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Temperature Tolerance of Hydrogenase Expression in *Alcaligenes eutrophus* Is Conferred by a Single Amino Acid Exchange in the Transcriptional Activator HoxA

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Expression of the soluble (SH) and membrane-bound (MBH) hydrogenases in the facultatively lithoautotrophic bacterium *Alcaligenes eutrophus* is dependent on the transcriptional activator HoxA and the alternative sigma factor σ^{54}. Deletion analysis revealed that a region 170 bp upstream of the transcriptional start of the SH operon is necessary for high-level promoter activity. Mobility shift assays with DNA fragments containing the SH upstream region and purified β-galactosidase–HoxA fusion protein isolated from *Escherichia coli* or authentic HoxA isolated by immunoaffinity chromatography from *A. eutrophus* failed to detect specific binding. In contrast, *A. eutrophus* extracts enriched for HoxA by heparin-Sepharose chromatography and ammonium sulfate fractionation produced a weak but discrete shift in the mobility of the target DNA. This effect was not observed with comparable extracts prepared from *hoxA* mutants. A similar experiment using antibodies against HoxA confirmed that HoxA was responsible for the observed mobility shift. Extracts prepared from a temperature-tolerant mutant of *A. eutrophus* gave a stronger retardation than did those from the wild type. Unlike the wild type, the *hox*(Tr) mutant is able to grow with hydrogen at temperatures above 33°C because of a mutation in the regulatory gene *hoxA*. In this paper, we show that a single amino acid substitution (Gly-468→Val) in the C-terminal part of HoxA is responsible for temperature tolerance. The SH upstream region also contains sequence motifs resembling the *E. coli* integration host factor (IHF) binding site, and purified *E. coli* IHF protein shifted the corresponding indicator fragment.

Alcaligenes eutrophus, a member of the β subgroup of the class *Proteobacteria*, can grow lithoautotrophically with hydrogen as the sole energy source and carbon dioxide as the carbon source (reviewed in reference 3). The bacterium harbors a large plasmid on which the genes required for hydrogen oxidation are located. The *hox* region encodes two hydrogenases: a cytoplasmic NAD-reducing enzyme (SH) and a membrane-bound electron transport-coupled protein (MBH). Both hydrogenases belong to the family of nickel-iron-containing proteins. The formation of these metalloproteins is an extremely complex and apparently highly conserved process requiring sets of ancillary functions in addition to the hydrogenase enzymes (reviewed in reference 17). The genes coding for these proteins are part of the *hox* gene complex in *A. eutrophus*. Their products are involved in nickel uptake (14), metal processing (9), and hydrogenase maturation (24).

Physiological experiments have demonstrated that the synthesis of the two hydrogenases of *A. eutrophus* is coordinately regulated. Tight repression is observed during growth of the cells on preferentially utilized carbon sources, whereas derepression occurs on poor substrates even in the absence of hydrogen (18). Moreover, hydrogenase synthesis in *A. eutrophus* is temperature sensitive and abolished above 33°C (19). Mutant analysis has shown that the expression of SH- and MBH-encoding genes in *A. eutrophus* is strictly dependent on two transcriptional components: the minor sigma factor σ^{54} of RNA polymerase (36, 54) and the *hoxA* gene product. Sequence comparisons revealed that the product of *hoxA* is a member of the family of two-component regulatory systems (12). Another regulatory *hox* gene, *hoxX*, has recently been identified immediately adjacent to *hoxA*. A mutation in *hoxX* lowered the expression level of both SH and MBH operons (28). Thus, the product of *hoxX* is a third transcriptional factor in the hydrogenase control circuit.

HoxA homologs have been reported for other hydrogenase-containing members of the class *Proteobacteria*. The products of *hydG* of *Escherichia coli* (47), $hupR_1$ of *Rhodobacter capsulatus* (35), and *hoxA* of *Bradyrhizobium japonicum* (53) share more than 50% sequence identity with HoxA of *A. eutrophus*.

In this report, we describe the results of a study of the in vitro binding of the hydrogenase-specific activator HoxA. Gel retardation assays carried out with DNA fragments containing the promoter region of the SH operon and partially purified HoxA demonstrated binding of the regulator. HoxA preparations from a temperature-tolerant mutant gave a stronger retardation than did corresponding preparations from the wild-type strain. The mutant HoxA protein contained a single amino acid exchange in the putative DNA binding domain. Phenotypically, this mutation caused a 4°C increase in the upper temperature limit for autotrophic growth. Moreover, the experiments clearly showed that purified *E. coli* integration host factor (IHF) binds to the SH operon DNA fragment. The data suggest positive regulation of the SH operon by HoxA and the involvement of an IHF-like protein.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. To facilitate the puri-

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fication of the regulator protein HoxA, we constructed an in-frame *lacZ-hoxA* gene fusion. A 3.6-kb *Xba*I fragment of pGE97 carrying the intact *hoxA* gene was inserted into vector pSS20*c (43), yielding plasmid pCH252. Excision of a 1.6-kb *Sal*I-*Bgl*II fragment followed by treatment with Klenow polymerase and ligation generated plasmid pCH351. This plasmid directed the synthesis of a β-galactosidase (β-Gal)–linker peptide–HoxA triprotein lacking the first 11 amino acids of HoxA. For regulatable expression of HoxA in *A. eutrophus*, the gene *hoxA* was placed under the control of the *E. coli lac* promoter in the broad-host-range vector pRK404 (10). The vector was cut with *Hin*dIII, treated with Klenow polymerase, recut with *Bam*HI, and dephosphorylated. A 3.6-kb *Xba*I fragment was excised from plasmid pGE98, end polished, and recut with *Bam*HI. The resulting fragment carrying the intact *hoxA* gene was ligated into the vector, yielding pGE208.

A plasmid-borne Φ(*hoxY-lacZ*) gene fusion was constructed as follows. A 0.6-kb *Bam*HI-*Bgl*II fragment of M13mp18 (30) containing the *lacZ*α sequences and part of the polylinker was inserted into the *Bam*HI site of the broad-host-range plasmid pIJ1363 (37) to give pGE185. A 5-kb *Bam*HI fragment of plasmid pGE15 containing the *hoxFUY′* region and upstream sequences was inserted into the *Bam*HI site, yielding plasmid pGE187. Plasmid pGE188 was obtained by cutting pGE187 with *Hin*dIII and religating.

Plasmid pCH116 carries the *hoxA*(Tr) allele of *A. eutrophus* HF117. A 2.0-kb *Eco*RI fragment from pCH116 carrying *hoxA*-(Tr) was inserted into the cloning vector pBluescript SK+ (Stratagene Cloning Systems) in both orientations, yielding plasmids pCH421 and pCH422. In order to generate an isogenic mutant carrying the 3′ terminus of the *hoxA* gene of HF117, a 253-bp *Pst*I fragment from pCH421 was inserted into the broad-host-range vector pSUP202 (45). The resulting plasmid was designated pCH453.

Media and growth conditions. *E. coli* strains were cultivated in LB medium (31) containing 0.5% (wt/vol) NaCl unless otherwise indicated. For heterotrophic growth of *A. eutrophus* strains, mineral salts medium (41) containing 0.4% (wt/vol) succinate (SN medium), 0.4% (wt/vol) fructose (FN medium), or 0.2% (wt/vol) fructose and 0.2% (wt/vol) glycerol (FGN medium) was employed. Lithoautotrophic cultures were grown on mineral salts medium under an atmosphere of hydrogen, carbon dioxide, and oxygen (8:1:1, vol/vol/vol). The following concentrations of antibiotics were used: 350 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 deletions in the *hoxF* upstream region. Plasmid pGE188 (Table 1) was linearized by cutting with *Hin*dIII. Linear plasmid DNA was treated with exonuclease III. Aliquots were withdrawn from the reaction mixture at various time intervals, and the reaction was stopped by adding S1 buffer. Subsequently, the exonuclease III-generated ends were polished by treatment with S1 nuclease and Klenow polymerase by using an Erase-a-Base kit (Promega) according to the manufacturer's instructions. After ligation and transformation, deletion clones were screened by restriction analysis and sequenced by the dideoxy chain termination method (39).

Purification of β-Gal–HoxA fusion protein. Overproduction and purification of a β-Gal–HoxA fusion protein were carried out according to the procedure described by Scholtissek and Grosse (43). *E. coli* WM1704(pCH351) was grown in NZCYM medium (38) with 15 μM thiamine at 27°C. At an A_{600} of 0.6, cultures were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubating for 30 min. The cells were harvested, resuspended in buffer A (20 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 600 mM NaCl; 10 mM mercaptoethanol; 5% [wt/vol] glycerol; 0.5 mg of lysozyme per ml), and homogenized by sonication, and the soluble fraction was recovered as the supernatant after centrifugation at 65,000 × *g* for 30 min. Proteins were precipitated by ammonium sulfate at 60% saturation, resuspended in buffer B (20 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 1.6 M NaCl; 10 mM mercaptoethanol; 5% [wt/vol] glycerol), and applied to an aminophenylthiogalactoside (APTG) (Sigma Chemical) agarose column. After a wash with buffer B, protein was eluted from the column with buffer C (100 mM sodium borate, pH 10.5; 10 mM $MgCl_2$; 1.6 M NaCl; 10 mM mercaptoethanol; 5% [wt/vol] glycerol) and immediately neutralized with 1 M Tris-HCl, pH 7.4. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% [wt/vol] polyacrylamide gels (26) and detected by silver staining (1). Protein concentrations were determined by the method of Bradford (5) by using bovine serum albumin as a reference.

For the isolation of the HoxA moiety from purified fusion protein, collagenase (type III from *Clostridium histolyticum*; Calbiochem) was added to the APTG eluate fractions without prior dialysis at a concentration of 1 μg/ml and the mixture was incubated at 30°C for 1 h. Proteins were precipitated with trichloroacetic acid and separated by preparative SDS-PAGE. After visualization of the proteins by soaking the gel in 0.25 M KCl, the band containing HoxA was excised and used for immunization.

Immunological techniques. Polyclonal anti-HoxA serum was raised by homogenizing gel slices containing β-Gal–HoxA fusion protein with an equal volume of Freund's incomplete adjuvant for primary immunization of rabbits. After 3 weeks, a series of two boosts with the purified HoxA moiety were given. The antiserum was purified with a soluble extract of megaplasmid-free *A. eutrophus* HF210. To this end, protein was coupled to CNBr-activated Sepharose beads (Pharmacia Biotech) according to the manufacturer's instructions. Crude antiserum was added to the conjugate at a ratio of 4:1 (vol/vol), and the mixture was incubated with gentle shaking for 2 h at room temperature. The supernatant was subsequently recovered and stored at −80°C. Immunoblot analysis was performed by standard methods (6, 51). An alkaline phosphatase-coupled goat anti-rabbit secondary antibody (Jackson ImmunoResearch) was used for detection (27). For mobility shift assays, the immunoglobulin G fraction of the anti-HoxA serum was purified by fast protein liquid chromatography on protein A-Sepharose (Pharmacia Biotech) according to the manufacturer's instructions.

Immunoaffinity chromatography. The immunoglobulin G fraction of the anti-HoxA serum was selectively bound to protein A-Sepharose (Pharmacia Biotech) and covalently crosslinked by dimethylsuberimidate (Sigma Chemical) as described elsewhere (20). The resulting beads were used to isolate HoxA from the soluble fraction of *A. eutrophus* cells by column chromatography.

Partial purification of HoxA from *A. eutrophus*. Cells were harvested, resuspended in buffer BP_{50} [4 mM Tris-HCl, pH 7.9; 12 mM *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid (HEPES), pH 7.9; 50 mM KCl; 1 mM EDTA; 12% (wt/vol) glycerol], homogenized by three passages through a French press, and centrifuged at 65,000 × *g* for 30 min. Proteins from this soluble extract were precipitated by ammonium sulfate at 60% saturation, resuspended in buffer BP_{50}, and separated by heparin-Sepharose (Pharmacia Biotech) column chromatography. Proteins were eluted with BP_{1000} buffer (4 mM Tris-HCl, pH 7.9; 12 mM HEPES, pH 7.9; 1,000 mM KCl; 1 mM EDTA; 12% [wt/vol] glycerol), and the peak fractions

TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain, phage, or plasmid	Characteristics	Source or reference
Strains		
A. eutrophus		
H16	MBH^+ SH^+	DSM 428; ATCC 17699
HF117	*hoxA*(Tr), 1330 T→C,[b] 1672 G→T	19
HF210	$pHG1^-$	24
HF294	*hoxA*::Tn*5*	12
HF367	*hoxA*(Tr), 1672 G→T	This work
E. coli		
HB101	*supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13* Δ(*mcrC-mrr*) *hsdS20* (r^- m^-)	4
S17-1	Tra^+ *recA pro thi hsdR chr*::RP4-2	45
WM1704	*araD139* Δ(*argF-lac*)*U169 proA*$^+$ Δ*lon hflA150 rpsL chr*::Tn*10*	29
XL-1 Blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac* (F′ *proAB lacI*^{q} *Z*ΔM15 Tn*10*)	Stratagene
Phage R408	Helper phage	Stratagene
Plasmids		
pBluescript SK+	Ap^r *lacZ′* T7 ϕ10 promoter f1 *ori*	Stratagene
pRK404	Tc^r Mob^+ RK2*ori lacZ*α	10
pRK2013	Km^r Tra^+	9
pIJ1363	Tc^r Mob^+ *′lacZ*	37
pSUP202	Ap^r Cm^r Tc^r ColE1*ori* Mob^+	45
pTZ18R	Ap^r *lacZ*α f1*ori*	Pharmacia
pSS20*c	Ap^r *lacZ*-collagenase linker sequence	43
pCH116	2.0-kb *Eco*RI *hoxA*(Tr) fragment from HF117 in pSUP202	13
pCH252	3.6-kb *Xba*I fragment of pGE97 in pSS20*c	This work
pCH292	1-kb *Bgl*II-*Hin*dIII fragment of pGE15 in pTZ18R	This work
pCH351	Ap^r Φ(*lacZ-hoxA*)	This work
pCH421	2.0-kb *Eco*RI fragment from pCH116 in pBluescript SK+	This work
pCH422	2.0-kb *Eco*RI fragment from pCH116 in pBluescript SK+, inverted orientation	This work
pCH453	235-bp *Pst*I fragment from pCH421 in pSUP202	This work
pGE15	Tc^r *hoxFUYH*	52
pGE97	Tc^r *hoxA*	12
pGE98	Derivative of pGE97 with 3-kb *Bam*HI fragment deleted	11
pGE185	0.6-kb *Bam*HI-*Bgl*II fragment of M13mp18 inserted into *Bam*HI site of pIJ1363 *lacZ*α oriented away from *lacZ* of pIJ1363	This work
pGE187	5.9-kb *Bam*HI fragment of pGE15 introduced into *Bam*HI site of pGE185 so as to give a *hoxY-lacZ* gene fusion	This work
pGE188	Derivative of pGE187 with a 2.5-kb *Hin*dIII fragment deleted	This work
pGE208	3.6-kb *Bam*HI-*Xba*I (Klenow-treated) fragment of pGE98 in pRK404	This work
pGE314	Deletion derivative of pGE188	This work
pGE315	Deletion derivative of pGE188	This work
pGE316	Deletion derivative of pGE188	This work
pGE317	Deletion derivative of pGE188	This work
pGE318	Deletion derivative of pGE188	This work

[a] Mob, mobilizability; Tra, transfer of mobilizable plasmids; *ori*, origin of replication.
[b] 1330 T→C, T-to-C exchange at position 1330.

were pooled and used for further experiments. For some experiments, an additional ammonium sulfate precipitation at 35% saturation was performed to remove some of the contaminating proteins.

DNA binding assay. For DNA manipulations, standard procedures were employed (38). A 284-bp fragment of plasmid pCH292 was used as the target sequence. Plasmid DNA was digested with *Hin*dIII and *Sca*I, dephosphorylated with shrimp alkaline phosphatase (United States Biochemicals), and cut with *Xho*I after heat inactivation of the phosphatase. The fragment was isolated from a 2% (wt/vol) agarose gel, and the 5′ termini were labelled with [γ-^{32}P]ATP and polynucleotide kinase (Boehringer Mannheim). Gel retardation experiments were carried out according to a standard protocol (2). DNA was incubated with a mixture containing 2.5 ng of poly(dI-dC) (Boehringer Mannheim) per ml, 150 mM KCl, 10 mM $MgCl_2$, and different amounts of partially purified protein extracts (see above) in buffer BP_{50} at 30°C for 30 min and separated in a nondenaturing 5% (wt/vol) polyacrylamide gel. DNA and DNA-protein complexes were detected by autoradiography. Purified *E. coli* IHF protein was a kind gift of S. Kustu, Berkeley, Calif.

Cloning and sequencing. Plasmid DNA was isolated by using DEAE columns (Qiagen). Genomic DNA was isolated from *A. eutrophus* according to the method of Chen and Kuo (7). DNA fragments were isolated from preparative agarose gels by using a GlassMAX kit (Bethesda Research Laboratories, Inc.). Single-stranded templates were sequenced according to the method of Sanger et al. (39) by using Sequenase 2.0 (United States Biochemicals) and [α-^{35}S]dATP (Amersham). DNA fragments obtained by PCR amplification were sequenced directly after NaOH denaturation. In both cases, the following

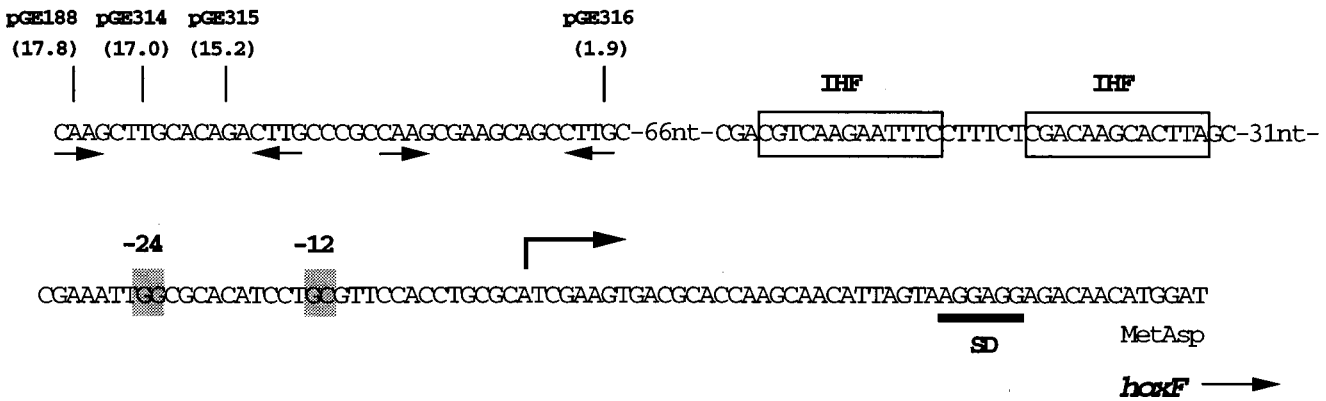

FIG. 1. Deletion analysis of the *hoxF* upstream region. Deletion derivatives of the Φ(*hoxY-lacZ*) plasmid were generated by treatment of *Hin*dIII-linearized plasmid pGE188 with exonuclease III and religation. The resulting derivatives were introduced into *A. eutrophus* H16, and β-Gal activity of cells grown in FGN medium was determined. The β-Gal activities (in units per milligram) are given in parentheses above the respective endpoints. The 5′ end of the *hoxF* gene is indicated. The transcription start site is marked by a bent arrow, and the Shine-Dalgarno sequence is underlined. Possible IHF binding sites are boxed, and the invariant dinucleotides of a putative −24/−12 promoter are shaded. A palindromic motif is indicated by arrows below the nucleotide sequence.

synthetic 17-bp oligonucleotides were used as primers (the coordinates of the first nucleotide in the published *hoxA* sequence [12] are indicated): BF205, 549-GGGGTTAACGAG GCGGG; BF206, 749-TTTCGAGCGCATTGTGC; BF207, 951-AACCTGCTCGAATCCGA; BF208, 1148-TTGGATAC CGGTCGATG; BF209, 1353-GGTCTGTATTTCGGTGG; BF210, 1556-GTCGGGTACGCTGCAGG; and BF211, 1753-TTTTGCTGAGGCAAGAC (noncoding strand). PCR amplification of the 3′ domain of *hoxA* from isolated genomic DNA was performed by following the standard protocols (2).

Mutagenesis by gene replacement. An isogenic mutant of *A. eutrophus* H16 with a point mutation in the region encoding the helix-turn-helix domain (position 1672) was obtained via a gene replacement protocol (25): plasmid pCH453 (Table 1) was introduced by conjugative transfer from *E. coli* S17-1 (45) into *A. eutrophus* H16. Homogenote recombinants were selected by incubation under lithoautotrophic growth conditions at 37°C and tested for kanamycin sensitivity of the resulting clones. The nucleotide sequences spanning positions 1330 and 1672 were verified by sequencing.

Enzyme assays. Determination of β-Gal activity was as described by Miller (31), with modifications. Cells were harvested and resuspended in 50 mM potassium phosphate buffer (pH 7.0). Aliquots of cells were added to Z buffer containing 10 μl of 0.5% (wt/vol) *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) and 10 μl of 0.75% (wt/vol) sodium deoxycholate to give a final volume of 1 ml. The reaction was initiated by the addition of 200 μl of *o*-nitrophenylgalactoside (ONPG) (4 mg/ml in 50 μM phosphate buffer, pH 7.0) and stopped by adding 500 μl of 1 M Na_2CO_3. SH activity was assayed by monitoring H_2-dependent NAD reduction in detergent-treated cells (16). MBH was determined by measuring H_2-dependent methylene blue reduction in membrane fractions (40).

RESULTS

Minimal sequences necessary for expression of the SH operon. Previous studies showed that a recombinant plasmid complemented *A. eutrophus* mutants devoid of SH protein to wild-type activity (52). The cloned DNA fragment contains 262 bp of the 5′ noncoding sequence. −24/−12 motifs characteristic of σ^{54} promoters are located upstream of the translational start of the first structural gene, *hoxF* (Fig. 1).

To determine the minimal sequence required for the expression of the SH operon, a set of plasmids with deletions extending from the *Hin*dIII site into the 5′ noncoding region was constructed. These deletion constructs were derived from plasmid pGE188, which carries the intact genes *hoxF* and *hoxU* and the 5′ part of *hoxY* fused in frame to the *lacZ* gene (Table 1). Representative deletion plasmids were selected for further analysis (Fig. 1). The deletion endpoints and the neighboring vector sequences were determined by sequence analysis. The resulting plasmids were transferred from *E. coli* to *A. eutrophus* by triparental mating by using the helper strain *E. coli* HB101(pRK2013) for mobilization (4). The expression of the SH operon was monitored by determining the β-Gal activity of cells grown under hydrogenase-derepressing conditions in FGN medium. Figure 1 demonstrates that the deletion of sequences up to position −170 with respect to the transcriptional start site was correlated with a dramatic decrease in β-Gal activity. These results indicate that more than 170 bp of upstream sequence is necessary for SH operon expression.

Isolation of a β-Gal–HoxA fusion protein from *E. coli* and preparation of an anti-HoxA serum. Sequencing revealed that *hoxA* encodes a member of the NtrC family of regulatory proteins (12), suggesting a model for hydrogenase regulation that involves transcriptional activation via binding of HoxA upstream of σ^{54} promoters and interaction with σ^{54} RNA polymerase. In order to test the binding of HoxA to *hox* upstream sequences in vitro, we attempted to purify HoxA. Initial attempts to overproduce HoxA in *E. coli* by using conventional expression vectors yielded scarcely detectable amounts of protein (data not shown). To obtain high-level expression and at the same time to take advantage of efficient purification techniques based on affinity chromatography, we constructed a plasmid (pCH351) carrying a *lacZ-hoxA* gene fusion under the control of the *lac* promoter. When cultures of *E. coli* harboring pCH351 were grown at 37°C and induced by addition of IPTG, a 165-kDa protein was produced. This protein was obviously the product of the plasmid-borne *lacZ-hoxA* gene fusion. Almost all of the fusion protein was present as inclusion bodies. Cultivation of cells at 27°C prior to induction led to the production of substantial amounts of soluble fusion protein (Fig. 2, lane 2). The fusion protein was isolated by affinity chromatography on an APTG column (Fig. 2, lane 3). Treatment of the purified fusion protein with collagenase yielded products of 116 and 49 kDa corresponding to the β-Gal and HoxA moieties (Fig. 2, lane 4). The latter polypeptide was isolated by preparative electrophoresis in an SDS–10% polyacrylamide gel and used to raise a polyclonal antiserum. Western blots (im-

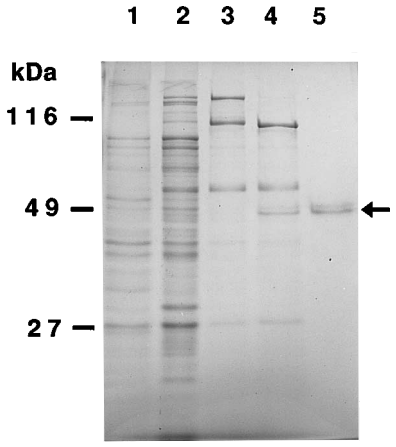

FIG. 2. Purification of the β-Gal–HoxA fusion protein and cleavage by collagenase treatment. A silver-stained SDS–10% PAGE gel of samples from various purification steps is shown. Lane 1, total protein from strain WM1704(pCH351) before induction; lane 2, soluble extract after induction by IPTG; lane 3, eluate after APTG-Sepharose chromatography; lane 4, fraction from lane 3 after cleavage of the fusion protein by collagenase; lane 5, HoxA after excision from a preparative SDS-PAGE gel following separation of the material shown in lane 4. HoxA is indicated by an arrow. A minor band resulted from collagenase cleavage at a second recognition site in the linker sequence.

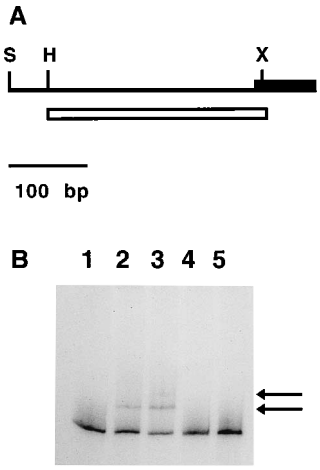

FIG. 4. Mobility shift assay with *A. eutrophus* extracts enriched for HoxA. (A) Simplified restriction map of the SH operon upstream region. The thicker bar indicates the 5′ end of the first structural gene, *hoxF*. The open bar indicates the *Hin*dIII-*Xho*I fragment used in the mobility shift experiments. Abbreviations for restriction sites: S, *Sma*I; H, *Hin*dIII; X, *Xho*I. (B) Autoradiogram of the mobility shift gel. A 2-μg amount of protein of each of the following strains was used: H16 (lane 2), H16(pGE208) (lane 3), HF294 (lane 4), and HF210(pRK404) (lane 5). Lane 1 contains no protein. The arrows indicate the retarded DNA fragments.

munoblots) stained with this antiserum gave a single, faint band corresponding to a protein of 49 kDa in the *A. eutrophus* wild-type strain and in a derivative harboring additional copies of *hoxA* (Fig. 3, lanes 1 and 3). No immunoreactive protein was observed in HoxA$^-$ strains (Fig. 3, lanes 2 and 4). These results indicate that the antiserum specifically detects the HoxA protein in *A. eutrophus* cells.

Mobility shift experiments with the upstream region of the SH operon. Neither the β-Gal–HoxA fusion protein purified from *E. coli* nor the HoxA moiety released by collagenase treatment showed binding activity in a gel shift assay with the SH promoter region as target sequence. Therefore, we isolated HoxA protein from *A. eutrophus* by immunoaffinity chromatography. The eluted protein preparations were subjected to a standard renaturation protocol (21) and tested for binding to *hox* upstream sequences in mobility shift assays. Various attempts with different target sequences and assay conditions did not reveal any binding activity, suggesting that the harsh elution conditions (pH 2.8 or pH 11.5, 4 M $MgCl_2$, or 6 M guanidinium-HCl) led to an irreversible loss of biological activity (data not shown).

As an alternative method, we tried the following approach. We partially purified HoxA from soluble extracts of *A. eutrophus* wild-type and control strains by heparin-Sepharose chromatography, eluting with buffer containing 1 M KCl. The eluate was then further fractioned with ammonium sulfate at 35% saturation. Western immunoblots showed that HoxA was significantly enriched in the eluate and was quantitatively precipitated by the ammonium sulfate step (data not shown). Aliquots of these protein fractions were screened in mobility shift assays. The target sequence was a ^{32}P-labelled fragment of the *hoxF* upstream region (Fig. 4A). In assays using protein fractions from the wild-type strain H16 and the HoxA-overexpressing strain H16(pGE208), some of the labelled fragment was retarded (Fig. 4B, lanes 2 and 3), whereas the corresponding test with proteins from HoxA-deficient control strains showed no effect (lanes 4 and 5). We also tested increasing amounts of protein from strain H16(pGE208), which contains higher levels of HoxA protein than the wild type does. In assays done with 4 μg of protein, a second band appeared above the first retarded species (Fig. 5, lane 3), indicating the presence of a second type of complex.

Temperature tolerance of *A. eutrophus* HF117. Strain HF117 is a derivative of H16 with a remarkable growth phenotype. Whereas wild-type H16 is unable to grow lithoautotrophically at temperatures above 33°C, strain HF117 thrives at tempera-

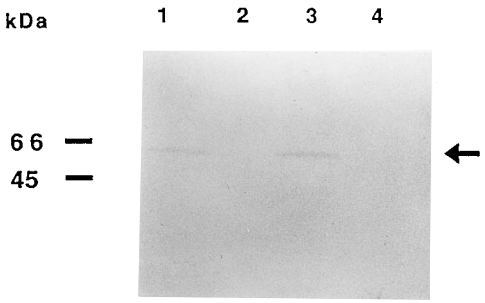

FIG. 3. Expression of HoxA in wild-type and mutant strains of *A. eutrophus*. A Western blot of soluble extracts from the following strains is shown: H16 (lane 1), HF294 (lane 2), H16(pGE208) (lane 3), and HF210(pRK404) (lane 4). A 250-μg amount of protein was loaded in each lane.

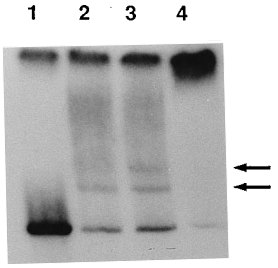

FIG. 5. Mobility shift assay with increasing amounts of extracts enriched for HoxA from H16(pGE208). Protein content per lane was as follows: 1, no protein; 2, 2 μg; 3, 4 μg; 4, 6 μg. The arrows indicate the retarded DNA fragments.

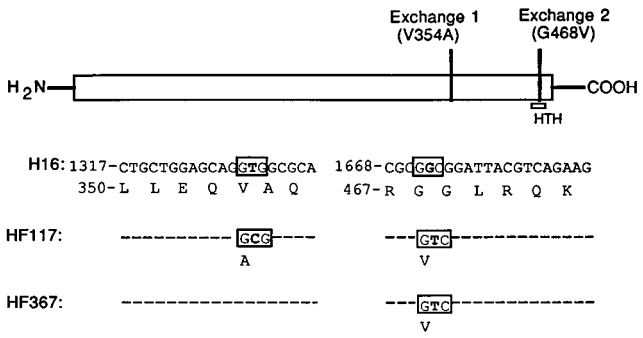

FIG. 6. Comparison of the wild-type and mutant alleles of *hoxA*. The nucleotide sequences of the relevant regions of the wild-type gene (H16) and the deduced amino acid sequences are shown. The relevant codons are boxed, and coordinates are given at the left. The corresponding codons of the mutant alleles of strains HF117 and HF367 and the deduced amino acid exchanges are shown below the wild-type sequence. The large open bar in the upper part of the figure represents the deduced gene product HoxA. The positions of the amino acid exchanges are marked with vertical bars. The helix-turn-helix (HTH) motif is indicated by a small box in the C-terminal region.

tures up to 37°C. Previous studies had shown that this effect is correlated with a mutant *hoxA* allele (12, 13). To investigate the exact basis of the Tr phenotype, we sequenced the *hoxA* allele from HF117. Two point mutations were found, one T→C at position 1330 resulting in a Val→Ala exchange in the protein and one G→T at position 1672 giving a Gly→Val exchange in the helix-turn-helix motif of the deduced gene product (Fig. 6). The rest of the sequence was identical to the published sequence of H16 *hoxA* (12).

To test the hypothesis that the mutation at position 1672 is alone responsible for temperature resistance, a strain of *A. eutrophus* that carried only the G→T exchange at position 1672 of *hoxA* was constructed. The mutation at position 1672 was introduced into strain H16 via gene replacement. Genomic DNA from the resulting clones was isolated, the 3′-terminal region of *hoxA* spanning nucleotides 1330 and 1672 was amplified by PCR, and the PCR products were sequenced. One of the isolates (designated HF367) carrying the mutated sequence was selected for further studies. At 37°C, this strain showed lithoautotrophic growth on hydrogen. These results show that the point mutation in the HoxA helix-turn-helix motif is sufficient to raise the maximum lithoautotrophic growth temperature by as much as 4°C.

In order to test the in vitro binding activity of HoxA from strain HF367, mobility shift assays were performed. Extracts enriched in HoxA were prepared from the wild-type and mutant strains, and aliquots were incubated with a ^{32}P-labelled fragment of the SH promoter region. Analysis by nondenaturing PAGE gave the following results (Fig. 7). Only a weak shift was observed with H16, even with increasing amounts of extract (Fig. 7, lanes 2 to 4). The same amounts of extract from HF117 showed a considerably stronger retardation and the appearance of a second retarded species (Fig. 7, lanes 5 to 7). Extracts from HF367 were comparable in binding activity to the HF117 extracts (Fig. 7, lanes 8 to 10). These findings provide evidence that HoxA is responsible for the observed retardation of the target fragment. Furthermore, they point to the formation of more stable DNA-protein complexes in vitro for HF117 and HF367 and suggest that increased affinity of the mutant HoxA proteins of HF117 and HF367 for the cognate binding sites in vivo is the basis of the temperature-tolerant lithoautotrophic growth. We cannot, however, exclude the possibility that the mutation in question simply alters the stability

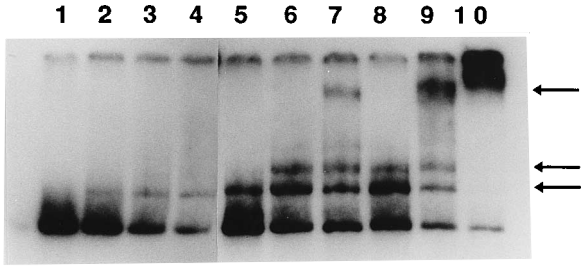

FIG. 7. Gel retardation assay with purified soluble extracts from H16, HF117, and HF367. The extracts were incubated with a ^{32}P-labelled 284-bp fragment of the *hoxF* promoter region. Lanes: 1, no protein added; 2 to 4, extract from H16; 5 to 7, extract from HF117; 8 to 10, extract from HF367. Lanes 2, 5, and 8 contained 2 μg of protein per assay; lanes 3, 6, and 9 contained 4 μg of protein; and lanes 4, 7, and 10 contained 6 μg of protein. The arrows indicate the retarded DNA-protein complexes.

of the mutant protein, thereby increasing the levels of HoxA in the cell and, hence, in the extracts used for the binding assay.

Mobility shift assays in the presence of antibodies against HoxA. To obtain further evidence for specific binding of HoxA, the mobility shift assays with the HoxA preparation from the temperature-tolerant mutant HF117 were repeated in the presence of antibodies against HoxA. Addition of antibody led to the formation of a high-molecular-weight complex which was not visible in the control reaction without antibody (Fig. 8, lane 2). Since this antibody was shown to be specific for HoxA (Fig. 3), we conclude that the formation of a DNA–HoxA–anti-HoxA complex is responsible for the supershift effect.

In vitro binding studies with *E. coli* IHF protein and *hoxF* upstream sequences. The activation of transcription from the *E. coli* σ^{54}-dependent promoters is often enhanced by the histone-like protein IHF. To test for the possible involvement of an IHF-like protein in the activation of transcription from the *hoxF* promoter, we examined the in vitro binding of purified *E. coli* IHF protein to the isolated DNA fragment containing 284 bp of the *hoxF* upstream region. As shown in Fig. 9, small amounts of IHF caused a marked retardation of the target DNA fragment, suggesting the formation of specific protein-DNA complexes.

DISCUSSION

Previous studies showed that the expression of the hydrogen-oxidizing enzymes of *A. eutrophus* is governed by the energy status of the cell (18) and that derepression of these enzymes is mediated at the transcriptional level (44). The available data suggest that transcriptional activation of the *hox*

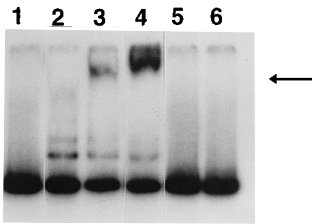

FIG. 8. Mobility shift assay with extracts enriched for HoxA from HF117 in the presence of purified HoxA antibodies. Standard binding reactions were carried out. Subsequently, antibody was added and samples were incubated at 30°C for an additional 45 min. Lanes: 1, no protein; 2, 4 μg of protein; 3, 4 μg of protein and 0.75 μg of anti-HoxA; 4, 4 μg of protein and 1.2 μg of anti-HoxA; 5, 0.75 μg of anti-HoxA; 6, 1.2 μg of anti-HoxA. The supershifted species is indicated by an arrow.

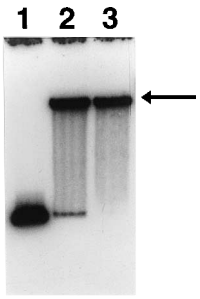

FIG. 9. Mobility shift assay with purified *E. coli* IHF and a DNA fragment containing the SH upstream region. Increasing amounts of protein were incubated together with ^{32}P-labelled fragment and subsequently separated in 5% PAGE gels. Lanes: 1, no protein; 2, 0.01 μg of protein; 3, 0.1 μg of protein. The retarded fragment is indicated by an arrow.

genes proceeds via a molecular mechanism similar to that postulated on the basis of extensive studies of the *glnAp2* promoter of *E. coli* (33, 34, 46, 55). The present study focuses on the promoter region of the operon encoding the *A. eutrophus* SH enzyme and examines two prerequisites for a regulatory model based on the *gln* paradigm: the presence of a positively acting regulatory site upstream of the SH promoter and the capacity of the activator protein HoxA to interact specifically with sequences in the vicinity of the target promoter.

Deletion analysis of the SH operon upstream region in a plasmid-borne gene fusion revealed that sequences between positions −198 and −170 with respect to the transcriptional start are required for wild-type levels of expression, indicating the presence of a regulatory signal in or overlapping the deleted segment. Upstream regulatory elements are a characteristic feature of bacterial σ^{54} promoters (8). These sequences are the binding sites of the cognate activator proteins. In the well-studied enterobacterial systems, the activator binding sites are typically located around −120 but may be as far upstream as −160 (8). Thus, the regulatory sequence identified upstream of the SH operon may well be a binding site for the hydrogenase regulator HoxA. A striking feature of the region highlighted by our deletion analysis is a tandem palindrome. The basic palindromic unit (CAAG-N_{9-10}-CTTG) is reminiscent of the binding sites of other NtrC-type activators (8).

The mobility shift experiments reported here provide the first evidence that HoxA binds to target sequences in the SH promoter region in vitro. Extracts from the *A. eutrophus* wild-type cells and cells harboring additional copies of the *hoxA* gene both showed a retardation of the indicator fragment, whereas extracts prepared from isogenic HoxA$^-$ mutants had no effect. Addition of HoxA-specific antibody to the assay mixture resulted in the formation of another, more strongly retarded complex, indicating that HoxA itself is responsible for the observed retardation.

Interestingly, extracts prepared from the *hox*(Tr) mutant HF117 gave a much stronger retardation than those from the wild-type strain did. This mutant is remarkable for its temperature-tolerant hydrogenase expression, which permits lithoautotrophic growth up to 37°C (13, 19). Increased stability of the complexes formed between HoxA and the recognition sequences upstream of *hox* promoters could provide an explanation for lithoautotrophic growth at elevated temperatures. It seemed likely that the mutation affecting the stability of DNA-protein complexes formed by HoxA would map in a part of the gene corresponding to the DNA binding domain of the protein. In the regulators of the NtrC family, a conserved helix-turn-helix motif is presumed to direct DNA binding (22, 32). This feature is also present in the derived amino acid sequence of *A. eutrophus* HoxA. Sequencing of the mutant *hoxA* allele cloned from mutant HF117 revealed base pair exchanges at positions 1330 and 1672. The latter mutation determines a Gly→Val replacement in the hypothetical recognition helix. While the two neighboring glycine residues in the wild-type protein would be expected to destabilize a helical conformation, thereby resulting in less stable DNA-protein complexes, the replacement of the Gly-468 residue by valine in the mutant HoxA (Fig. 6) should improve the stability of a helical conformation and concomitantly increase the stability of DNA binding. Striking support for this rationalization comes from studies of the closely related lithoautotroph *Alcaligenes hydrogenophilus*. Hydrogenase expression in natural isolates of *A. hydrogenophilus* is strictly H_2 dependent and temperature tolerant (42). Sequencing of the *hoxA* gene of *A. hydrogenophilus* revealed that the derived amino acid sequence contains a valine at the same position as in the mutant HoxA of HF117 (48).

Surprisingly, the *hoxA* allele of HF117 revealed a second base pair exchange at position 1330. This mutation determines a Val→Ala exchange in a nonconserved part of the protein. In order to clarify the respective contributions of the two mutations to the temperature tolerance phenotype, we generated an isogenic HoxA$^-$ mutant containing only the C-terminal mutation at position 1672. This mutant, HF367, was able to grow with hydrogen at 37°C. Extracts from HF367 and HF117 showed the same enhanced binding activity in mobility shift assays.

Taken together, the above results are compatible with the current molecular model for the activation of σ^{54}-dependent promoters. According to this model, direct protein-protein contact between activator and σ^{54} RNA polymerase holoenzyme correlated with looping of the DNA in the promoter region is required for the formation of an open complex. The binding of the histonelike protein IHF has been shown to enhance loop formation and stimulate transcription (8, 23). Our observation that heterologous IHF binds specifically to sequences in the SH promoter region suggests that *A. eutrophus* contains an IHF homolog and that this protein is involved in the activation of *hox* promoters. Two sequence elements (5′-CGTCAAGAATTTC-3′ and 5′-CGACAAGCACTTA-3′) resembling the *E. coli* IHF consensus binding site (15) are located at positions −86 and −67 with respect to the transcription start. IHF binding sites are typically positioned between positions −80 and −40, and a distance of 80 bp from the activator binding site seems to be important (8). The sequence motif at position −86 in the SH operon upstream region is thus a reasonable candidate for the IHF binding site. Recently, an IHF-like protein was identified in the purple phototroph *R. capsulatus*. The purified *R. capsulatus* IHF binds upstream of the *hup* promoter, and hydrogenase activity is significantly reduced in an IHF$^-$ mutant (49, 50). These results document the role of IHF in *hup* gene expression.

Future efforts will aim at precise definition of the HoxA binding site by using purified protein in footprinting experiments.

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