}$ (pGE319)
H16	+	2.1 ± 0.1	0.6 ± 0.1	12,961 ± 960	14,445 ± 773
HF457 (H16 *hoxTΩR-B2*)	−	0.3 ± 0	0.0	1,115 ± 32	942 ± 32
HF491 (H16 *hoxKΔ171-R4*)	−	ND	ND	4,039 ± 48	3,033 ± 158

[a] Single colonies were picked and suspended in phosphate buffer. The optical density of the suspension was normalized, and the same volume of each was streaked on minimal salts plates. The plates were incubated for 3 days under a standard gas mixture consisting of 80% H$_2$, 10% CO$_2$, and 10% O$_2$.
[b] FGN cultures; mean values ± standard errors; $n = 5$. ND, not determined.
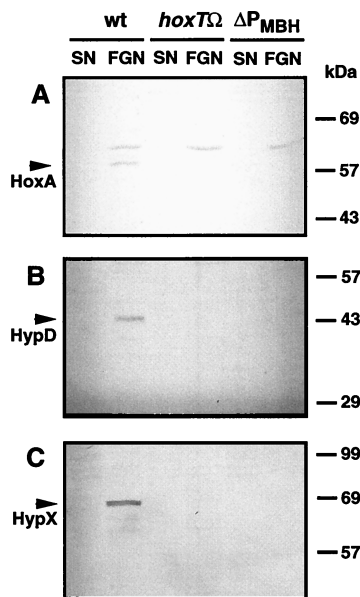
FIG. 5. Cellular levels of HoxA, HypD, and HypX in strains of *A. eutrophus* under different growth conditions. Cultures of the parental strain *A. eutrophus* H16 (wild type [wt]) and of the isogenic derivatives HF457 (*hoxTΩ*) and HF491 (ΔP_MBH) were grown under hydrogenase-repressing (SN) and hydrogenase-derepressing (FGN) conditions. Western immunoblots were stained with antisera directed against HoxA (A), HypD (B), and HypX (C). Arrows indicate immunoreactive species. Positions of molecular mass standards are given at the right.

introduced the promoter assay plasmids pGE319 and pGE320 into the mutant and wild-type strains and monitored the β-galactosidase activity in the resulting transconjugants under hydrogenase-derepressing conditions (Table 3). The indicator plasmids produced 10-fold less β-galactosidase in the *hoxTΩR-B2* background, indicating a drastic reduction in the activity of both hydrogenase promoters. The reduction in promoter activity can account for the reduction in SH activity and can in turn be explained by a curtailment of HoxA expression. The above data provide key evidence that the interposon in HF457 curtails transcription of the gene *hoxA*, which originates from a remote promoter. An important prediction follows from this conjecture: if transcription of *hoxA* in HF457 is reduced enough to cause a drastic decrease in hydrogenase activity, then the cellular levels of HoxA must be measurably lower in this strain. A Western analysis of the wild-type and mutant strains showed that this is indeed the case (Fig. 5). Not only was the HoxA content of the HF457 cells below the detection threshold of our system, but the proteins HypD and HypX were likewise undetectable. The lower levels of the Hyp proteins support our hypothesis, since a polar mutation in *hoxT* which exerts an effect on *hoxA* must necessarily also influence the expression of the genes located in between.

The results described so far indicate that a remote promoter is responsible for elevated expression of *hoxA* under derepressing conditions. This promoter must be σ[54] dependent and controlled by HoxA. P_MBH, the promoter which directs transcription of the MBH structural genes, was an obvious candidate. However, a transcript originating at P_MBH and encompassing the coding sequence of *hoxA* would be unusually large (16,903 nt). To resolve this question, we generated a mutant with a 171-bp deletion in the *hoxK* upstream region (*hoxKΔ171-R4*). This deletion encompasses the invariant dinucleotide at position −24 of the σ[54]-dependent promoter (44).

The resulting mutant, designated HF491, was strongly retarded in lithoautotrophic growth. We introduced the indicator plasmids pGE319 and pGE320 into HF491 and monitored the activity of P_SH and P_MBH under hydrogenase-derepressing conditions. The test system showed significantly reduced activities of the two promoters (4,039 and 3,033 U versus 12,961 and 14,445 U, respectively, for the wild-type background). This shows unequivocally that P_MBH contributes to the transcription of *hoxA*. Interestingly, activities of the two promoters were higher in HF491 than in the polar mutant HF457. This may be due to read-through transcription from upstream promoters or to the activity of a yet unidentified promoter in the MBH region. Despite the significant transcriptional activity, HF491 showed no autotrophic growth after 3 days of incubation and only slight growth after prolonged incubation. This suggests that the combined effects of lowered expression of the *hyp* genes, which may limit the capacity of the maturation machinery, and lowered expression of the regulator are responsible for the curtailment of autotrophic growth.

DISCUSSION

The data presented above fit into a coherent model describing expression of the regulator gene *hoxA* and the hydrogenase structural genes controlled by the promoters P_SH and P_MBH (Fig. 6). In the repressed state, low-level expression of HoxA is mediated by both a proximal (P_A) and distal promoters in the *hyp* region. This weak expression is below the detection threshold of the immunological assay but can be detected in vivo using the plasmid-borne translational fusions. This basal expression ensures that some regulator is present under all conditions. Thus, the system is poised to respond to the relevant
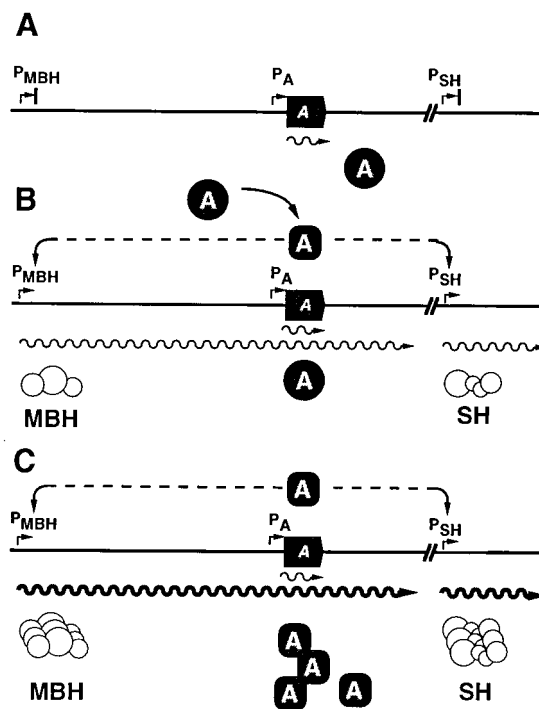

FIG. 6. Molecular model for the derepression of the *hox* regulon. The schematic shows a simplified map of the *hox* gene clusters, with the *hoxA* gene represented by a solid arrow. Gene products are represented as circles; transcripts are represented as wavy lines. The activated form of HoxA is symbolized as an oval. See text for details.

physiological signal. In the absence of this cue, P_{SH} and P_{MBH} are inactive (Fig. 6A). Under derepressing conditions regulator molecules become competent to activate transcription at P_{SH} and P_{MBH} and synthesis of the hydrogenases commences (Fig. 6B). Apparently the amount of regulator present at the outset of induction is limiting and does not support levels of hydrogenase synthesis required for normal lithoautotrophic growth rates. Transcription from P_{MBH}, however, augments expression of *hoxA*, leading to an increase in the pool of regulator, which in turn raises the rate of transcription from the cognate promoters (Fig. 6C). The result is a gradual amplification of the expression of both regulator and hydrogenase enzymes. This amplification is curtailed if *hoxA* is transcriptionally isolated from P_{MBH} (as it is in the interposon mutant HF457) or if P_{MBH} is eliminated altogether (as in mutant HF491).

Transcriptional feedback mechanisms of this type are not uncommon in two-component regulatory systems, the classical example being the first system of this type to be described: the *glnALG* (*ntrABC*) operon of *E. coli*. The response regulator GlnG (NtrC; NRI) is phosphorylated in response to nitrogen limitation and activates transcription from *glnAp2*. This results in a 10-fold increase in the levels of GlnG over the basal level produced by transcription from *glnLp* (32). A similar situation is found in the *bvg* virulence control system of *Bordetella pertussis*. A weak constitutive promoter P_2 provides a small cellular pool of activator protein BvgA. Phosphorylation of BvgA activates the stronger promoter P_1, increasing the transcription of the *bvgA* gene (36). Positive autoregulation is by no means limited to two-component systems. An example from another class of transcriptional activator is the *Pseudomonas aeruginosa regAB* operon (42). The expression of some σ factors is controlled by a similar mechanism (12a). Positive feedback control is also found in regulatory systems which are not dependent on transcriptional activation, such as the operons encoding phosphotransferase system transporters (3, 38).

The *A. eutrophus hox* system differs from the examples of positive transcriptional feedback named above in an important respect: transcription of the gene *hoxA* by P_{MBH} implies a transcript of at least 16,903 nt. Bacterial mRNAs in this size range are unusual but not unprecedented. A 17,000-nt transcript from the *Erwinia amylovora ams* locus was demonstrated by Northern mapping (5). The *fla/che* operon of *Bacillus subtilis* contains more than 30 genes and is at least 26 kb long (28a). A recent study on *Bacillus brevis* suggests that even larger transcriptional units exist in bacteria (31).

Membrane-bound hydrogenases are found in a wide variety of gram-negative bacteria. In several cases including *A. eutrophus*, the hydrogenase gene clusters have been sequenced (reviewed in reference 14). The overall arrangements of genes in these clusters are remarkably similar, pointing to conserved mechanisms of expression. In two organisms, *Rhodobacter capsulatus* and *Bradyrhizobium japonicum*, a positive regulator homologous to HoxA is encoded downstream of a promoter under its own control (35, 46). This situation is suggestive of a positive transcriptional feedback mechanism like the one reported here. In *E. coli*, a *hyp* operon consisting of the genes *hypA*, *hypB*, *hypC*, *hypD*, and *hypE* is transcribed from two promoters. The promoter proximal to *hypA* is dependent on σ^{54} and the positive regulator FhlA, which is encoded immediately downstream of *hypE* (27). FhlA is controlled by its own promoter but may also be susceptible to transcription from the FhlA-dependent *hyp* promoter.

Our findings also sketch a picture of the transcriptional organization of the *A. eutrophus hyp* region. Promoter mapping data suggest that the genes *hypB1*, *hypF1*, *hypC*, *hypD*, and

hypE are expressed as a polycistronic transcript. *hypA1* is controlled by a separate promoter as is *hypX*. We can at present only speculate on the significance of this configuration. Since almost nothing is known about the specific functions of the various proteins in the maturation of the *A. eutrophus* hydrogenases, we have little to go on. The former gene products may act as a set. The products of *hypA1* and *hypX* might be required in different amounts and/or at different times and thus be subject to independent expression. However, major differences in promoter activity were not apparent under the conditions tested. The data for the *hypA1* promoter test construct do not rule out the existence of two promoters: one of the σ^{70} type and one σ^{54} promoter.

hypX is also expressed from a separate promoter, suggesting that the product can act independently of the other Hyp proteins. The deduced sequence of HypX reveals motifs characteristic of N_{10}-formyltetrahydrofolate-dependent enzymes and of enoyl-coenzyme A-hydratases/isomerases (6). It has been suggested that proteins of the HypX family are not involved in donating nickel to the nascent hydrogenase, the function postulated for the other Hyp proteins, but rather mediate the insertion of the diatomic ligands CO and CN (34). In an *A. eutrophus hypX* null mutant, hydrogenase activity was reduced by about 50%, whereas knockout mutants for the other *hyp* genes (with the exception of the two copies of *hypA*) blocked hydrogenase maturation (6, 7, 47). This suggests a conditional requirement for HypX in contrast to the other Hyp proteins. In *B. japonicum*, the arrangement of the *hyp* genes is similar to that in *A. eutrophus*, and indirect data suggest that the *hypX* gene is expressed from its own promoter (9).

Interestingly, activity of the *hoxA* promoter was higher in the HoxA⁻ and RpoN⁻ backgrounds (Table 2). The data for the HoxA⁻ strain could be explained assuming that the promoter is negatively autoregulated. Since a σ^{54}-dependent promoter (i.e., P_{MBH}) is responsible for high-level expression of *hoxA* under hydrogenase-derepressing conditions, mutations in *rpoN* should have an effect similar to that of mutations in *hoxA*. The data show that this is in fact the case.

The expression of the *A. eutrophus hyp* genes under hydrogenase-repressing and -derepressing conditions alike seems at first paradoxical but is explained by the requirements for the synthesis of the H_2-sensing apparatus. The actual H_2-sensing molecule is a dimer consisting of the products of the genes *hoxB* and *hoxC* (3a, 24). HoxB and HoxC are homologous to the small and large subunits, respectively, of the dimeric hydrogenases (24). Moreover, HoxBC is, like the true hydrogenases, a nickel metalloenzyme and requires the Hyp proteins for normal maturation (6, 6a). Active HoxBC must be present in the cell at all times to enable the organism to respond to the availability of H_2. This, in turn, necessitates the constitutive expression of the *hyp* genes.

REFERENCES

1. **Albracht, S. P. J.** 1994. Nickel hydrogenases: in search of the active site. Biochim. Biophys. Acta **1199:**167–204.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York, N.Y.
3. **Bardowski, J., S. D. Ehrlich, and A. Chopin.** 1994. BglR protein, which belongs to the BglG family of transcriptional antiterminators, is involved in

β-glucoside utilization in *Lactococcus lactis*. J. Bacteriol. **176:**5681–5685.

3a.**Bernhard, M., and B. Friedrich.** Unpublished data.

4. **Bernhard, M., E. Schwartz, J. Rietdorf, and B. Friedrich.** 1996. The *Alcaligenes eutrophus* membrane-bound hydrogenase gene locus encodes functions involved in maturation and electron transport coupling. J. Bacteriol. **178:**4522–5429.

5. **Bugert, P., and K. Geider.** 1995. Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovora*. Mol. Microbiol. **15:**917–933.

6. **Buhrke, T., and B. Friedrich.** 1998. *hoxX* (*hypX*) is a functional member of the *Alcaligenes eutrophus hyp* gene cluster. Arch. Microbiol. **170:**460–463.

6a.**Buhrke, T., and B. Friedrich.** Unpublished data.

7. **Dernedde, J., T. Eitinger, N. Patenge, and B. Friedrich.** 1996. *hyp* gene products in *Alcaligenes eutrophus* are part of a hydrogenase-maturation system. Eur. J. Biochem. **235:**351–358.

8. **Dernedde, J., M. Eitinger, and B. Friedrich.** 1993. Analysis of a pleiotropic gene region involved in formation of catalytically active hydrogenase in *Alcaligenes eutrophus* H16. Arch. Microbiol. **159:**545–553.

9. **Durmowicz, M. C., and R. J. Maier.** 1997. Roles of HoxX and HoxA in biosynthesis of hydrogenase in *Bradyrhizobium japonicum*. J. Bacteriol. **179:**3676–3682.

10. **Eberz, G., and B. Friedrich.** 1991. Three *trans*-acting functions control hydrogenase expression in *Alcaligenes eutrophus*. J. Bacteriol. **173:**1845–1854.

11. **Eberz, G., C. Hogrefe, C. Kortlüke, A. Kamienski, and B. Friedrich.** 1986. Molecular cloning of structural and regulatory hydrogenase genes (*hox*) of *Alcaligenes eutrophus* H16. J. Bacteriol. **168:**636–641.

12. **Eitinger, T., and B. Friedrich.** 1991. Cloning, nucleotide sequence, and heterologous expression of a high-affinity nickel transport gene from *Alcaligenes eutrophus*. J. Biol. Chem. **266:**3222–3227.

12a.**Estacio, W., S. Santa Anna-Arriola, M. Adedipe, and L. M. Márquez-Magaña.** 1998. Dual promoters are responsible for the transcription initiation of the *fla/che* operon in *Bacillus subtilis*. J. Bacteriol. **180:**3548–3555.

13. **Frey, M.** 1998. Nickel-iron hydrogenases: structural and functional properties. Struct. Bonding **90:**97–126.

14. **Friedrich, B., and E. Schwartz.** 1993. Molecular biology of hydrogen utilization in aerobic chemolithotrophs. Annu. Rev. Microbiol. **47:**351–383.

15. **Friedrich, B., M. Bernhard, J. Dernedde, T. Eitinger, O. Lenz, C. Massanz, and E. Schwartz.** 1996. Hydrogen oxidation by *Alcaligenes*, p. 110–117. *In* M. E. Lindstrom and F. R. Tabita (ed.), Microbial growth on C1 compounds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

16. **Friedrich, C. G.** 1982. Derepression of hydrogenase during limitation of electron donors and derepression of ribulosebisphosphate carboxylase during carbon limitation of *Alcaligenes eutrophus*. J. Bacteriol. **149:**203–210.

17. **Friedrich, C. G., B. Bowien, and B. Friedrich.** 1979. Formate and oxalate metabolism in *Alcaligenes eutrophus*. J. Gen. Microbiol. **115:**185–192.

18. **Friedrich, C. G., B. Friedrich, and B. Bowien.** 1981. Formation of enzymes of autotrophic metabolism during heterotrophic growth of *Alcaligenes eutrophus*. J. Gen. Microbiol. **122:**69–78.

19. **Gerischer, U., and P. Dürre.** 1992. mRNA analysis of the *adc* gene region of *Clostridium acetobutylicum* during the shift to solventogenesis. J. Bacteriol. **174:**426–433.

20. **Hübner, P., J. C. Willison, P. M. Vignais, and T. A. Bickel.** 1991. Expression of regulatory *nif* genes in *Rhodobacter capsulatus*. J. Bacteriol. **173:**2993–2999.

21. **Kortlüke, C., K. Horstmann, E. Schwartz, M. Rohde, R. Binsack, and B. Friedrich.** 1992. A gene complex coding for the membrane-bound hydrogenase of *Alcaligenes eutrophus* H16. J. Bacteriol. **174:**6277–6289.

22. **Kortlüke, C., and B. Friedrich.** 1992. Maturation of membrane-bound hydrogenase of *Alcaligenes eutrophus* H16. J. Bacteriol. **174:**6290–6293.

23. **Krüger, N., and A. Steinbüchel.** 1992. Identification of *acoR*, a regulatory gene for the expression of genes essential for acetoin catabolism in *Alcaligenes eutrophus* H16. J. Bacteriol. **174:**4391–4400.

24. **Lenz, O., and B. Friedrich.** 1998. A novel multicomponent regulatory system mediates H$_2$ sensing in *Alcaligenes eutrophus*. Proc. Natl. Acad. Sci. USA **95:**12474–12479.

25. **Lenz, O., E. Schwartz, J. Dernedde, M. Eitinger, and B. Friedrich.** 1994. The *Alcaligenes eutrophus* H16 *hoxX* gene participates in hydrogenase regulation. J. Bacteriol. **176:**4385–4393.

26. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.

27. **Lutz, S., R. Böhm, A. Beier, and A. Böck.** 1990. Characterization of divergent NtrA-dependent promoters in the anaerobically expressed gene cluster coding for hydrogenase 3 components of *Escherichia coli*. Mol. Microbiol. **4:**13–20.

28. **Maier, T., and A. Böck.** 1996. Nickel incorporation into hydrogenases, p. 173–192. *In* R. P. Hausinger, G. L. Eichhorn, and L. G. Marzilli (ed.), Advances in inorganic biochemistry: mechanisms of metallocenter assembly. VHC Publishers, Inc., New York, N.Y.

28a.**Márquez-Magaña, L. M., and M. J. Chamberlin.** 1994. Characterization of the *sigD* transcriptional unit of *Bacillus subtilis*. J. Bacteriol. **176:**2427–2434.

29. **Massanz, C., V. M. Fernandez, and B. Friedrich.** 1997. C-terminal extension of the H$_2$-activating subunit, HoxH, directs maturation of the NAD-reducing hydrogenase in *Alcaligenes eutrophus*. Eur. J. Biochem. **245:**441–448.

30. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

31. **Mootz, H. D., and M. A. Marahiel.** 1997. The tyrocidin biosynthesis operon of *Bacillus brevis*: Complete nucleotide sequence and biochemical characterization of functional internal adenylation domains. J. Bacteriol. **179:**6843–6850.

32. **Pahel, G., D. M. Rothstein, and B. Magasanik.** 1982. Complex *glnA-glnL-glnG* operon of *Escherichia coli*. J. Bacteriol. **150:**202–213.

33. **Pierik, A. J., M. Schmelz, O. Lenz, B. Friedrich, and S. P. J. Albracht.** 1998. Characterization of the active site of a hydrogen sensor from *Alcaligenes eutrophus*. FEBS Lett. **438:**231–235.

34. **Rey, L., D. Fernández, B. Brito, Y. Hernando, J.-M. Palacios, J. Imperial, and T. Ruiz-Argüeso.** 1996. The hydrogenase gene cluster of *Rhizobium leguminosarum* bv. *viciae* contains an additional gene (*hypX*), which encodes a protein with sequence similarity to the N$_{10}$-formyltetrahydrofolate-dependent enzyme family and is required for nickel-dependent hydrogenase processing and activity. Mol. Gen. Genet. **252:**237–248.

35. **Richaud, P., A. Colbeau, B. Toussaint, and P. M. Vignais.** 1991. Identification and sequence analysis of the *hupR1* gene, which encodes a response regulator of the NtrC family required for hydrogenase expression in *Rhodobacter capsulatus*. J. Bacteriol. **173:**5928–5932.

36. **Scarlato, V., A. Prugnola, B. Aricò, and R. Rappuoli.** 1990. Positive transcriptional feedback at the *bvg* locus controls expression of virulence factors in *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA **87:**6753–6757.

37. **Schink, B., and H. G. Schlegel.** 1978. Mutants of *Alcaligenes eutrophus* defective in autotrophic metabolism. Arch. Microbiol. **117:**123–129.

38. **Schnetz, K., and B. Rak.** 1988. Regulation of the *bgl* operon of *Escherichia coli* by transcriptional antitermination. EMBO J. **7:**3271–3277.

39. **Schwartz, E., U. Gerischer, and B. Friedrich.** 1998. Transcriptional regulation of *Alcaligenes eutrophus* hydrogenase genes. J. Bacteriol. **180:**3197–3204.

40. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance gene cassette for site-specific insertion and deletion mutagenesis. BioTechniques **15:**831–834.

41. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–791.

42. **Storey, D. G., T. L. Raivio, D. W. Frank, M. J. Wick, S. Kaye, and B. Iglewski.** 1991. Effect of *regB* on expression from the P1 and P2 promoters of the *Pseudomonas aeruginosa regAB* operon. J. Bacteriol. **173:**6088–6094.

43. **Thiemermann, S., J. Dernedde, M. Bernhard, W. Schroeder, C. Massanz, and B. Friedrich.** 1996. Carboxyl-terminal processing of the cytoplasmic NAD-reducing hydrogenase of *Alcaligenes eutrophus* requires the *hoxW* gene product. J. Bacteriol. **178:**2368–2374.

44. **Thöny, B., and H. Hennecke.** 1989. The −24/−12 promoter comes of age. FEMS Microbiol. Rev. **63:**341–358.

45. **Towbin, N., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.

46. **Van Soom, C., P. de Wilde, and J. Vanderleyden.** 1997. HoxA is a transcriptional regulator for expression of the *hup* structural genes in free-living *Bradyrhizobium japonicum*. Mol. Microbiol. **23:**967–977.

47. **Wolf, I., T. Buhrke, J. Dernedde, A. Pohlmann, and B. Friedrich.** 1998. Duplication of *hyp* genes involved in maturation of [NiFe] hydrogenases in *Alcaligenes eutrophus* H16. Arch Microbiol. **170:**451–459.

48. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.