JOURNAL OF BACTERIOLOGY, July 1991, p. 3993–3999
0021-9193/91/133993-07$02.00/0
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Vol. 173, No. 13

Common *cis*-Acting Region Responsible for Transcriptional Regulation of *Bradyrhizobium japonicum* Hydrogenase by Nickel, Oxygen, and Hydrogen†

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Received 28 February 1991/Accepted 22 April 1991

***Bradyrhizobium japonicum* expresses hydrogenase in microaerophilic free-living conditions in the presence of nickel. Plasmid-borne *hup-lacZ* transcriptional fusion constructs were used to study the regulation of the hydrogenase gene. The hydrogenase gene was transcriptionally induced under microaerobic conditions (0.1 to 3.0% partial pressure O_2). The hydrogenase gene was not transcribed or was poorly transcribed in strictly anaerobic conditions or conditions above 3.0% O_2. Hydrogen gas at levels as low as 0.1% partial pressure induced hydrogenase transcription, and a high level of transcription was maintained up to at least 10% H_2 concentration. No transcription was observed in the absence of H_2. Hydrogenase was regulated by H_2, O_2, and Ni when the 5′-upstream sequence was pared down to include base number −168. However, when the upstream sequence was pared down to base number −118, the regulatory response to O_2, H_2, and Ni levels was negated. Thus, a common *cis*-acting regulatory region localized within 50 bp is critical for the regulation of hydrogenase by hydrogen, oxygen, and nickel. As a control, the *B. japonicum hemA* gene which codes for δ-aminolevulinic acid synthase was also fused to the promoterless *lacZ* gene, and its regulation was tested in the presence of various concentrations of O_2 and H_2. *hemA-lacZ* transcription was not dependent on levels of Ni, O_2, or H_2. Two different *hup-lacZ* fusions were tested in a Hup$^-$ background, strain JH47; these *hup-lacZ* constructs in JH47 demonstrated dependency on nickel, O_2, and H_2, indicating that the hydrogenase protein itself is not a sensor for regulation by O_2, H_2, or nickel.**

In soybean root nodules, the *Bradyrhizobium japonicum* bacterium undergoes a morphological transformation into N_2-fixing bacteroids. Within the nodule, conditions are microaerobic because of an O_2 diffusion barrier and O_2-binding leghemoglobins; because of this, the O_2 concentration at the bacteroid surface is estimated to be 11 nM (1). Many new proteins are expressed in the O_2-limited nodule environment. In addition to those encoded by the nitrogen fixation genes, a membrane-bound H_2-oxidizing hydrogenase enzyme that utilizes the H_2 evolved by nitrogenase is expressed. The Ni-containing hydrogenase (3, 32, 33) activates H_2 and sends the electrons through an energy-generating respiratory electron transport chain (reviewed in reference 27). Therefore, it is thought to be a component responsible for maintaining efficient N_2 fixation in the nodules.

Studying the regulation of bacteroid hydrogenase in response to environmental stimuli supplied to the root nodules is difficult if not physically impossible. Conditions have been worked out so that *B. japonicum* hydrogenase activity can be induced in the free-living form in an H_2- and CO_2-containing atmosphere (16–18). It is within these controlled circumstances that most hydrogenase regulation studies have been done (16–19, 22, 26, 35, 36).

The extent of supercoiling of the chromosome, DNA gyrase activity, and expression of oxygen-regulated genes appear to be related in some bacteria. Previously, our laboratory provided evidence that DNA gyrase is required for the expression of hydrogenase in free-living *B. japonicum* (26). When gyrase inhibitors such as novobiocin, coumermycin, or nalidixic acid were introduced to the cells at the onset of derepression in a microaerobic environment, hydrogenase was not synthesized. Thus, it was proposed that O_2 regulates hydrogenase at the transcriptional level (26).

Repression of hydrogenase activities by oxygen has been observed in many bacteria. These include *Aquaspirillum autotrophicum* (2), *Alcaligenes latus* (9), *Alcaligenes eutrophus* 17707 (6), *Escherichia coli* (28), and *B. japonicum* (18, 19). Whether this regulation occurs at the transcriptional level in these systems has not been addressed. Another possible regulator of hydrogenase expression in *B. japonicum* is the substrate, H_2. Hydrogenase expression requires derepression of cells in an H_2-containing atmosphere (18, 35). H_2-dependent hydrogenase expression has been observed in other bacteria as well. In the hydrogen bacteria *Alcaligenes hydrogenophilus* (11), *Paracoccus denitrificans* (31), and *Alcaligenes latus* (9) and in the methanotroph *Methylosinus trichosporium* (7), incubation of cultures with high H_2 levels resulted in high hydrogenase activities. Also, enhancement of hydrogenase activities in *Azotobacter vinelandii* has been observed by incubation of the cells with H_2 (29). However, transcriptional regulation by H_2 has not been addressed thus far in any of the above systems.

Plasmid-borne constructs that contain the hydrogenase promoter transcriptionally fused to a promoterless β-galactosidase (β-gal) gene were mobilized into *B. japonicum* to test for transcriptional induction by various environmental stimuli. In a previous report, we demonstrated that Ni was a transcriptional regulator of hydrogenase (14). Here, we report on the critical *cis*-acting sequence necessary for proper regulation by O_2, H_2, and nickel. The results presented here demonstrate that the same *cis*-acting 5′-upstream site is necessary for transcriptional regulation by all three components.

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† Publication no. 1466 from the Department of Biology and the McCollum-Pratt Institute.

TABLE 1. Fusion constructs used in this study

Construct	Hydrogenase gene promoter (reference or source)
pSY7	−650 to +1630 bases (14)
pGK2	−650 to +140 bases (14)
pKG4	Reverse of pGK2 (+140 to −650 bases) (14)
pGN1	−239 to +140 bases (14)
pGHh1	−190 to +20 bases (14)
pGHp1	−168 to +140 bases (14)
pGR1	−118 to +140 bases (14)
pGHf1	−63 to +140 bases (this study)
pGBs3	−48 to +140 bases (this study)
pGBB2	−239 to −83 bases (this study)
pGNSdB	−239 to +140 bases with region −83 to −48 deleted (this study)
δ-Aminolevulinic acid synthase gene promoter	
pBJ3-1	−347 to +382 bases

MATERIALS AND METHODS

Materials. All glassware used for nickel-free cultures were routinely soaked in 2 M nitric acid for at least 12 h and rinsed three or four times with double-deionized water. Controlled pore glass-8-hydroxyquinoline was purchased from Pierce Chemical Co. High-purity metal salts (Puratronic grade) used to make nickel-free trace element stock were purchased from Morton Thiokol, Alfa Products (Danvers, Mass.), and were certified to be 99.99% pure or greater. Puratronic grade nickel was also purchased from the same company. All other chemicals for culture media used were of reagent grade. Gases were purchased from Linde Gases or Potomac Air Gas Co. (Baltimore, Md.).

All enzymes for biochemical manipulation of DNA were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). A Sequenase sequencing kit was purchased from United States Biochemicals (Cleveland, Ohio). ^{35}S-ATP for labeling sequencing reactions was purchased from Dupont, NEN Research Products (Boston, Mass.). Plasmid pKS was purchased from Stratagene (La Jolla, Calif.). 2-Nitrophenyl-β-D-galactopyranoside was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Strains. *B. japonicum* LO is derived from the parent strain USDA DES 122 and is considered to be wild type in this study. Strain JH47 is derived from the parent strain USDA DES 110 with a Tn*5* mutation in the 33-kDa subunit of the hydrogenase gene (13, 14). Its phenotype is Hup$^-$. Fragments of DNA cloned into pKS were harbored in *E. coli* JM101 or XL-1 Blue. DNA fragments cloned into pGD499 to form fusions with *lacZ* were maintained in strain HB101 and subsequently mated into *B. japonicum*.

***lacZ* fusions.** The hydrogenase gene upstream deletion fragments shown in Table 1 were cloned into pGD499, which contains an *E. coli* promoterless *lacZ* gene, to create transcriptional fusions (4, 8, 20). Some of these constructs have been reported previously (14). The deletions were created by searching for convenient restriction sites that will render deletions of upstream regions and leave the 3′ end extended beyond the transcription initiation site. The ends of the fragments were verified by DNA sequence analysis. The fragments were cloned into the polylinker region of the pKS plasmid. Double digestion with *Bam*HI and *Hin*dIII, which flank the inserts of these subclones, produced the fragments with modified ends that were adaptable to the *Bam*HI-*Hin*dIII site of pGD499.

Of special mention are pSY7, pGHh1, and pBJ3-1. Plasmid pSY7, which is a 2.4-kb *Bam*HI-*Pst*I fragment of hydrogenase, covers 650 bases of upstream sequence plus all of the 33-kDa subunit gene and part of the 65-kDa subunit gene. This was cloned into the *Bam*HI-*Hin*dIII site of pGD499, resulting in a transcriptional fusion to β-gal. pGD499 harbors an ampicillin resistance gene and an *E. coli* kanamycin resistance gene promoter which are bound at the 5′ end by *Bam*HI and at the 3′ end by *Hin*dIII sites. This segment of DNA was replaced with the promoter of interest in constructing the fusions. pGHh1 is a fragment which contains −190 bases upstream of the transcription initiation site (30) and 20 bases downstream of it. The transcription initiation site has previously been mapped (30). pBJ3-1 is a 729-base *Sal*I-*Xho*I fragment of the *B. japonicum* δ-aminolevulinic acid synthase gene composed of 347 bases of upstream sequence extending into 382 bases of the transcript cloned into the pGD499 *Bam*HI-*Hin*dIII site (21).

Triparental mating. *E. coli* HB101 cells harboring the fusion constructs were grown to log phase, as well as HB101 cells that harbor the helper plasmid, pRK2013. These two components were mixed with either *B. japonicum* LO or JH47, allowed to conjugate at 30°C on a filter for 6 days, and subsequently plated out on plates containing MB medium plus 50 μg of tetracycline per ml, and the transconjugants were isolated (13).

Growth and derepression media. Growth (modified Bergersen's, MB) (5) and derepression (no carbon) (34) media were treated to remove nickel by passing through a controlled pore glass-8-hydroxyquinoline column, which removes all divalent cations (10). Ultrapure trace elements, including calcium, iron, cobalt, copper, manganese, zinc, and molybdenum, were then added after this purification step.

Growth and derepression conditions. *B. japonicum* harboring promoter fusions was grown aerobically to 3×10^8 to 4×10^8 cells per ml in nickel-free MB medium plus 50 μg of tetracycline per ml with shaking at 150 rpm in a 29°C incubator. They were harvested by centrifugation at 5,000 rpm for 25 min. The pellet was washed once with medium containing no carbon and centrifuged again at 5,000 rpm for 25 min. The pellet was then resuspended in the original volume of medium without carbon. The suspension was supplemented with 5 μM nickel and derepressed for hydrogenase with various amounts of H_2 and O_2 as indicated. The derepression experiments were performed with small culture volumes (10 ml), a large gas phase (240 ml), relatively low cell concentrations (3×10^8 to 4×10^8 cells per ml), and rapid shaking so that rapid gas equilibration with the liquid would occur (19).

For studying H_2 regulation, 10 ml of culture was sparged with N_2 for 15 min in 250-ml stoppered bottle. After sparging, 5% CO_2, 1% O_2, and the indicated amount of H_2 were injected. To prevent the formation of internal pressure, the various gases were injected after an equal volume of nitrogen gas was removed. The bottles were shaken at 150 rpm in a 29°C constant-temperature incubator for 12 h and assayed for both hydrogenase and β-gal activities.

For studying O_2 regulation, 10 ml of culture was sparged with an anaerobic gas mixture which was composed of 10% H_2, 5% CO_2, and 85% N_2. The indicated levels of O_2 were injected (see figures and tables), again after an equal volume of gas was removed so as not to create any pressure within the bottle. These bottles were also shaken at 150 rpm in a 29°C incubator for 12 h and assayed for both hydrogenase and β-gal activities.

Hydrogenase activity assays. Samples of the whole cells were assayed amperometrically for hydrogenase as described previously (22, 37).

β-Gal assays. The β-gal assays were done as described previously (23), with 2-nitrophenyl-β-D-galactopyranoside as the chromogenic substrate. The cells were made permeable by adding 50 μl of chloroform and 35 μl of 0.1% sodium dodecyl sulfate. The reactions were done at 37°C. The units are Miller units, which are expressed per 10^8 cells.

RESULTS

The hydrogenase promoter fragments that were fused to *lacZ* were mobilized into *B. japonicum* LO (wild type) and JH47 (Hup^-). Their induction levels were assayed in response to changes in the O_2 and H_2 concentrations. These data were generated from plasmid-borne fusions and as such could be influenced by the copy number or the DNA context of the plasmid. However, in previous time course assays performed with these *hup-lacZ* fusion constructs, it was clear that β-gal activity was induced in a correlative manner with the endogenous (chromosomal) hydrogenase activity in wild-type cells. Therefore, these plasmid-borne constructs were suitable physiological probes of the strength and specificity of the hydrogenase promoter in response to various environmental parameters.

Regulation by oxygen. *B. japonicum* LO containing the plasmid pSY7, which contains 650 bases of upstream sequence and extends 1630 bases beyond the transcription initiation site, was assayed for β-gal activity from cultures that were grown aerobically, and the O_2 level was adjusted before the start of the 12-h derepression incubation. Under anaerobic conditions, the hydrogenase promoter was not induced (Fig. 1A) (β-gal activity of 29 U). As the controlled environment was adjusted to include more O_2, the promoter was more fully induced. The maximal level (about 200 U) was achieved at 0.2 to 0.4% O_2, but hydrogenase was still actively transcribed when cultures were incubated in O_2 levels above 0.4%. Hydrogenase activities determined directly (19, 37) were also greater when cultures were incubated in O_2 levels up to the 0.2 to 0.4% O_2 range (data not shown). As the atmosphere within the derepression bottles was changed to include levels of O_2 greater than 2% (Fig. 1B), the hydrogenase promoter was strongly repressed such that there was little β-gal activity at 8.0% or greater O_2 partial pressure.

A smaller sequence with which to study this regulation was desirable to ensure that the regulation was indeed due to a 5′-upstream area. Plasmid pGHhl contains 190 bases of upstream sequence that extend 20 bases into the transcribed region (Table 1). Similar to the results with LO(pSY7), LO(pGHhl) induction occurred in the 0.05 to 1% O_2 range (Fig. 1A). As O_2 levels were further increased beyond 2.0 or 3.0% O_2 partial pressure, the β-gal level began to diminish, until at 8.0% O_2, the β-gal activity reached the same low level that was observed in completely anaerobic conditions. From the results of Fig. 1, the hydrogenase promoter appears to be not expressed when cells are under completely anaerobic conditions as well as highly aerobic conditions (O_2 concentrations greater than 8.0%).

There was a concern that because *B. japonicum* is an obligate aerobe, the conditions used in which the amount of added O_2 was very low may provide such limited O_2 levels that gene expression in general may be inhibited. The decrease in β-gal levels would then be due to a decrease in energy levels within the cell rather than to specific transcriptional regulation. To test for this, we used a control gene, *B. japonicum hemA*. *hemA* codes for δ-aminolevulinic acid synthase, which is the first gene in the heme biosynthetic pathway. In a previous work (14), it was demonstrated that the δ-aminolevulinic acid synthase mRNA was synthesized constitutively in the presence of various concentrations of Ni as demonstrated by both RNA hybridization studies and transcriptional fusions. The control plasmid, pBJ3-1, contains 347 bases of upstream sequence fused to β-gal (Table 1). Figure 1 shows that the effect of O_2 levels on the transcriptional activity of pBJ3-1 harbored in LO was minimal. Therefore, the β-gal activity expressed through the fusions appears to truly reflect regulation and not merely limitations of energy levels within the cell.

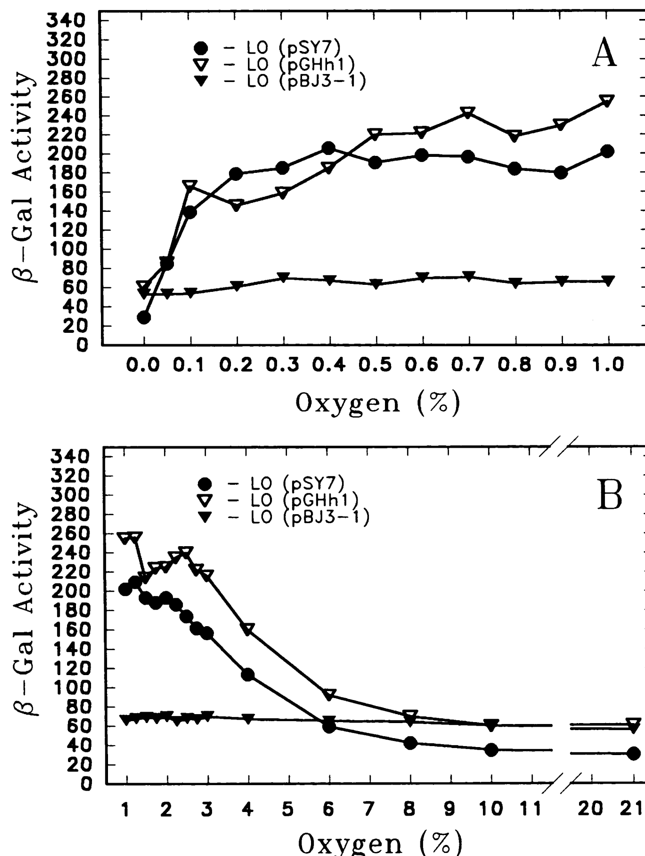

FIG. 1. β-Gal activities of merodiploid strains of *B. japonicum* LO containing pSY7, pGHhl, and pBJ3-1 in response to O_2 levels supplied during the 12-h derepression. The cells were derepressed in the presence of 10% H_2 and 5 μM Ni for 12 h. (A) β-Gal activity response to incremental changes of O_2 concentration from 0% up to 1% partial pressure. (B) β-Gal activity response to higher (1 to 21%) concentrations of O_2. Hydrogenase activities (determined amperometrically) were expressed to the same O_2-dependent levels in all three strains (constructs). Each point in the figure represents the mean of at least three independent assays.

Purified *B. japonicum* hydrogenase can bind both O_2, which inhibits its activity (3), and molecular H_2, which is the physiological substrate. In fact, H_2 binding can result in a conformational change of the enzyme (24). Therefore, the regulatory gas O_2 or H_2 could be sensed in the cell by a basal pool of hydrogenase that is available in noninducing conditions. Therefore, it was possible that hydrogenase could sense these regulators and then activate its own gene. To test

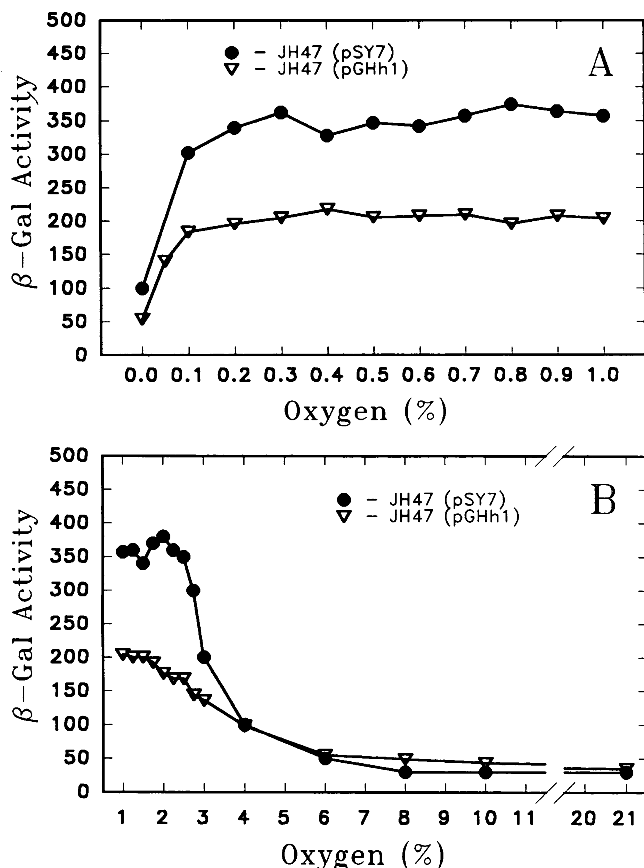

FIG. 2. β-Gal activities of merodiploid strains of *B. japonicum* JH47 containing pSY7 and pGHhl in response to O_2 levels supplied during the 12-h derepression incubation. All conditions were as described in the legend to Fig. 1.

for this possibility, we mobilized the plasmids pSY7 and pGHhl into a mutant strain (JH47) that contains Tn*5* near the 5′ end of the hydrogenase gene. These cells harboring pSY7 and pGHhl were then derepressed as was done for LO (Fig. 1), with variable concentrations of O_2 present during the derepression time period (Fig. 2).

Significant levels of induction were achieved when the O_2 partial pressure in derepressing cultures was raised from 0 to 0.1% for both pSY7 and pGHhl in JH47. The change (increase) in transcriptional activity by incubating cultures in anaerobic conditions versus 0.2% O_2 was about fourfold for both pSY7 and pGHhl (Fig. 2A). From analyzing numerous experiments, it was consistently observed that the onset of strong hydrogenase transcription (β-gal activity) occurred at 0.1% O_2 partial pressure, just as in the wild-type LO background. From the results of Fig. 2, we conclude that hydrogenase per se is not a prerequisite for environmental sensing of O_2 and H_2. Consequently, there must exist a unique factor(s) that physiologically senses these effectors and induces hydrogenase transcription.

The upstream region of the hydrogenase gene was progressively deleted (Table 1) to determine the area required for O_2- and H_2-dependent regulation. The fusion constructs were then mobilized into strain LO. Based on this analysis, both induction by microaerobic conditions and repression by the aerobic state were assigned to the same *cis*-acting area. The fold changes (Table 2) of less than 1.4, encompassing 5′

TABLE 2. Promoter activity response to different O_2 levels provided during derepressions

Fusion construct	Miller units/10^8 cells[a] at an O_2 concn (%) of:						Fold change	
	0	0.5	1.0	4.0	10.0	21.0	Microaerobic induction[b]	Aerobic repression[c]
pSY7	29	191	202	114	35	30	7.0	6.7
pGK2	60	230	260	210	85	54	4.3	4.8
pKG4	67	83	82	79	74	73	1.2	1.1
pGN1	158	480	552	383	162	148	3.5	3.7
pGHhl	61	220	255	160	60	61	4.2	4.2
pGHpl	92	201	202	127	88	89	2.2	2.3
pGR1	80	101	104	91	84	85	1.3	1.2
pGHfl	75	97	103	89	77	82	1.4	1.3
pGBs3	118	157	154	128	117	117	1.3	1.3
pGBB2	63	76	78	77	71	64	1.3	1.2
pGNSdB	136	220	204	156	125	119	1.6	1.7
pBJ3-1	53	63	66	67	60	56	1.2	1.2

[a] The units of activity of the values expressed from the β-gal fusions is in Miller units per 10^8 cells.
[b] The fold change for microaerobic induction is based on comparing 0% O_2 with either 0.5 or 1.0% O_2, whichever is greater.
[c] The fold change for aerobic repression is based on comparing the 21% O_2 added value with the 1% O_2 level.

deletions of −118 and less, were judged to be no longer regulated by O_2. The sequence of DNA up to −168 (pGHpl) was necessary for regulation by O_2, but the sequence of DNA that included bases up to −118 (pGR1) was no longer regulated by O_2. Even though the fold change was only slightly more than twofold in one of the deletion constructs (pGHpl), the experiments were repeated enough times to be confident that this was truly different from the constructs with a shorter upstream region.

When the hydrogenase promoter was inserted in the opposite orientation (pKG4), only an insignificant (about 1.2-fold) change was observed, which is consistent with the unidirectionality of transcription. pGBB2 contains 5′-upstream DNA that does not traverse the transcription initiation site. This construct also exhibited no response to O_2. Interestingly, plasmid pGNSdB containing bases −239 to +140 but with the area from −83 to −48 deleted appeared to be somewhat responsive to O_2-dependent regulation. Thus, the 35-base region from −83 to −48 appears not to be essential for some transcriptional expression but still seems to be important for regulation.

Regulation by hydrogen. It was previously reported that the expression of hydrogenase activity required incubation of cells with H_2 during the hydrogenase induction conditions (18). Figure 3A shows that the H_2 level positively regulated transcription of hydrogenase, as demonstrated by β-gal activities of pSY7 and pGHhl in wild-type *B. japonicum* LO. The hydrogenase promoter was highly sensitive to low levels of H_2; even a partial pressure of 0.05% H_2 significantly induced β-gal activity. Progressively more transcriptional activity was obtained as a function of H_2 concentration up to 1.0% partial pressure (Fig. 3A), with a slight increase in activity above 1.0% partial pressure (Fig. 3B). The maximum fold increase was approximately sevenfold for pSY7 and approximately fivefold for pGHhl. No marked inhibition of hydrogenase transcription was observed at the higher H_2 levels. Chromosomal hydrogenase was monitored during the H_2 incubation inducing conditions and correlated very well with the transcriptional assays (data not shown).

Like the experiments with O_2, it was possible that hydrog-

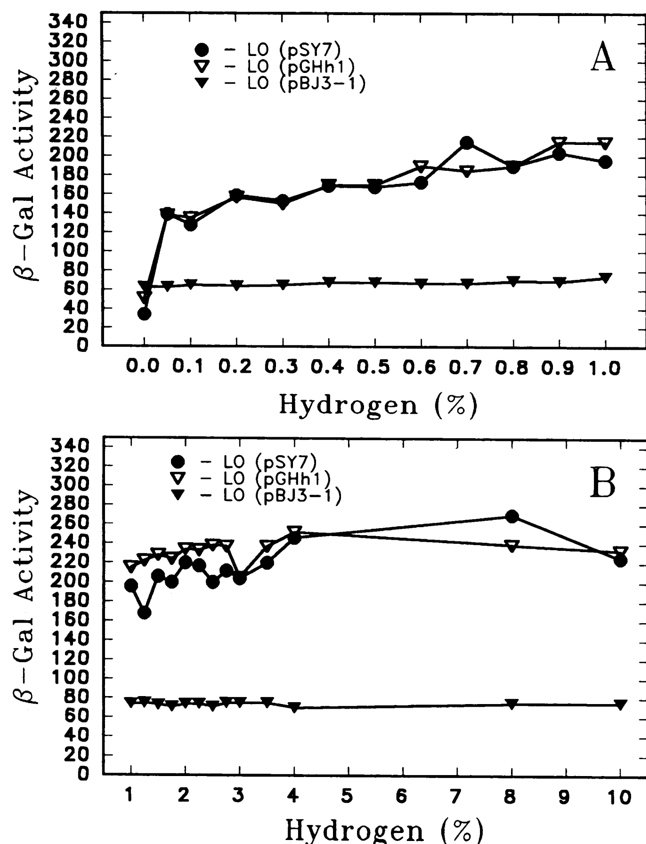

FIG. 3. β-Gal activities of merodiploid strains of *B. japonicum* LO containing pSY7, pGHhl, and pBJ3-1 in response to H_2 levels. LO cells harboring these gene fusion plasmids were derepressed in the presence of 1% O_2, 5 μM Ni, and the indicated H_2 partial pressure for 12 h. (A) Effect of concentrations of H_2 between 0 and 1%. (B) Effect of higher concentrations of H_2 (1 to 10%). Endogenous hydrogenase activity was also monitored amperometrically, and it was expressed to the same H_2-dependent levels in all three strains. Each point in the figure represents the mean of at least three independent assays.

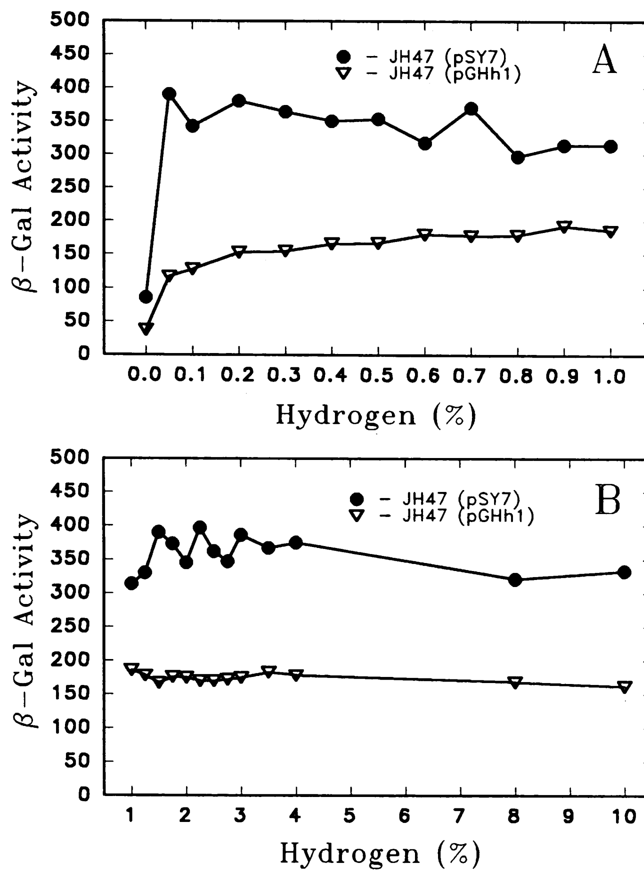

FIG. 4. β-Gal activities of merodiploid strains of *B. japonicum* JH47 containing pSY7 and pGHhl in response to H_2 levels. All conditions are like those described in the legend to Fig. 3, with each point representing the mean of at least three independent assays.

enase itself is the environmental sensor for H_2 levels. Therefore, we tested the transcriptional activity of hydrogenase in the Hup^- strain JH47 (Fig. 4). In the absence of endogenous hydrogenase, pSY7 in JH47 was expressed maximally at 0.05% H_2, which was a fourfold increase (as in LO) from the β-gal activity observed at 0% H_2. pGHhl in the Hup^- background showed a pattern similar to that of pSY7 with respect to H_2 induction (Fig. 4). Data from the fusion constructs placed in the JH47 background indicate that the hydrogenase enzyme plays no noticeable role in regulation of its own gene mediated by either H_2 or O_2.

The same deleted fragments used for the O_2 study were also used to study their response to H_2-dependent regulation (Table 3). Hydrogen gas induced the clones which contained the sequence upstream of and including base number −168 that were necessary for induction by O_2; fusion construct pGHpl extending to −168 was H_2 inducible, but not construct pGR1 (−118 to +140). All the fusions not inducible by H_2 were also not inducible by O_2. Again, as with O_2 as a regulator, pGNSdB, containing an internal deletion between −83 and −48, demonstrated slightly less than a twofold increase in β-gal activity with H_2 compared to that without H_2.

Previously, we reported that the DNA sequence necessary for Ni-dependent induction of hydrogenase comprised up to base −190 and that a fragment containing the 5′-upstream sequence up to base number −168 was not inducible (14). However, the levels of β-gal induction on which to base a

TABLE 3. Promoter activity response to various H_2 levels provided during derepression

Fusion construct	Miller units/10^8 cells[a] at an H_2 concn (%) of:					Fold change[b]
	0	0.5	1.0	4.0	10.0	
pSY7	34	168	196	246	225	7.2
pGK2	65	239	238	248	241	3.8
pKG4	76	84	81	76	77	1.1
pGN1	121	423	447	528	494	4.4
pGHhl	100	279	298	287	270	3.0
pGHpl	82	164	204	197	194	2.5
pGR1	83	98	95	100	95	1.2
pGHfl	69	80	100	94	95	1.4
pGBs3	98	119	131	127	131	1.3
pGBB2	67	70	73	79	76	1.2
pGNSdB	121	203	200	183	187	1.7
pBJ3-1	63	68	74	70	75	1.2

[a] The unit of activity of the values expressed from the β-gal fusions is in Miller units per 10^8 cells.

[b] The fold change is based on comparing 0% H_2 with either 1% or 4% H_2 added, whichever is greater.

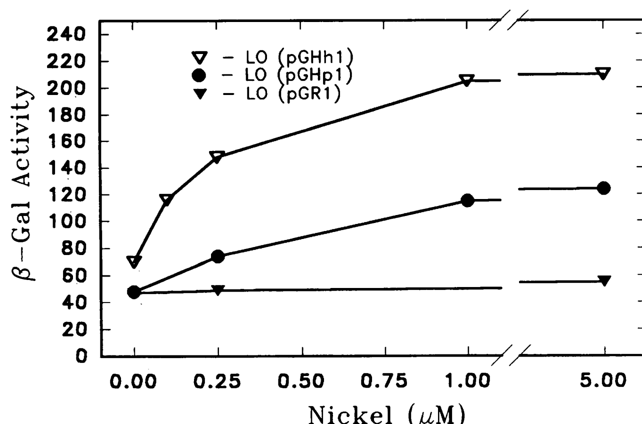

FIG. 5. β-Gal activities of merodiploid strains of *B. japonicum* LO containing pGHh1, pGHp1, and pGR1 in response to exogenous nickel. LO cells harboring these gene fusion plasmids were derepressed in an anaerobic gas mixture containing 1% O_2 and 10% H_2. Endogenous hydrogenase activity was also assayed amperometrically and found to be expressed to the same (Ni-dependent) levels in all three strains. Each point in this figure represents the mean of at least three independent assays.

decision on whether or not expression occurs become equivocal as the window for the necessary *cis*-acting area is progressively narrowed (i.e., deleted). Consequently, upon further experimentation (Fig. 5) with many replicates, we found that the −190 to −168 area is indeed needed for Ni-dependent regulation. pGHh1 and pGHp1 were clearly Ni regulated (Fig. 5), and pGR1 (−118 to +140) clearly was not Ni regulated.

The results with the upstream-deleted subclones show that a common hydrogenase 5′-upstream area exists that is subject to microaerophilic activation, O_2 repression, and H_2 activation. The boundary for this regulatory site is within a window of 50 bases from −168 to −118. The site of Ni-dependent transcriptional regulation is also at this juncture.

DISCUSSION

We determined that O_2 and H_2 regulate hydrogenase transcriptionally. This is the first demonstration of transcriptional regulation of hydrogenase by O_2 or H_2 in any bacterium. From these results combined with recent results on Ni-dependent regulation (14), it is clear that all these conditions—0.2 to 3% O_2, greater than 0.1% H_2 gas, and at least 1 μM Ni—must be present for the hydrogenase gene to be fully expressed. If any one of these three components is entirely absent, the gene is minimally expressed. For example, low O_2 and excess H_2 but an environment lacking nickel resulted in no expression. Conversely, excess H_2 and an abundance of nickel but high O_2 levels resulted in no expression. Similarly, completely anaerobic conditions but with ordinarily inducible levels of H_2 and nickel also were not permissible for hydrogenase transcription. Microaerobic induction, O_2 repression, and H_2- and nickel-dependent transcription all require the same specific upstream area between bases −168 and −118 with respect to transcription initiation. Therefore, these three components appear to regulate the hydrogenase gene through a common *cis*-acting region. The conclusion that the −168 to −118 area is critical does not exclude the probable importance of areas further upstream. That a specific *cis*-acting region is recognized and shared by these three components lends cogency to a regulatory mechanism through a single DNA-binding component.

A simple model to hypothesize how regulation such as this could occur is that the O_2 and H_2 gases, when diffused into the cell, may affect the redox state of Ni atoms bound to a Ni-containing DNA-binding protein, which in turn leads to transcriptional regulation of the hydrogenase message. A signal-transducing pathway (25) is very attractive for Ni-dependent regulation, because a membrane-bound protein could be imagined to be involved in the detection of nickel and a second component could detect the redox-dependent condition within the cell cytoplasm to gauge whether the hydrogenase would be expressed. Oxygen and hydrogen presumably would not require a membrane-bound sensor since they can readily permeate the cell. Ni bound to polypeptides has the capacity to change its valence as it reacts with O_2 or H_2 (15). Valence changes by metalloregulatory enzymes involving O_2 sensing have been proposed (12).

Some spontaneously generated as well as chemically mutagenized mutant *B. japonicum* strains whose hydrogenase expression appears to be free from regulation by nickel, H_2, or O_2 were described previously (22). Mutants that are hypersensitive to repression by O_2 were also described (19). The study of these mutants should present further insight into the mechanism of hydrogenase regulation.

ACKNOWLEDGMENTS

We thank Fred Moshiri and Changlin Fu for helpful discussions. We thank Hyonchu Kim for excellent technical assistance. We are grateful to Gary Ditta for the gift of pGD499. pBJ110 was supplied by the late Barry Chelm.

This work was supported by Public Health Service grant GM40079 (to R.J.M.) from the National Institutes of Health.

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