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Roles of HoxX and HoxA in Biosynthesis of Hydrogenase in *Bradyrhizobium japonicum*

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In-frame deletion mutagenesis was used to study the roles of two *Bradyrhizobium japonicum* proteins, HoxX and HoxA, in hydrogenase biosynthesis; based on their sequences, these proteins were previously proposed to be sensor and regulator proteins, respectively, of a two-component regulatory system necessary for hydrogenase transcription. Deletion of the *hoxX* gene resulted in a strain that expressed only 30 to 40% of wild-type hydrogenase activity. The inactive unprocessed form of the hydrogenase large subunit accumulated in this strain, indicating a role for HoxX in posttranslational processing of the hydrogenase enzyme but not in transcriptional regulation. Strains containing a deletion of the *hoxA* gene or a double mutation (*hoxX* and *hoxA*) did not exhibit any hydrogenase activity under free-living conditions, and extracts from these strains were inactive in gel retardation assays with a 158-bp fragment of the DNA region upstream of the *hupSL* operon. However, bacteroids from root nodules formed by all three mutant types (*hoxX*, *hoxA*, and *hoxX hoxA*) exhibited hydrogenase activity comparable to that of wild-type bacteroids. Bacteroid extracts from all of these strains, including the wild type, failed to cause a shift of the hydrogenase upstream region used in our assay. It was shown that HoxA is a DNA-binding transcriptional activator of hydrogenase structural gene expression under free-living conditions but not under symbiotic conditions. Although symbiotic hydrogenase expression is still σ^{54} dependent, a transcriptional activator other than HoxA functions presumably upstream of the HoxA binding site.

Bradyrhizobium japonicum, the slow-growing, nitrogen-fixing symbiont of the soybean plant, expresses a hydrogen uptake hydrogenase that oxidizes hydrogen into its constituent protons and electrons. During symbiosis, the nickel-containing hydrogenase is thought to help improve the efficiency of nitrogen fixation in root nodules by passing H_2-derived electrons to the electron transport chain, thereby recycling energy lost during nitrogen fixation (21). In *B. japonicum*, hydrogenase activity can also be induced under free-living, microaerobic conditions. Most studies of hydrogenase expression in this organism have been done under these conditions (21).

Studies of the regulation of hydrogenase expression have shown that the hydrogenase structural genes, *hupS* and *hupL*, are regulated at the transcriptional level by hydrogen, oxygen, and nickel (14). Subsequently, a 50-bp region from base −99 to base −149 upstream of the transcriptional start site that is necessary for this regulation was identified (16). In addition, the hydrogenase promoter is σ^{54} dependent and also uses integration host factor for full induction (4). Because the same *cis*-acting genetic region is responsible for regulation by the three environmental components, it is thought that the three signals are somehow integrated and passed on to a single activating factor that then binds within the 50-bp regulatory region to affect transcription (15).

Previous sequencing work revealed the presence of two genes, *hoxX* and *hoxA*, approximately 10 kb downstream of the *B. japonicum* hydrogenase structural genes (35). These genes are in an area of the *hup* gene cluster previously known to be necessary for hydrogenase activity under free-living conditions (18) and have been proposed, based on homology to regulatory elements, to encode a two-component regulatory system that affects the transcription of hydrogenase (35). Bacterial two-component regulatory systems form a superfamily, consisting of (i) a family of sensor proteins, all of which have homology in a carboxy-terminal transmitter domain, and (ii) a family of regulator proteins, all of which have homology in an amino-terminal receiver domain (10). Signals from the environment are passed via phosphorylation from the sensor protein(s) to the regulator protein in order to control a wide range of diverse cellular processes, including chemotaxis, symbiosis, sporulation, bacterial pathogenesis, nitrogen metabolism, and responses to osmolarity changes and nutrient deprivation.

The predicted HoxX gene product has extensive homology with the predicted HoxX gene product from *Alcaligenes eutrophus*, which has been postulated to be the sensor protein of the HoxX-HoxA pair (6). However, except for a potential membrane-spanning domain and a histidine kinase domain (24), HoxX has no homology with other known sensors of the NtrB family, which is considered the paradigm of sensor proteins (10). A homolog of HoxX, HypX, has recently been identified in the pea symbiont *Rhizobium leguminosarum*; it contains sequences similar to N_{10}-formyl tetrahydrofolate-dependent enzymes, which are involved in the transfer of one-carbon units, and sequences conserved in the enoyl-coenzyme A hydratase/isomerase family of enzymes (28). HypX has been shown to have a role in posttranslational processing of the hydrogenase enzyme; a possible role in metallocluster assembly has been proposed (28). The high degree of sequence similarity between the predicted HoxX and the predicted HypX suggests that HoxX has a similar (i.e., processing) role in *B. japonicum.*

The predicted HoxA gene product has extensive homologies with transcriptional activators of hydrogenase expression from several other organisms, including HoxA from *A. eutrophus* (61.9% similarity) (6), $HupR_1$ from *Rhodobacter capsulatus* (53.3% similarity) (29), and HydG from *Escherichia coli* (48.7% similarity) (32). Each of these proteins belongs to the family of NtrC-like response regulators (10). Each protein in this family has an invariant aspartic acid residue in the N-terminal end of the protein that is phosphorylated by the cognate sensor pro-

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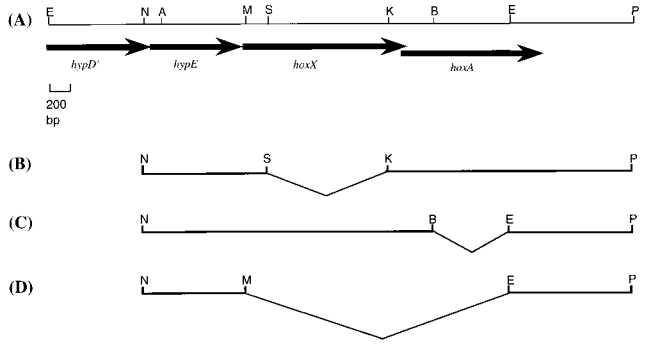

FIG. 1. (A) Restriction map of the region of cosmid pFAJ1010 (35) containing *hoxX* and *hoxA*. (B to D) Maps of the genetic regions deleted in the *hoxX*, *hoxA*, and *hoxX hoxA* mutants, respectively. These constructs were subcloned into the mobilizable vector pLO1 and mated into *B. japonicum* JH. The deletion constructs replaced the normal chromosomal regions via a double recombination event. E, *Eco*RI; N, *Not*I; A, *Apa*LI; M, *Mam*I; S, *Sma*I; K, *Kpn*I; B, *Bam*HI; and P, *Pst*I.

tein, as well as a conserved lysine residue and another aspartic acid residue in this region (10). Interestingly, no protein with homology to HoxA has been identified in *R. leguminosarum*. The hydrogenase enzyme in this organism, however, is not capable of being expressed under free-living, microaerobic conditions. Hydrogenase activity is expressed only under symbiotic conditions, and the hydrogenase genes have been shown to be coexpressed with nitrogen fixation genes (5, 26).

In this work, we show that a HoxX mutant of *B. japonicum* has the same phenotype as a HypX mutant of *R. leguminosarum*. In both organisms, HoxX is involved in posttranslational processing of the hydrogenase enzyme. The *hoxA* gene, which is cotranscribed with *hoxX*, is shown to encode a transcriptional activator of hydrogenase gene expression, but HoxA performs this function only under free-living, microaerobic conditions. Under symbiotic conditions, some other protein is presumably responsible for the activation of transcription from the hydrogenase promoter, as HoxA mutants are fully hydrogenase active in symbiosis.

MATERIALS AND METHODS

Materials. All the glassware used for nickel-free experiments was soaked in 10% nitric acid overnight and rinsed four times with double-deionized water. Controlled pore glass-8-hydroxyquinoline was purchased from Pierce Chemical Company (Rockford, Ill.). High-purity metal salts were purchased from Morton Thiokol, Alfa Products (Danvers, Mass.). Gases were purchased from Specialty Gases, Welder's Supply Company (Baltimore, Md.). Soybean seeds and vermiculite were purchased from Meyer Seed Company (Baltimore, Md.).

All the enzymes used in DNA manipulations were obtained from New England Biolabs, Inc. (Beverly, Mass.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). [α-^{32}P]dATP was purchased from DuPont-NEN (Boston, Mass.). All other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.).

Bacterial strains and plasmids. *B. japonicum* JH, a derivative of strain USDA110, was considered the wild type in these studies. *B. japonicum* JHΔX is a derivative of JH in which a 1.4-kb *Sma*I-*Kpn*I fragment containing a majority of the *hoxX* gene has been deleted and replaced by a 10-bp *Bgl*II DNA linker, creating an in-frame deletion of *hoxX*. Both strains JHΔA and JHΔXA were derived from JH by deleting specific gene fragments and performing blunt-end ligations to keep remaining chromosomal regions in frame. In JHΔA, an 886-bp *Bam*HI-*Eco*RI fragment containing most of the *hoxA* gene was deleted, and in JHΔXA, a 2.8-kb *Mam*I-*Eco*RI fragment containing all of the *hoxX* gene and a majority of the *hoxA* gene was deleted. All of these mutants were constructed via a double recombination event with subclones (Fig. 1) in the double-suicide vector pLO1 (19) and selected as described previously (25). Plasmids pRKX1 and pRKX2 contain a 3.8-kb *Apa*LI-*Bam*HI fragment from cosmid pFAJ1010 (35) that includes the *hoxX* gene as well as 804 bp of DNA upstream of this gene. This fragment was subcloned into vector pRK415 in the opposite (pRKX1) or same (pRKX2) orientation as that of the vector *lac* promoter. Plasmid pRKXA is a 5.2-kb *Not*I-*Pst*I fragment containing DNA from 1 kb upstream of *hoxX* to about 1.1 kb downstream of *hoxA* also subcloned into the broad-host-range vector pRK415 (13). Plasmid pSY7 was used in β-galactosidase transcriptional assays and contains a fragment from base -631 to base $+1649$ of the hydrogenase promoter fused to a promoterless *lacZ* gene (16).

Growth of cells and induction of hydrogenase activity. All strains were grown in modified Bergerson's medium (3). The standard conditions for the induction of hydrogenase activity consisted of incubation for 18 to 20 h in no-carbon medium (22) supplemented with 5 μM nickel under an atmosphere of 84% N_2–10% H_2–5% CO_2–1% O_2. The medium used for the growth and induction of nickel-free cultures was passed through a controlled pore glass-8-hydroxyquinoline column to remove all divalent cations as previously described (7). Ultrapure trace elements were then added.

Hydrogen uptake assay. For free-living cultures, samples of whole cells were assayed amperometrically for hydrogenase activity as previously described (33, 37), with oxygen as the terminal electron acceptor. For bacteroid samples, 1 ml of bacteroid preparation was mixed with 6 ml of deionized water and then assayed amperometrically with oxygen as the terminal electron acceptor.

Preparation of cell extracts. Cell extracts were prepared from 200 ml of cultures incubated as described above. Cultures were centrifuged at 8,281 $\times$ *g*, washed in Dixon's phosphate buffer once, and resuspended in 2 ml of Dixon's buffer with phenylmethylsulfonyl fluoride added to a final concentration of 1 mM. Samples were then passed through a French pressure cell three times at 20,000 lb/in^2. Cellular debris was removed by a low-speed centrifuge spin at 11,950 $\times$ *g* for 10 min.

Gel retardation assays. A *Hin*dIII-*Eco*RI fragment from plasmid pKBB2 (16) encompassing base -221 to base -64 upstream of the transcriptional start site of the hydrogenase structural genes (Fig. 2) was end labeled with [α-^{32}P]dATP in a Klenow fill-in reaction. The probe was purified with a Nuc-trap push column from Stratagene (La Jolla, Calif.). Binding reaction mixtures containing an amount of DNA corresponding to 10,000 cpm of incorporated nucleotide, 3 μg of poly(dI-dC), 10% glycerol, 4 mM Tris [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol, 300 ng of bovine serum albumin per ml, 5 mM $MgCl_2$, 20 mM KCl, and designated amounts of cell extract were carried out in a total volume of 20 μl for 25 min at room temperature. Samples were loaded onto a 1.5% agarose gel made with low-ionic-strength buffer (6.75 mM Tris-HCl [pH 8.0], 3.3 mM sodium acetate [pH 7.9], and 1 mM EDTA [pH 8.0] [2]) and run in the same buffer at 100 V for 2.5 to 3 h. The gel was then dried without heat for 1 h before exposure to autoradiographic film.

β-Galactosidase activity assays. Plasmid pSY7 (16) was mobilized into strains JH, JHΔX, and JHΔA via triparental mating (16). β-Galactosidase transcriptional assays were performed as previously described (23). Cells were permeabilized by the addition of 35 μl of chloroform and 50 μl of 0.1% sodium dodecyl sulfate and incubated at 37°C with *o*-nitrophenyl-β-D-galactopyranoside as the chromogenic substrate. Activities were expressed in Miller units per 10^8 cells.

Immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 25 μg of total cell protein from cell extracts. Samples were boiled for 5 min and separated on an 8% resolving gel. Proteins were electrophoretically transferred to a nitrocellulose membrane as previously described (34). Immunoblotting was carried out as previously described (8) with antibody to the large subunit of *B. japonicum* hydrogenase at a 1:500 dilution.

Growth and inoculation of soybeans. Soybean seeds were surface sterilized by soaking in acidified mercuric chloride as previously described (20). Sterilized seeds were incubated on water agar plates at 30°C for 5 to 7 days. Seedlings were then planted, root down, in prewatered vermiculite. *B. japonicum* strains were grown on modified Bergerson's agar plates. Each *B. japonicum* strain was resuspended in sterile water that had been added to an agar plate, and 0.5 ml of suspension (approximately 10^9 cells/ml) was then inoculated onto each seed. The plants were watered approximately every other day by filling the saucer under-

-221 CGATCCAGCACATCGTGCGCTCGTTCGATCCCT

-188 GCATGGTGTGCACCGCGCACTGACCGGAAACTT

-155 CGTTGC**CGGCTGCAAATCGAATTGTTTCCGCTT**

-122 **CTCGTAATCTCCAGCGTCCTCGTGTA**CCGCTCC

-90 TGCGAGCAATTCAAACACCCAACGTTC

FIG. 2. Sequence of the upstream region of the hydrogenase structural gene operon. This genetic region extending from -64 to -221 bp upstream of the transcriptional start site of the hydrogenase structural genes was used as the probe in gel retardation assays. The 50-bp region implicated in transcriptional regulation is in bold type.

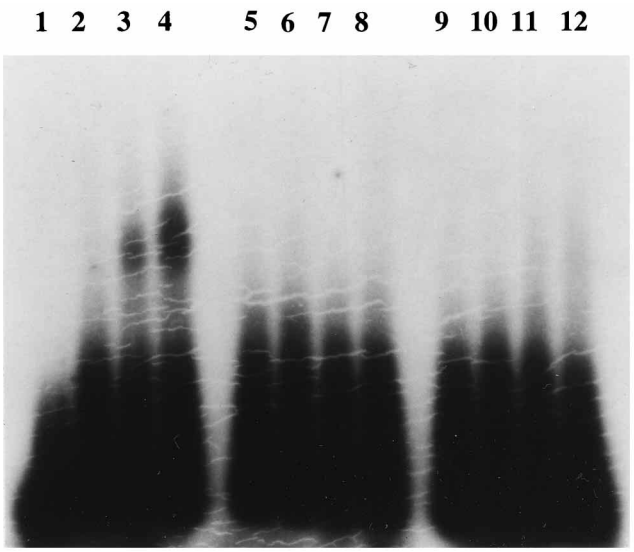

FIG. 3. Nickel- and microaerobic-condition-dependent gel retardation assay. Cultures were grown to mid-log phase and incubated in no-carbon medium for 20 h under the conditions listed below. Extracts were prepared, and binding reactions were carried out as described in Materials and Methods. For lanes 3 through 12, 5 μg of cell extract was used in the first lane of every pair and 10 μg was used in the second lane. Lane 1, free DNA; lane 2, JH (heterotrophically grown); lanes 3 and 4, JH (standard hydrogenase induction conditions); lanes 5 and 6, JH (94% N_2, 5% CO_2, 1% O_2; 5 μM nickel); lanes 7 and 8, JH (85% N_2, 10% H_2, 5% CO_2; 5 μM nickel); lanes 9 and 10, JH (84% N_2, 10% H_2, 5% CO_2, 1% O_2); and lanes 11 and 12, JH (air; 5 μM nickel).

neath each pot with water (12). Once a week, 10 ml of concentrated Jensen's medium (36) was added to saucers.

Preparation of bacteroids and bacteroid extracts. After 5 to 6 weeks of growth, soybean plants were uprooted and nodules were picked off roots. To obtain bacteroids, equal amounts of nodules formed by all strains were weighed out and crushed in 8 ml of 50 mM phosphate buffer with a mortar and pestle (12). The solution was strained through two layers of cheesecloth and centrifuged at 119 × *g* for 1 min to remove plant debris. The supernatant was then centrifuged at approximately 20,000 × *g* for 10 min. The pellet from this spin was resuspended in 10 ml of Dixon's buffer and assayed for hydrogenase activity.

Bacteroid extracts were prepared by centrifuging the bacteroid preparation for 10 min at 12,000 × *g* and resuspending cells in 2 ml of Dixon's buffer with phenylmethylsulfonyl fluoride (1 mM [final concentration]). Samples were passed through a French pressure cell at 20,000 lb/in^2 three times and centrifuged again at approximately 12,000 × *g* for 10 min to remove cell debris.

RESULTS

Nickel-dependent gel retardation. Previously, truncated versions of transcriptional fusions of the *hup* promoter were used to show that a 50-bp area of the promoter region is responsible for the regulation of expression of the hydrogenase structural genes by hydrogen, oxygen, and nickel (16). We have correlated the data from earlier β-galactosidase transcriptional assays of the hydrogenase promoter region with the shift of a 158-bp fragment (Fig. 2) of the upstream region of the hydrogenase structural genes. This fragment encompasses the 50-bp regulatory region previously identified between bases −149 and −99 but does not include either the integration host factor or the σ^{54} binding sites also found upstream of the *hupSL* operon. Cultures grown heterotrophically and then incubated in no-carbon medium under conditions without hydrogen, under anaerobic conditions, or under high-oxygen conditions (20% partial pressure) did not exhibit any hydrogenase activity (data not shown); extracts from these cultures failed to produce a shift of the DNA band corresponding to the hydrogenase upstream region (Fig. 3). In addition, extracts from cultures grown and incubated without nickel (and lacking hydrogenase activity) did not cause a shift of the 158-bp fragment (Fig. 3). Only strain JH incubated under standard hydrogenase activation conditions demonstrated a shift of the upstream region. In order to further establish the shift of this 158-bp fragment as a reliable assay for transcription of the hydrogenase structural genes, we also tested a previously described Hup$^-$ mutant strain, JH47 (11), in gel retardation assays (data not shown). JH47 is a hydrogenase structural gene mutant that has an intact promoter region and thus is transcriptionally active in β-galactosidase assays (14). This strain also caused a shift of the hydrogenase upstream region, as expected.

Mutagenesis of *hoxX*. Based on sequence homology, the *hoxX* gene was originally proposed to encode the sensor protein of a two-component system involved in the regulation of hydrogenase expression. We generated an in-frame deletion of *hoxX* in order to more clearly determine its role in hydrogenase expression. The deletion was constructed with a subclone in vector pLO1 (Fig. 1B), which was mobilized into strain JH and recombined into the chromosome. Assayed for hydrogenase activity under standard activation conditions, strain JHΔX exhibited 30 to 40% of wild-type activity (see below). Like the wild-type activity, strain JHΔX activity was nickel dependent and reached its maximum level at low-nickel (0.1 μM) concentrations (Fig. 4). However, even when JHΔX was derepressed in the presence of up to 100 μM added nickel, its activity never rose above approximately 30% of wild-type hydrogenase activity. Hydrogenase activity was restored to wild-type levels (181 nmol of H_2/h/10^8 cells) in the *hoxX* mutant by the addition of either plasmid pRKX1 (177 nmol of H_2/h/10^8 cells) or pRKX2 (291 nmol of H_2/h/10^8 cells), containing DNA from 800 bp upstream of *hoxX* to the end of the *hoxX* gene subcloned in the opposite and same orientations, respectively, as that of the *lac* promoter on the vector. This indicates that the promoter region for *hoxX* is located within 800 bp upstream of the begin-

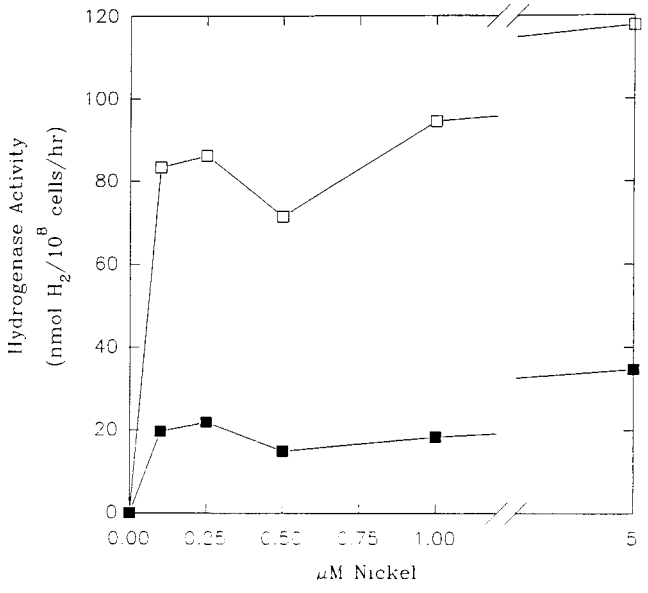

FIG. 4. Nickel titration of wild-type and HoxX mutant strains. Previously nickel-starved wild-type (open) and HoxX mutant (solid) strains were grown to mid-log phase in nickel-free medium and incubated under standard induction conditions, except for the amount of nickel added, for 19 h. The nickel concentrations in the induction period ranged from 0 to 5 μM. Hydrogenase activities were measured with whole-cell samples.

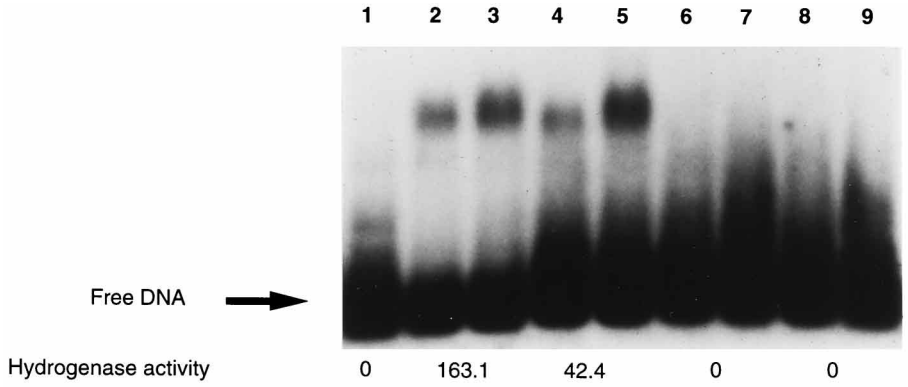

FIG. 5. Activity and gel retardation assays of wild-type and mutant strains. Cultures were grown, and the expression of hydrogenase was induced under standard conditions for 20 h. Hydrogenase activity was measured with cultures of whole cells. Activity data (in nanomoles of H_2 oxidized per 10^8 cells per hour) are averages of three separate experiments. After activity measurement, a gel retardation assay was conducted with cell extracts as described in Materials and Methods. Lane 1, JH (heterotrophically grown); lane 2, JH (2 μg); lane 3, JH (5 μg); lane 4, JHΔX (2 μg); lane 5, JHΔX (5 μg); lane 6, JHΔA (2 μg); lane 7, JHΔA (5 μg); lane 8, JHΔXA (2 μg); and lane 9, JHΔXA (5 μg).

ning of *hoxX*, not at the promoter for the immediately adjacent *hyp* operon, as has been proposed for the *hoxX* homolog *R. leguminosarum hypX* (27).

In a gel shift assay with extracts of the same cultures, a shift of the hydrogenase upstream region was detectable for both wild-type (JH) and mutant (JHΔX) strains (Fig. 5, lanes 4 and 5). The shift by JHΔX extracts did not seem to be noticeably diminished, even though the amount of hydrogenase activity in this strain was much smaller than that in the wild type. In order to quantify the level of *hup* transcription in each strain, plasmid pSY7 (which contains the hydrogenase promoter region in addition to the *hupS* gene and a portion of the *hupL* gene fused to a promoterless *lacZ* gene) was mobilized into each strain and β-galactosidase transcriptional assays were done. As shown in Table 1, the level of transcription in strain JHΔX was the same as that in the wild-type strain, JH. Therefore, the phenotype of the *hoxX* mutant is not due to a decrease in the transcription of the hydrogenase structural genes.

The levels of hydrogenase protein in strain JHΔX were also investigated. Western blots with extracts from JH and JHΔX and with a polyclonal antibody to the 66-kDa large subunit of hydrogenase showed that the unprocessed, inactive form of the large subunit accumulated in the *hoxX* mutant (Fig. 6). In the mutant strain containing the complementing plasmid pRKX2, wild-type levels of the active form of hydrogenase were restored (Fig. 6, lane 5). This indicates that the HoxX protein has a role in processing the hydrogenase enzyme, not in the regulation of transcription. A *hypX* mutant of *R. leguminosarum* also has this phenotype, and a role in processing the hydrogenase enzyme has been proposed for this gene product (28).

TABLE 1. Hydrogenase and β-galactosidase activities[a] of *B. japonicum* wild-type and mutant strains containing the *hup-lacZ* promoter fusion plasmid pSY7

Strain	Hydrogenase activity (nmol of H_2/10^8 cells/h)	β-Galactosidase activity (Miller units/10^8 cells)
JH(pSY7) (aerobic)	0	60 ± 3
JH(pSY7)	501 ± 45	409 ± 9
JHΔX(pSY7)	114 ± 25	589 ± 53
JHΔA(pSY7)	10 ± 9	52 ± 5

[a] Activity data are averages and standard deviations of three separate experiments.

Mutagenesis of *hoxA* and phenotype of a HoxA$^-$ strain under free-living conditions. Based on its sequence, *hoxA* has been proposed to be the DNA-binding regulatory component of a two-component system that transcriptionally regulates hydrogenase expression. We used in-frame deletion mutagenesis to study its exact role in the transcription of the *B. japonicum* hydrogenase structural genes. Two mutants, a *hoxA* deletion mutant and a *hoxX hoxA* double deletion mutant, were constructed by using constructs in vector pLO1 (Fig. 1C and D, respectively). Grown aerobically and subsequently incubated in no-carbon medium under free-living, microaerobic conditions, neither mutant exhibited any hydrogenase activity (Fig. 5, lanes 6 to 9). In complementation studies, a plasmid containing only the *hoxA* gene cannot restore hydrogenase activity

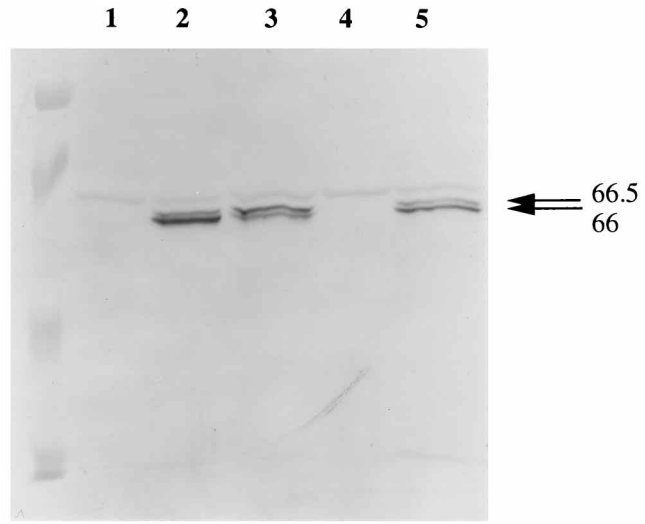

FIG. 6. Western blot of wild-type and mutant cell extracts. Cultures were grown to mid-log phase and, except in one case (lane 1), incubated for 20 h in no-carbon medium under microaerobic conditions. Twenty-five micrograms of each cell extract was electrophoresed on an 8% polyacrylamide gel and transferred to a nitrocellulose filter which was then probed with polyclonal antibody to HupL, the large subunit of hydrogenase. Arrows indicate the positions of the 66.5-kDa unprocessed, inactive form of HupL and the 66-kDa active form of HupL. Lane 1, JH (heterotrophically grown); lane 2, JH; lane 3, JHΔX; lane 4, JHΔA; lane 5, JHΔX(pRKX2).

to strain JHΔA (data not shown). In addition, attempts to complement both the *hoxA* mutant and the *hoxX hoxA* double mutant with plasmid pRKXA, which contains both genes (including 1 kb of DNA upstream of *hoxX*), were not successful. Since these mutations are in-frame deletions, it is unlikely that the inability to restore hydrogenase activity is due to polar effects on genes downstream.

Extracts from strains JHΔA and JHΔXA did not cause a detectable shift of the hydrogenase upstream region in gel retardation assays (Fig. 5, lanes 6 to 9). This indicated that the HoxA protein plays a role in the transcriptional regulation of hydrogenase expression. In order to confirm this role, β-galactosidase assays were performed with strain JHΔA containing *hup-lacZ* plasmid pSY7. As shown in Table 1, the levels of transcription in strain JHΔA were only at background levels, like those of strain JH grown heterotrophically. In addition, no hydrogenase large subunit was detected in extracts from strain JHΔA when Western blot analysis was carried out with antibody to the 66-kDa subunit (Fig. 6, lane 4). Therefore, the HoxA protein is a transcriptional activator of hydrogenase expression under free-living, microaerobic conditions.

Phenotype of *hoxA* mutant under symbiotic conditions. In order to investigate the roles of HoxX and HoxA in hydrogenase regulation under symbiotic conditions, soybean seeds were inoculated with the wild-type strain, JH, as well as each of the deletion mutant strains, JHΔX, JHΔA, and JHΔXA. Bacteroids harvested from the nodules of these plants were then assayed for hydrogenase activity. Bacteroids from all three mutant strains, including JHΔA, which is Hup^- under free-living conditions, exhibited hydrogenase activities (13 ± 2, 22 ± 3, and 15 ± 3 nmol of H_2/min/mg of protein for JHΔX, JHΔA, and JHΔXA, respectively) comparable to that seen in wild-type bacteroids (19 ± 3 nmol of H_2/min/mg of protein). Strains with mutations in the genes for the alternative sigma factor (17) σ^{54} were tested for symbiotic expression of hydrogenase. Bacteroids from each of the single σ^{54} mutants still demonstrated full hydrogenase activity. However, bacteroids from the RpoN1 RpoN2 double mutant did not have detectable hydrogenase activity (data not shown).

Since the HoxA protein does not have a role in the regulation of hydrogenase transcription under symbiotic conditions, we wanted to determine if some other protein binds upstream of the hydrogenase structural genes to affect transcription; such a protein would then cause a different gel shift pattern (i.e., a different size) relative to the shift caused by HoxA. Gel shift assays were carried out with extracts from bacteroids of all three mutant types. None of the bacteroid samples, including wild-type bacteroids, exhibited any shift of the upstream region from base −221 to base −64 (data not shown), even though all of them had some hydrogenase activity. Nevertheless, as the alternative sigma factor σ^{54} is still involved in the regulation of hydrogenase expression under symbiotic conditions, there must be a DNA-binding protein other than HoxA that acts as a symbiotic transcriptional activator. This activator must bind outside (upstream) of the region encompassed by the fragment used as a probe in our gel shift assays.

DISCUSSION

Previous sequencing work and homology searches indicated that the *hoxX* and *hoxA* genes encode sensor and regulator proteins, respectively, of a two-component regulatory system responsible for the regulation of expression of the hydrogenase structural genes. Deletion of the *hoxX* gene resulted in a strain able to express only 30 to 40% of the wild-type hydrogenase activity. This activity could not be recovered by adding excess nickel to the no-carbon medium, but full hydrogenase activity was recovered by the presence of a plasmid containing the *hoxX* gene and 800 bp of upstream sequence. This construct complemented hydrogenase activity to wild-type levels regardless of the orientation of the insert relative to that of the *lac* promoter on the vector, indicating that the promoter region for *hoxX* is located within 800 bp upstream of the start of this gene. This is in contrast to what has been found for the *hypX* gene in *R. leguminosarum*, a *hoxX* homolog that is believed to be transcribed from the promoter for the large *hyp* operon (28). Since the end of the *hypE* gene is only 9 bp from the start of *hoxX*, it is possible that important sequences for *hoxXA* transcription are located within *hypE*. The shift of a 158-bp fragment of the hydrogenase upstream region is a valid assay for transcription of the hydrogenase genes in *B. japonicum* under free-living conditions. This assay has been used as further evidence of transcriptional regulation of the hydrogenase structural genes by hydrogen, oxygen, and nickel.

Though hydrogenase activity was decreased in the HoxX mutant, the level of *hup* transcription in this strain was the same as that in the wild-type strain. HoxX is involved in the formation of active hydrogenase enzyme but not in the transcriptional activation of the hydrogenase promoter, as was previously thought (35).

The *R. leguminosarum hypX* gene product has been shown to be involved in the processing of the large subunit of hydrogenase (28). Western blots of extracts from our *B. japonicum hoxX* deletion mutant showed that as in a *hypX* mutant, the inactive unprocessed form of the large subunit accumulated. The predicted amino acid sequences of *hoxX* and *hypX* are highly homologous to N_{10}-formyl tetrahydrofolate-dependent enzymes and to enzymes in the enoyl-coenzyme A hydratase/isomerase family. These homologies as well as the data presented here indicate that the role of HoxX in the expression of hydrogenase is related to posttranslational processing of this enzyme, possibly metallocluster assembly.

The *B. japonicum hoxA* gene shows extensive homologies to genes encoding several well characterized DNA-binding transcriptional activators that belong to the family of two-component regulatory systems, as well as several known transcriptional activators of hydrogenase in other bacteria. Because of these homologies, it seemed likely that the *hoxA* gene product was the protein binding in the 50-bp regulatory region to affect transcription. Indeed, when *hoxA* was deleted, there was no hydrogenase activity under free-living conditions and no shift of the upstream region of the hydrogenase structural genes was observed. In addition, no hydrogenase transcription occurred in this strain under free-living conditions. These data do not rule out the possibility that another protein, whose expression is controlled by HoxA, binds in the hydrogenase upstream region to affect transcription of the structural genes. When the data are considered in conjunction with the homologies, they strongly indicate that HoxA is indeed a DNA-binding activator for free-living hydrogenase transcription in *B. japonicum*. However, when bacteroids from soybean plants inoculated with the *hoxA* deletion strain were assayed for hydrogenase activity, full activity was observed. This suggests that the HoxA protein is not the transcriptional regulator of hydrogenase expression under symbiotic conditions. Further evidence that HoxA is not a regulator of symbiotic hydrogenase expression came from gel shift assays; bacteroid extracts from both wild-type and *hoxA* mutant strains did not cause a shift of the hydrogenase upstream region we used in gel retardation assays.

There could be several interesting scenarios for this observation. First, it could be that there is no upstream activator for

hydrogenase expression under symbiotic conditions. This is unlikely, however, since the σ^{54} form of RNA polymerase is necessary for hydrogenase expression under both free-living and symbiotic conditions and all σ^{54}-dependent promoters require an activating protein that binds upstream of the transcriptional start site. The second, and more probable, reason that extracts from these bacteroids did not exhibit a shift of the hydrogenase upstream region could be that the binding site for the activator of the hydrogenase genes symbiotically is not entirely included on the probe that we use in gel retardation assays. The DNA-binding protein binds either within 64 bp of the transcription start site or farther upstream than 221 bp from the start site. It is possible that our probe contains only a partial site for such a protein.

In *R. leguminosarum*, hydrogenase is expressed only symbiotically (26). Interestingly, no homolog to HoxA has been found in this bacterium and the hydrogenase genes have been shown to be regulated by the nitrogen fixation regulatory protein NifA (30). We have not found any obvious nitrogen fixation regulatory protein binding motifs, such as NifA sites, upstream of the hydrogenase structural genes. Nevertheless, symbiotic regulation of hydrogenase expression by NifA in *B. japonicum* is a possibility that we are studying. Another protein that could be involved in the regulation of hydrogenase under symbiotic conditions is FixK (1). Despite being homologous to nitrogen fixation regulatory proteins in other bacteria, strains with mutations in this gene are still Nif^+ (1). Nevertheless, Anthamatten et al. (1) have suggested that this protein is involved in the regulation of other oxygen-responsive systems, such as hydrogenase.

Differential control of the expression of hydrogenase in *B. japonicum* makes this system considerably more complicated than previously thought. It is possible that these two mechanisms exist because the environmental signals that activate hydrogenase biosynthesis are different. For instance, under free-living conditions, the main signal for hydrogenase expression may be the absence of a carbon source other than carbon dioxide and the onset of chemoautotrophic growth. In fact, ribulose-biphosphate carboxylase activity is known to be coordinately expressed with hydrogenase activity under free-living conditions, but not in nodules (27, 31). Nitrogenase has been expressed in free-living *B. japonicum* strains with hydrogenase under exceptionally unique conditions (9). Under symbiotic conditions, however, hydrogenase is closely tied to nitrogen fixation and the expression of hydrogenase to remove the hydrogen produced by nitrogen fixation is a key metabolic concern; thus, the main signal for *hup* expression may be most related to *nif* control.

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ADDENDUM IN PROOF

A recent study (C. Van Soom, P. de Wilde, and J. Vanderleyden, Mol. Microbiol. **23:**967–977, 1997) describes *B. japonicum* HoxA as a transcriptional regulator of hydrogenase.

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