JOURNAL OF BACTERIOLOGY, Nov. 1994, p. 7102–7106
0021-9193/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 176, No. 22

Sequences and Characterization of *hupU* and *hupV* Genes of *Bradyrhizobium japonicum* Encoding a Possible Nickel-Sensing Complex Involved in Hydrogenase Expression

LORI K. BLACK, CHANGLIN FU, AND ROBERT J. MAIER*
Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218

Received 30 June 1994/Accepted 12 September 1994

A 2.7-kb DNA fragment of *Bradyrhizobium japonicum* previously shown to be involved in hydrogenase expression has been sequenced. The area is located just upstream of the *hupSLCDF* operon and was found to contain two open reading frames, designated *hupU* and *hupV*; these encode proteins of 35.4 and 51.8 kDa, respectively. These proteins are homologous to *Rhodobacter capsulatus* HupU, a possible repressor of hydrogenase expression in that organism. *B. japonicum* HupU is 54% identical to the N terminus of *R. capsulatus* HupU, and HupV is 50% identical to the C terminus of *R. capsulatus* HupU. HupU and HupV also show homology to the [Ni-Fe] hydrogenase small and large subunits, respectively. Notably, HupV contains the probable nickel-binding sites RxCGxC and DPCxxCxxH, which are located in the N- and C-terminal portions, respectively, of the large subunit of hydrogenases. Hydrogenase activity assays, immunological assays for hydrogenase subunits, and β-galactosidase assays on mutant strain JHCS2 (lacking a portion of HupV) were all indicative that HupV is necessary for transcriptional activation of hydrogenase. A physiological role as a possible nickel- or other environmental (i.e., oxygen or hydrogen)-sensing complex is proposed for HupU and HupV.

Hydrogenases, which can catalyze both the uptake and evolution of hydrogen, play key roles in energy metabolism of many bacteria. In nitrogen-fixing bacteria, hydrogenase oxidizes the nitrogenase-evolved hydrogen, generating ATP. This process, called hydrogen cycling, recovers some of the energy lost during the energy-expensive nitrogen fixation reaction. The [Ni-Fe] hydrogenases are a well-conserved family of membrane-bound hydrogenases that function as hydrogen-uptake enzymes (for recent reviews, see references 23, 29, and 32). These heterodimeric proteins, composed of a small subunit (~30 kDa) and a large subunit (~65 kDa), contain nickel and iron-sulfur clusters.

Bradyrhizobium japonicum, the nitrogen-fixing symbiont of soybean, contains a [Ni-Fe] hydrogenase composed of two subunits, a 33-kDa subunit and a 65-kDa subunit (1, 26), encoded by the *hupSL* genes (24). In addition to the structural genes, some other loci on the hydrogenase gene cluster of *B. japonicum* have been found to be involved in hydrogenase processing (9), nickel metabolism (7), and regulation (16, 17, 19). *B. japonicum* SR139, a Hup$^-$ (hydrogen-uptake-deficient) mutant, has been characterized and localized to a region between less than 0.5 kb and 2.5 kb upstream from the transcriptional start site of the small subunit of hydrogenase (22). To further understand this mutant, a 3-kb area spanning this region has been sequenced. Two open reading frames, *hupU* and *hupV*, have been found encoding proteins of 35.4 and 51.8 kDa, respectively. These proteins are homologous to the N and C termini, respectively, of HupU in *Rhodobacter capsulatus* (5), a putative repressor of hydrogenase expression in that organism. Interestingly, HupU and HupV also show homology to the small and large subunits of hydrogenase, respectively. HupV contains the proposed nickel-binding sites (23) found within the large subunit of hydrogenase. Studies of a deletion mutation in *hupV* show that the gene plays a role in transcriptional activation of hydrogenase expression, possibly as a nickel sensor, especially in view of the fact that *B. japonicum* hydrogenase has been shown to be transcriptionally regulated by nickel (16, 17).

Bacterial strains, plasmids, and growth of cells. *B. japonicum* JH (12) is a derivative of USDA 110 and is considered the wild type for these studies. *B. japonicum* JHCS2 is a derivative of JH in which a 3.2-kb *Bam*HI fragment containing the hydrogenase structural genes and regulatory region has been replaced by the spectinomycin (Ω) cassette (10). Plasmid pRY12 (22) is a 7.6-kb *Bgl*II fragment cloned from pSH22 into the broad-host-range vector pRK290 (4) (Fig. 1). Plasmids pLD9 and pLD22 (11) are about 6- and 8-kb *Hin*dIII fragments, respectively, cloned into pVK101 (18), a derivative of pRK290 (Fig. 1). For β-galactosidase assays, the plasmids pSY7 and pBJ3-1 (16) were mobilized into strains JH and JHCS2 as described previously (16). pSY7 is a 2.4-kb *Bam*HI-*Pst*I fragment of hydrogenase including 680 bp of the promoter region cloned into pGD499 (3), creating a *hup-lacZ* transcriptional fusion. pBJ3-1 is a 729-bp *Sal*I-*Xho*I fragment of the *B. japonicum hemA* gene, encoding δ-aminolevulinic acid synthase and cloned into pGD499, creating a *hem-lacZ* transcriptional fusion. This *hem-lacZ* fusion plasmid has been shown to be unregulated by any of the environmental sensors—nickel, hydrogen, or oxygen—and thus serves as our negative control. Strains were grown in modified Bergerson's medium (2), which had been made nickel free by passage through a controlled pore glass-8-hydroxyquinoline column to remove all divalent cations (6). Derepression of hydrogenase activity occurred by incubation in a nickel-free, no-carbon medium (26) for 24 h. Standard conditions include 5 μM nickel and a gas mix of 1% oxygen, 10% hydrogen, and 5% carbon dioxide with the balance as nitrogen.

Sequencing and analysis. A 2.7-kb DNA fragment of pSH22 (14) (Fig. 1) located upstream of the *hupSLCDF* operon (11) was used for generating nested deletions with the exonuclease

* Corresponding author. Phone: (410) 516-8276. Fax: (410) 516-5213. Electronic mail address: maier_rj@jhuvms.hcf.jhu.edu.

FIG. 1. Genetic and physical map of the *hup* gene cluster in cosmid pSH22 (14). The shaded boxes, *hupU* and *hupV*, indicate the two genes sequenced in this study. Plasmid pRY12 (22), a 7.6-kb *Bgl*II fragment, was used to make subclones for sequencing. Plasmids pLD9 and pLD22 (11) are about 6.0- and 8.0-kb *Hin*dIII fragments, respectively, cloned into the broad-host-range vector pVK101. The arrow above the pSH22 map represents the area deleted and replaced by the spectinomycin (Ω) cassette in the mutant JHCS2 (10). The *hupSLCDF* genes are organized as an operon that includes the structural genes of hydrogenase, *hupS* and *hupL*. Other genes involved in hydrogenase activity are not shown on the map. E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII.

III-mung bean nuclease deletion kit from Stratagene (La Jolla, Calif.). Most of the sequencing was done by the DNA Analysis Service of the Johns Hopkins University Core Facility by the fluorescent dideoxy terminator method of cycle sequencing (20, 25). The remaining sequencing was done with the U.S. Biochemical Corp. Sequenase kit (Cleveland, Ohio) with Sequenase version 2.0. Oligonucleotides were synthesized with phosphoramidite chemistry in order to sequence across junctions. Sequencing was carried out on both strands; the region of the *hupU* and *hupV* junction was sequenced twice in each orientation.

Analysis of the sequence of this region revealed two open reading frames, designated *hupU* and *hupV* (Fig. 2), oriented in the same direction as the *hupSL* genes. Potential ribosome binding sites are located 3 bp upstream of the ATG start codon of *hupU* and 6 bp upstream of the *hupV* start codon (Fig. 2). The termination codon of *hupU* overlaps the start site of *hupV* in an ATGA motif, suggesting that these two genes are part of an operon and are likely to be translationally coupled. There is a potential stem-loop structure located at the end of *hupV*, indicating a possible transcription termination point.

B. japonicum HupU and HupV are composed of 338 and 479 amino acids, respectively, with predicted molecular masses of 35.4 and 51.8 kDa. The proteins show homology with the N-terminal and C-terminal portions of *Rhodobacter capsulatus* HupU (5). *B. japonicum* HupU is 54% identical to the N-terminal portion of the *R. capsulatus* HupU protein, while *B. japonicum* HupV is 50% identical to the C-terminal portion of the *R. capsulatus* HupU protein (Fig. 3). The HupU and HupV proteins also show low homology to the small and large subunits, respectively, of [Ni-Fe] hydrogenases, as does *R. capsulatus* HupU. It has been suggested that the *R. capsulatus hupU* gene is an in-frame fusion of the *hupSL* structural genes (5). The best match to HupU and HupV is the [Ni-Fe-Se] hydrogenase of *Desulfovibrio baculatus* (21, 30). The small subunit of *D. baculatus* and HupU are 30% homologous in a 293-amino-acid overlap, and the large subunit of *D. baculatus* is 26.5% homologous to HupV in a 513-amino-acid overlap. The proposed nickel-binding sites RxCGxC and DPCxxCxxH, which are located in the N- and C-terminal portions, respectively, of the large subunit of hydrogenases (23), are also found in HupV and in the corresponding locations within *R. capsulatus* HupU (underlined in Fig. 3).

To determine the role of HupV in hydrogenase expression, a previously constructed mutant, JHCS2 (11), was utilized. This mutant lacks the C-terminal 187 amino acids of HupV as

FIG. 2. Nucleotide and deduced amino acid sequences of *hupU* and *hupV*. Possible ribosome binding sites are underlined. The inverted arrow represents a potential transcription termination loop. The boldface A indicates the transcriptional start site of the *hupSL-CDF* operon (15). The GenBank accession number is L33773.

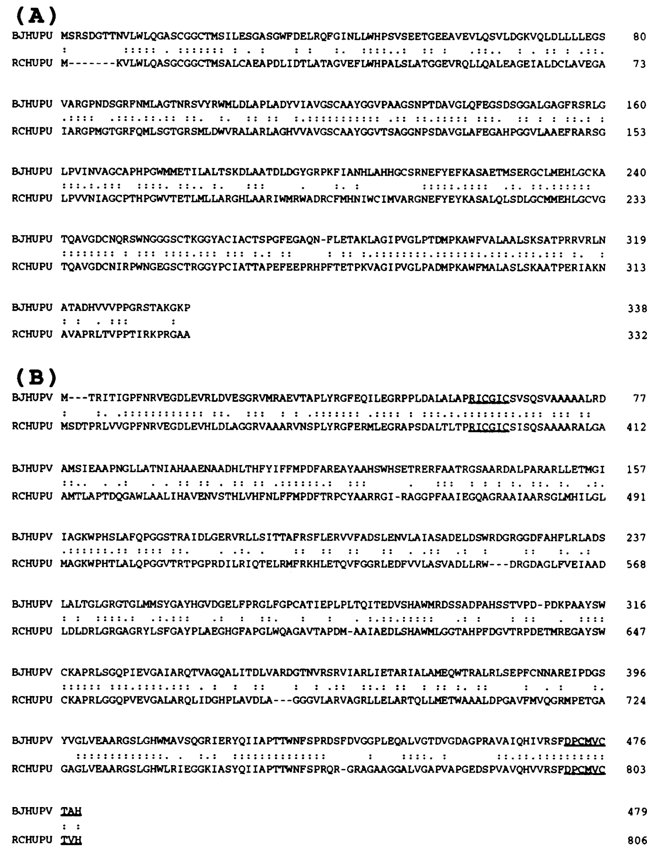

FIG. 3. Alignment of the deduced amino acid sequences of *B. japonicum* (BJ) HupU (A) and HupV (B) with the N-terminal and C-terminal portions of *R. capsulatus* (RC) HupU, respectively. Double dots indicate identical residues, and single dots indicate conservative substitutions. The underlined areas represent the putative nickel-binding sites RxCGxC and DPCxxCxxH of the large subunit of hydrogenase, which are conserved in these proteins.

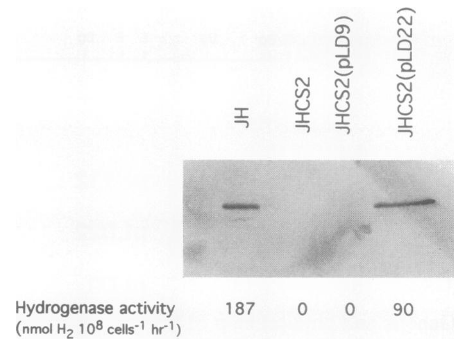

FIG. 4. Immunoblotting and hydrogenase activity of wild-type JH, mutant strain JHCS2, and the mutant strain complemented with pLD9 and pLD22. Western blots of cell extracts were probed with antibody against the large subunit of *B. japonicum* hydrogenase. The hydrogenase activity of whole cells was measured amperometrically (13, 31). The data for the activity assay are averages of data from duplicate experiments.

well as the hydrogenase structural genes. It has previously been shown with mutant JH47 (a Tn*5* insertion into the small subunit of hydrogenase) that *B. japonicum* hydrogenase is not autoregulated (16). Therefore, the JHCS2 mutant can be used to evaluate the role of HupV in regulation of hydrogenase expression.

Mutant analysis. Western blot (immunoblot) analysis (8) with an antibody against the *B. japonicum* hydrogenase large subunit was carried out on wild-type strain JH, mutant strain JHCS2, and JHCS2 containing plasmids pLD9 and pLD22 (Fig. 4). As expected, JHCS2 does not make hydrogenase (Fig. 4, lane 2). Also, this mutant strain does not have any hydrogenase activity, as shown when whole cells were assayed for hydrogen uptake (Fig. 4, lane 2). When complemented with a plasmid, pLD9, that contains the structural genes for hydrogenase but not the gene for HupV, there is still no hydrogen uptake activity and no hydrogenase is made (Fig. 4, lane 3). In contrast, when a plasmid, pLD22, containing the structural genes and the genes for HupUV is mated into JHCS2, hydrogenase is synthesized and there is recovery of 48% of the hydrogenase activity of the wild type (Fig. 4, lane 4). Similar results have been observed with cell extracts for methylene blue-dependent hydrogenase activity (data not shown). These data are in accordance with previous work done on *B. japonicum* SR139, which contained a point mutation of unspecified location within the HupUV locus. This mutant did not have hydrogenase activity, although a small amount of the large subunit was detectable by immunoblots (22). We can conclude from these data that a functional *hupV* gene is necessary for expression of hydrogenase.

Hydrogenase has been shown to be regulated by nickel, hydrogen, and oxygen (16, 17). It was concluded that these three regulators use some common factor(s) to exert their transcriptional regulatory effects. To determine whether hydrogenase transcriptional regulation by these three regulators is affected in the HupV mutant, plasmids pSY7 (*hup-lacZ*) and pBJ3-1 (*hem-lacZ*) were mobilized into strain JH and the mutant strain JHCS2 for the purpose of conducting β-galactosidase assays as described previously (16, 17). In strain JH(pSY7), the promoter region is regulated by nickel (9.4-fold induction), oxygen (22.5-fold induction, followed by 27.4-fold repression under high oxygen), and hydrogen (23.9-fold induction) (Table 1). Strain JHCS2(pSY7) showed no induction of β-galactosidase activity under any of these conditions (Table 1); the Miller units for the mutant correspond to those of the strains containing the control plasmid pBJ3-1 (data not shown). It has previously been shown that the activity of β-galactosidase from the strain containing a mutation in the hydrogenase structural genes, JH47(pSY7), is induced to the same levels as the wild-type strain, JH(pSY7), by all three environmental sensors (16). Thus, we can conclude that HupV is necessary for transcriptional activation of hydrogenase.

It is very doubtful that HupV is the transcriptional activator per se, because it has no DNA-binding motifs and has no homology with previously sequenced transcriptional activators. Also, on the basis of homology with other hydrogenase activators, a possible transcriptional activator of free-living *B. japonicum* hydrogenase, *hoxA*, has been sequenced (27). In *R. capsulatus*, HupU has been suggested to play a role in repression of hydrogenase synthesis (28). A mutation within HupU resulted in constitutive expression of hydrogenase in *R. capsulatus* (28). This is not the case for *B. japonicum* HupUV, because both mutants, SR139 and JHCS2, are Hup$^-$, not Hup^c (constitutive), and as we have shown, JHCS2 has no hydrogenase promoter activity.

It is interesting to note that while both *R. capsulatus* HupU and *B. japonicum* HupU and HupV are homologous to their own hydrogenases, these proteins share much greater homology with the hydrogenase of *D. baculatus*. Although it is

TABLE 1. Comparison of β-galactosidase activity in the wild-type strain and a mutant strain

Strain (plasmid)	β-Galactosidase activity (Miller units)[a] or fold change with the factor:									
	Ni[b]		Fold[e] change	O_2[c]			Fold change	H_2[d]		Fold change
	0 μM	5 μM		0%	1%	20%		0%	10%	
JH(pSY-7)	104	977	9.4	56	1,259	46	22.5	44	1,052	23.9
JHCS2(pSY-7)	44	47	1.1	51	57	56	1.1	40	39	0.9

[a] Units are expressed per 10^8 cells; each number is an average of four separate experiments.
[b] Derepression was done in 1% O_2–5% CO_2–10% H_2 and with the balance of gas as N_2 ± 5 μM Ni.
[c] Derepression was done in variable O_2–5% CO_2–10% H_2 and with the balance of gas as N_2 plus 5 μM Ni.
[d] Derepression was done in 1% O_2–5% CO_2 with variable H_2 and with the balance of gas as N_2 plus 5 μM Ni.
[e] The fold change is based on comparison of the standard condition with its zero counterpart. For example, 5 μM Ni represents the standard condition and is compared with 0 μM Ni.

possible that a gene duplication occurred within each organism, this does not explain the similarity of these proteins to the *D. baculatus* hydrogenase or the similarity of the *R. capsulatus* HupU protein to the *B. japonicum* HupU and HupV proteins. To determine if this has any evolutionary significance awaits further study. With HupU and HupV resembling the [Ni-Fe] hydrogenases, containing potential nickel-binding sites, and the involvement in transcriptional regulation of hydrogenase expression, a role for these proteins as a nickel-sensing complex could be proposed. Another possibility is that the bound nickel could serve as a redox indicator, and this complex could function as an oxygen or hydrogen sensor. Further work involving expressing, purifying, and characterizing the proteins is necessary to determine the exact role they play in regulation of hydrogenase.

We are grateful to Choonbal Yu and Jon Olson for their contribution to part of this work and Tom Ng for computer analysis. We appreciate the help of the Johns Hopkins University Core Facility, especially Roxann Ashworth, for assistance in sequencing.

This work was supported by grant DE-FG02-89ER14011 from the Department of Energy.

REFERENCES

1. **Arp, D. J.** 1985. *Rhizobium japonicum* hydrogenase: purification to homogeneity from soybean nodules and molecular characterization. Arch. Biochem. Biophys. **237**:504–512.
2. **Bishop, P., J. G. Guevarra, J. A. Engelke, and H. J. Evans.** 1976. Relation between glutamine synthetase and nitrogenase activities in the symbiotic associations between *Rhizobium japonicum* and *Glycine max*. Plant Physiol. **57**:542–546.
3. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.-W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13**:149–153.
4. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
5. **Elsen, S., P. Richaud, A. Colbeau, and P. M. Vignais.** 1993. Sequence analysis and interposon mutagenesis of the *hupT* gene, which encodes a sensor protein involved in repression of hydrogenase synthesis in *Rhodobacter capsulatus*. J. Bacteriol. **175**:7404–7412.
6. **Eskew, L., R. M. Welch, and E. E. Cary.** 1984. A simple plant nutrient solution purification method for effective removal of trace metals using controlled pore glass-8-hydroxyquinoline chelation column chromatography. Plant Physiol. **76**:103–105.
7. **Fu, C., S. Javedan, F. Moshiri, and R. J. Maier.** 1994. Bacterial genes involved in incorporation of nickel into a hydrogenase enzyme. Proc. Natl. Acad. Sci. USA **91**:5099–5103.
8. **Fu, C., and R. J. Maier.** 1992. Nickel-dependent reconstitution of hydrogenase apoprotein in *Bradyrhizobium japonicum* Hup[c] mutants and direct evidence for a nickel metabolism locus involved in nickel incorporation into the enzyme. Arch. Microbiol. **157**:493–498.
9. **Fu, C., and R. J. Maier.** 1993. A genetic region downstream of the hydrogenase structural genes of *Bradyrhizobium japonicum* that is required for hydrogenase processing. J. Bacteriol. **175**:295–298.
10. **Fu, C., and R. J. Maier.** 1993. Rapid and efficient selection of recombinant site-directed mutants of *Bradyrhizobium japonicum* by colony hybridization. FEMS Microbiol. Lett. **109**:33–38.
11. **Fu, C., and R. J. Maier.** 1994. Sequence and characterization of three genes within the hydrogenase gene cluster of *Bradyrhizobium japonicum*. Gene **141**:47–52.
12. **Graham, L. A., L. W. Stults, and R. J. Maier.** 1984. Nitrogenase-hydrogenase relationships in *Rhizobium japonicum*. Arch. Microbiol. **140**:243–246.
13. **Hanus, F. J., K. R. Carter, and H. J. Evans.** 1980. Techniques for measurement of hydrogen evolution by nodules. Methods Enzymol. **69**:731–739.
14. **Hom, S. S. M., L. A. Graham, and R. J. Maier.** 1985. Isolation of genes (*nif/hup* cosmids) involved in hydrogenase and nitrogenase activities in *Rhizobium japonicum*. J. Bacteriol. **161**:882–887.
15. **Kim, H., C. Gabel, and R. J. Maier.** 1993. Expression of hydrogenase genes in Hup[c] strains of *Bradyrhizobium japonicum*. Arch. Microbiol. **160**:43–50.
16. **Kim, H., and R. J. Maier.** 1990. Transcriptional regulation of hydrogenase synthesis by nickel in *Bradyrhizobium japonicum*. J. Biol. Chem. **265**:18729–18732.
17. **Kim, H., C. Yu, and R. J. Maier.** 1991. Common *cis*-acting region responsible for transcriptional regulation of *Bradyrhizobium japonicum* hydrogenase by nickel, oxygen, and hydrogen. J. Bacteriol. **173**:3993–3999.
18. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8**:45–54.
19. **Lambert, G. R., A. R. Harker, M. A. Cantrell, F. J. Hanus, S. A. Russell, R. A. Haugland, and H. J. Evans.** 1987. Symbiotic expression of cosmid-borne *Bradyrhizobium japonicum* hydrogenase genes. Appl. Environ. Microbiol. **53**:422–428.
20. **McCombie, W. R., C. Heiner, J. M. Kelly, M. G. Fitzgerald, and J. D. Gocayne.** 1992. Rapid and reliable fluorescent cycle sequencing of double stranded templates. DNA Sequence **2**:289–296.
21. **Menon, N. K., H. D. Peck, Jr., J. Le Gall, and A. E. Przybyla.** 1987. Cloning and sequencing of the genes encoding the large and small subunits of the periplasmic (NiFeSe) hydrogenase of *Desulfovibrio baculatus*. J. Bacteriol. **169**:5401–5407. (Erratum, **170**:4429, 1988.)
22. **Novak, P. D., and R. J. Maier.** 1989. Identification of a locus upstream from the hydrogenase structural genes that is involved in hydrogenase expression in *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. **55**:3051–3057.
23. **Przybyla, A. E., J. Robbins, N. Menon, and H. D. Peck, Jr.** 1992. Structure-function relationships among the nickel-containing hydrogenases. FEMS Microbiol. Rev. **88**:109–136.
24. **Sayavedra-Soto, L. A., G. K. Powell, H. J. Evans, and R. O. Morris.** 1988. Nucleotide sequence of the genetic loci encoding subunits of *Bradyrhizobium japonicum* uptake hydrogenase. Proc. Natl. Acad. Sci. USA **85**:8395–8399.
25. **Smith, L. M., J. Z. Sander, R. J. Kaiser, P. Hughes, C. Dodd, C. R.**

Connel, C. Heiner, S. B. Kent, and L. E. Hood. 1986. Fluorescence detection in automated DNA sequence analysis. Nature (London) **321:**674–679.

26. **Stults, L. W., F. Moshiri, and R. J. Maier.** 1986. Aerobic purification of hydrogenase from *Rhizobium japonicum* by affinity chromatography. J. Bacteriol. **166:**795–800.
27. **Van Soom, C., C. Verreth, M. J. Sampaio, and J. Vanderleyden.** 1993. Identification of a potential transcriptional regulator of hydrogenase activity in free-living *Bradyrhizobium japonicum* strains. Mol. Gen. Genet. **239:**235–240.
28. **Vignais, P. M., and B. Toussaint.** 1994. Molecular biology of membrane-bound H_2 uptake hydrogenases. Arch. Microbiol. **161:** 1–10.
29. **Voordouw, G.** 1992. Evolution of hydrogenase genes. Adv. Inorg. Chem. **38:**397–422.
30. **Voordouw, G., N. K. Menon, J. LeGall, E. S. Choi, H. D. Peck, Jr., and A. E. Przybyla.** 1989. Analysis and comparison of nucleotide sequences encoding the genes for [NiFe] and [NiFeSe] hydrogenases from *Desulfovibrio gigas* and *Desulfovibrio baculatus*. J. Bacteriol. **171:**2894–2899.
31. **Wang, R.** 1980. Amperometric hydrogen electrode. Methods Enzymol. **69:**409–412.
32. **Wu, L.-F., and M. A. Mandrand.** 1993. Microbial hydrogenases: primary structure, classification, signatures and phylogeny. FEMS Microbiol. Rev. **104:**243–270.