Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5099–5103, May 1994
Microbiology

Bacterial genes involved in incorporation of nickel into a hydrogenase enzyme

(nickel-containing enzyme/nickel metabolism/nitrogen fixation)

CHANGLIN FU, SAM JAVEDAN, FARHAD MOSHIRI, AND ROBERT J. MAIER*

Department of Biology, The Johns Hopkins University, Baltimore, MD 21218

Communicated by Christian B. Anfinsen, February 24, 1994

ABSTRACT **Nickel is an essential component of all H_2-uptake hydrogenases. A fragment of DNA that complements a H_2-uptake-deficient but nickel-cured mutant strain (JHK7) of *Bradyrhizobium japonicum* was isolated and sequenced. This 4.5-kb DNA fragment contains four open reading frames designated as ORF1, *hupN*, *hupO*, and *hupP*, which encode polypeptides with predicted masses of 17, 40, 19, and 63.5 kDa, respectively. The last three open reading frames (*hupNOP*) are most likely organized as an operon with a putative σ^{54}-type promoter. Based on its hydropathy profile, HupN is predicted to be a transmembrane protein. It has 56% identity to the previously described HoxN (high-affinity nickel transport protein) of *Alcaligenes eutrophus*. A subclone (pJF23) containing the *hupNOP* genes excluding ORF1 completely complemented (in trans) strain JHK7 for hydrogenase activity in low nickel conditions. pJF26 containing only a functional *hupN* complemented the hydrogenase activity of mutant strain JHK7 to 30–55% of the wild-type level. Mutant strain JHK70, with a chromosomal deletion in *hupP* but with an intact *hupNO*, showed greater activities than pJF26-complemented JHK7 but still had lower activities than the wild type at all nickel levels tested. pJF25, containing the entire *hupO* and *hupP*, but without *hupN* (a portion of *hupN* was deleted), did not complement hydrogenase activity of mutant strain JHK7. The results suggest that the products of the *hupNOP* operon are all involved in nickel incorporation/metabolism into the hydrogenase apoprotein. Based on (previous) nickel transport studies of strain JHK7, the *hupNOP* genes appear not to be involved in nickel transport by whole cells. The deleterious effects on hydrogenase expression are most pronounced by lack of the HupN product.**

Nickel is an essential metal for proper functioning of several microbial enzymes, such as CO dehydrogenase, urease, methylcoenzyme M reductase, and hydrogenase (1). Bacteria sequester nickel via a magnesium transport system and/or a high-affinity nickel-specific transport protein. Nickel-specific transport operons have been reported in *Alcaligenes eutrophus* (2, 3) and *Escherichia coli* (4). Genes involved in intracellular nickel functions for *E. coli* hydrogenase (5) and *Klebsiella aerogenes* urease (6) have recently been described. GTP-binding domains in HypB of *E. coli* were identified and are thought to be important in nickel metabolism (5). Specific functions of the gene products in intracellular nickel metabolism pathways are not known. Identification of additional genes or products would be helpful for understanding bacterial intracellular nickel metabolism.

Bradyrhizobium japonicum, a N_2-fixing symbiont of soybean, possesses a nickel-containing hydrogenase capable of recycling H_2 evolved by the nitrogenase reaction. H_2 is an obligate product of nitrogenase catalysis, and its oxidation is important in regenerating energy for nitrogen fixation. Nickel not only serves as an essential component of the *B. japonicum* hydrogenase enzyme (7) but also affects hydrogenase synthesis in wild-type strains of the bacterium (8). From use of inhibitors of ^{63}Ni uptake in *B. japonicum* JH, it has been suggested that the bulk of nickel is transported via a magnesium transport system (9). A nickel metabolism locus has been identified within the hydrogenase gene cluster of *B. japonicum* (10). A mutation within this locus resulted in strains that require higher levels of nickel than the wild type for hydrogenase activity, and that produce an (apo)hydrogenase deficient in nickel content (10, 11). The mutant strain is not deficient in nickel transport (10). In this study, we have isolated a fragment of DNA which complements the nickel-metabolism locus-deleted mutant strain. Here we report the cloning, sequence,† and characterization of the role of these nickel-metabolism genes from *B. japonicum*. Complementation and mutant analyses indicate that each of the three genes (*hupNOP*) is needed for complete nickel incorporation into the hydrogenase enzyme. *hupO* and *hupP* lack homology to known genes. Of particular comparison interest is the product of *hupN*, which has a predicted 56% identity to the previously described HoxN (high-affinity nickel-specific transport protein) of *A. eutrophus* (3).

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *B. japonicum* JH (12) was derived from strain USDA 110. Strain JHK7 (10) lacked a large area of genetic information (*hupNOP*) (see text) for nickel metabolism. Strain JHK70 is a mutant lacking a 0.6-kb *Eco*RI fragment (within the *hupP* gene) and was created by kanamycin-cassette replacement (13). *E. coli* HB101, XL1-Blue (Stratagene), and S17-1 (14) were used for genetic manipulations.

Cosmid pSH22 was isolated by Hom *et al.* (15) from a *B. japonicum* gene bank that was constructed by Russell *et al.* (16). pSH22 contains 23 kb of *B. japonicum* chromosomal DNA. It complements a variety of Hup$^-$ mutants and has been shown to contain the hydrogenase structural genes and other H_2 uptake-related genes. pJF18-4, containing a 4.5-kb *Bgl* II fragment, was subcloned from a cosmid (map not shown) which was isolated from a *B. japonicum* strain 110 gene bank (17). pJF23, containing the *hupNOP* putative operon, was subcloned from pJF18-4. DNA manipulation, Southern blotting, and conjugation of plasmids from *E. coli* strains into *B. japonicum* were previously described (10).

Growth Conditions. *B. japonicum* JH, JHK7, and JHK70 and mutant strains containing conjugant plasmids were grown in modified Bergersen's (MB) (18) medium at 30°C with shaking at 150 rpm. Tetracycline (Tc) (75 μg/ml) was

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: ORF, open reading frame.

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L24432).

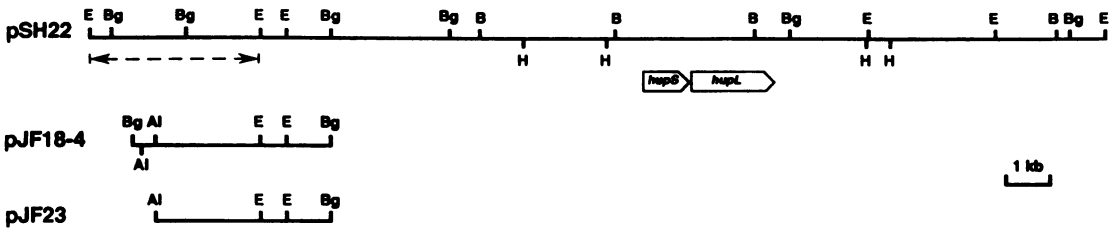

FIG. 1. Restriction map of plasmids pSH22, pJF18-4, and pJF23. *hupS* and *hupL* indicate the hydrogenase structural genes for the small and large subunits, respectively. The dashed-line arrows below pSH22 indicate the region that was found not to be genetically linked to the rest of the DNA on pSH22. Restriction sites: E, *Eco*RI; Bg, *Bgl* II; B, *Bam*HI; H, *Hin*dIII; Al, *Alw*NI.

added if the mutant contained plasmid. Nickel-free growth medium and derepression medium (also nickel-free) were prepared as described (19). Nickel chloride was added to the derepression medium at levels indicated in the figures. *E. coli* cells containing cloned plasmid were grown in LB medium supplemented with antibiotics when necessary (10).

DNA Sequence and Analysis. For DNA sequencing, the 4.5-kb *Bgl* II fragment of pJF18-4 was cloned into the *Bam*HI site of pBluescript II KS(+) (Stratagene) in two orientations, forming pB2 and pB3. For convenient generation of nested deletion mutants, subclones were made from pB2 at appropriate restriction sites. Overlapping nested deletions were generated with the exonuclease III/mung bean deletion kit from Stratagene. Nested deletion clones that were to be used for sequencing were transformed into *E. coli* HB101 or XL1-Blue. Double-stranded template DNA was prepared by use of the Qiagen plasmid kit (Qiagen, Chatsworth, CA) or Magic minipreps (Promega) system. In some cases, single-stranded template DNA was prepared from phagemids by infection of *E. coli* XL1-Blue with helper phage M13 (with the protocol supplied by Stratagene). DNA templates were sequenced by the Core Facility at the Johns Hopkins University, using an automated DNA sequencer (Applied Biosystems). Some sequencing was also done by use of a Sequenase II kit (United States Biochemical) using [α-[^{35}S]thio]dATP (New England Nuclear). In some cases oligonucleotide primers were synthesized and used for sequencing junctions. Both strands were sequenced. The nucleotide sequence data were analyzed with the PC/GENE software package (IntelliGenetics). The deduced amino acid sequences were compared with the Swiss-Prot protein sequence databank (release 26).

Construction of Deletion Clones. In order to understand the roles of each gene product, deletion plasmids were constructed. pJF25 contained *hupO* and *hupP* but without the entire *hupN* gene (a 742-bp *Pvu* I fragment in the *hupN* region was deleted; the deletion of the *Pvu* I fragment in the *hupN* coding region yielded a premature stop codon, but the reading frames of the *hupO* and *hupP* were not affected). pJF26 contained a functional *hupN* only (*hupOP* genes were disrupted after deletion of an *Xho* II–*Aat* II DNA fragment in the *hupO* region). pJF27 contained the entire *hupP* gene only (a 808-bp *Sal* I fragment in the *hupN* and *hupO* regions was deleted without changing the *hupP* reading frame).

Derepression of Hydrogenase and Enzyme Assay. To derepress hydrogenase activity of *B. japonicum* JH or its derived mutants, cells were heterotrophically grown in the nickel-free MB medium until midlogarithmic phase, then harvested and washed in carbon-free, nickel-free derepression medium (10). The pelleted cells were finally resuspended in fresh carbon-free, nickel-free derepression medium to cell densities of 3–5 $\times$ 10^8 cells per ml, and 10-ml aliquots were dispensed into 150-ml serum-stoppered bottles. The appropriate amount of nickel chloride (as indicated in the figures) was added. The bottles were then tightly closed with butyl rubber stoppers and aluminum crimps. The gas phase was adjusted to 1% O_2/5% CO_2/10% H_2/84% N_2 as described (10). The bottles were then incubated at 30°C with shaking at 150 rpm for 20 hr for derepression of hydrogenase activity. Hydrogenase activity was measured amperometrically with O_2 (air) as electron acceptor (10). The cell number was determined after derepression from a standard curve of viable cell number versus optical density at 540 nm. Data points are the average of duplicates.

RESULTS

Isolation of a Clone That Complements the Nickel Metabolism Locus-Deleted Mutant JHK7. We previously identified a locus (0.6-kb *Eco*RI fragment) within the hydrogenase gene cluster (pSH22) of *B. japonicum* that was involved in nickel incorporation into the hydrogenase apoprotein but not involved in nickel transport by cells (10, 11). Since the cosmid (pSH22) did not complement (in trans) mutant strain JHK7 (10), we probed a different gene bank of *B. japonicum* strain 110 (17) by using the 0.6-kb *Eco*RI fragment from pSH22 as a probe. A few positive clones were identified; a 4.5-kb *Bgl* II fragment encompassing the 0.6-kb *Eco*RI fragment from one positive clone was subcloned into the *Bgl* II site of pVK101 (20) (but without the 1.7-kb *cos*-site *Bgl* II fragment), yielding pJF18-4 (Tc^r) (Fig. 1). pJF18-4 was conjugated into the mutant JHK7. Hydrogenase activity was determined in the presence of increasing amounts of nickel. Hydrogenase activity of the mutant (JHK7) was complemented to 94–100% of wild type (JH) by pJF18-4 in the presence of 0.2–5.0 μM nickel (Fig. 2), indicating that pJF18-4 contains all the necessary nickel metabolism genes required for hydrogenase activity.

JHK7 Lacks All Three Nickel Metabolism Genes (*hupNOP*). Because the *Bgl* II fragment of pJF18-4 (4.5 kb) was larger than that covering the presumably same area of pSH22 (Fig. 1), we isolated more clones and subsequently did Southern analysis (data not shown). It was found that a 3.8-kb *Eco*RI fragment on pSH22 (Fig. 1; the dashed-line arrows) was not

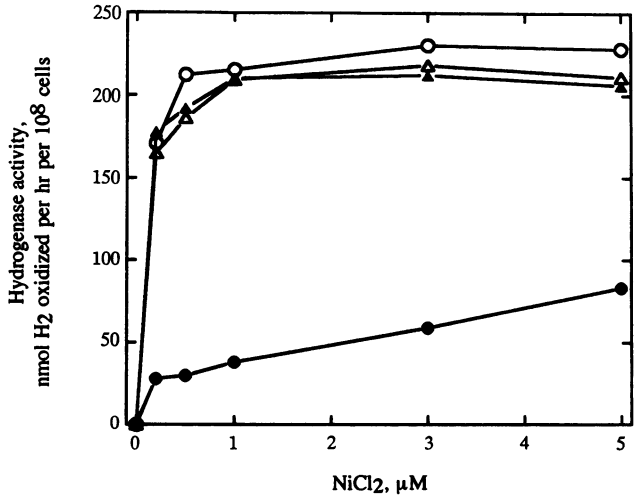

FIG. 2. Derepression of hydrogenase activity in strains JH (○), JHK7 (●), JHK7(pJF18-4) (△), and JHK7(pJF23) (▲) as a function of nickel concentration.

```
Bj  HupN   MLPFSMTGLEKDHTRGVLILANAHRRSERSRTASCAGPAVLFGGLITANIVAWAWAFALF   60
Ae  HoxN                          MFQLLAGVRMNSTGRPRAKIILLYALLIAFNIGAWLCALAAF   42

Bj  HupN   ADRPVVMATALLAWVFGLRHAVDADHIAAIDNVVRSLMQTGGTPRSAGLYFALGHSSVVV  120
Ae  HoxN   RDHPVLLGTALLAYGLGLRHAVDADHLAAIDNVTRKLMQDGRRPITAGLWFSLGHSSVVV  102

Bj  HupN   VATMLLALGVVSLGGD-GLLKEIGSFIGASVSALFLLVIAAINLAIFASLWRTFRKAREQ  179
Ae  HoxN   LASVLIAVMATTLQERLDRFHEVGSVIGTLASALFLFAIAAINLVILRSAYRAFRRVRRG  162

Bj  HupN   GIRDAAGLDALLAHRGILVRLLGPMFRLVTKPWHMYPLGFLFGLGFDTATEIGLLSISAS  239
Ae  HoxN   GIYVEEDFDLLFGNRGFLARIFRPLFRFITRSWHMYPLGMLFALGFDTATEVALLGISTM  222

Bj  HupN   EAARGASLADVMVFPALFAAGMALVDTADSTLMVSAYRWAFVDPMRKLWYNLTITGASVA  299
Ae  HoxN   EASRGVPIWSILVFPALFTAGMALIDTIDSILMCGAYAWAYAKPVRKLYYNMTITFVSAI  282

Bj  HupN   VALFIGGIEALGLIGNRLDLSGGVWTLIDALNESLANVGLAVIALFAIAWLLSIVLYRRL  359
Ae  HoxN   VALIVGGIETLASWPTSSC                                          301

Bj  HupN   IAGSSGLADTEVLECADATEAV                                        381
```

FIG. 3. Alignment of the amino acid sequence of *B. japonicum* HupN with *A. eutrophus* HoxN. Vertical lines indicate identical amino acids, and colons indicate conservative substitutions. Histidines conserved between HupN and HoxN are shown in bold type.

genetically directly linked to the rest of the DNA on pSH22. It is possible that this was due to DNA rearrangement events, but this possibility was not investigated. After sequence analysis, therefore, the previously created mutant strain JHK7 (and similar mutants; see refs. 10 and 11) lacked the entire nickel metabolism operon (*hupNOP*).

DNA Sequence Analysis. To characterize the nickel metabolism genes, we sequenced the entire 4.5-kb *Bgl* II fragment of pJF18-4. Analysis of the nucleotide sequence revealed that there were four open reading frames (ORFs), designated as ORF1, *hupN*, *hupO*, and *hupP*, oriented in the same direction as *hupSL* (hydrogenase structural genes). All ORFs started with ATG and terminated with TGA except for *hupP*, which terminated with TAG. Each of these ORFs was preceded by a putative ribosome binding site.

ORF1 had 474 bp, encoding 158 aa with a predicted mass of 17 kDa. ORF1 was separated by 290 bp from the start codon (ATG) of *hupN*. A possible transcriptional termination loop was present between the end of ORF1 and the beginning of *hupN*. Apparently, ORF1 is located within a different operon. A computer search (Swiss-Prot, release 26) of the ORF1-predicted product did not reveal any highly homologous proteins.

The *hupN*, *hupO*, and *hupP* genes had 1143, 537, and 1752 bp, respectively, encoding proteins with predicted molecular masses of 40, 19, and 63.5 kDa. *hupN* shared 20 bp with *hupO*, whereas *hupO* and *hupP* were separated by 20 bp. Analysis of the 290-bp intergenic space between ORF1 and *hupN* identified a sequence, 5′-CGGATCGCGCATTGCA-3′, at positions 794–809, that was similar to the common σ^{54}-binding consensus sequence 5′-TGGYRYRN4TTGCA-3′ (21).

Most notably, HupN (40 kDa) had 56% identity with the previously described HoxN (high-affinity nickel transport protein) in *A. eutrophus* (3). Analysis by the method of Klein *et al.* (22) showed that the HupN would be classified as an integral membrane protein with six transmembrane segments. Seven histidines, potential nickel ligands, were present in the predicted HupN sequence. Four of those were conserved between HupN and the HoxN protein of *A. eutrophus* (Fig. 3).

HupO (19 kDa) did not show significant homology to any of the gene products in the Swiss-Prot database. Based on the Kyte–Doolittle scale (23), HupO contained an 11- to 15-aa stretch close to the N terminus exhibiting a hydrophobic domain (data not shown). This hydrophobic domain may serve as a membrane-spanning anchor for the protein to the membrane. Possible nickel-binding sites, Cys-Arg-Arg-Cys at the N-terminus and His-Ala-His at the C terminus, were present in the HupO sequence. HupP (63.5 kDa) did not have significant homology to any of the proteins in the Swiss-Prot database. The motifs His-Gln-His, His-Cys, and Cys-Xaa$_9$-Cys were also identified in this protein.

The *hupNOP* Genes Are Required for Insertion of Nickel into the Enzyme. Based on the nucleotide sequences, *hupN*, *hupO*, and *hupP* (excluding ORF1) are most likely organized as an operon and it was possible that only these three products of the locus were involved in incorporation of nickel into the enzyme. To establish this, a 4-kb *Alw*NI–*Bgl* II subclone (containing only *hupNOP*) was constructed in the *Bgl* II-blunted site of pVK101, yielding pJF23 (Figs. 1 and 4). pJF23 was conjugated into the mutant JHK7. Hydrogenase activity was determined after derepression in the presence of various levels of nickel. pJF23, like pJF18-4, almost com-

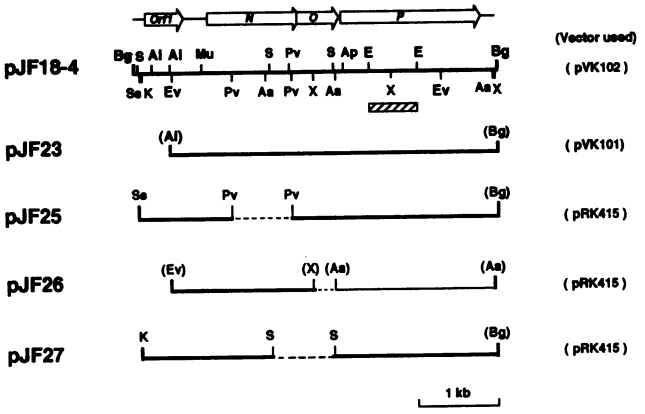

FIG. 4. Restriction map of pJF18-4 and subclones (deletion clones). Organization of the nickel metabolism genes (*hupNOP*) is shown above pJF18-4. The hatched box below pJF18-4 indicates a region that was deleted in mutant strain JHK70. Dashed lines in pJF25, pJF26, and pJF27 indicate the areas deleted to form the constructs. Restriction sites: E, *Eco*RI; Bg, *Bgl* II; S, *Sal* I; Ev, *Eco*RV; K, *Kpn* I; Bs, *Bss*HII; Ap, *Apa* I; H, *Hin*dIII; P, *Pst* I; Sm, *Sma* I; Xb, *Xba* I; N, *Not* I; Sc, *Sac* I; Ss, *Ssp* I; Al, *Alw*NI; Mu, *Mun* I; Pv, *Pvu* I; Aa, *Aat* II; X, *Xho* II. Restriction sites in parentheses indicate the sites which have been modified in the course of cloning and cannot be cut by these enzymes. Not all restriction sites are shown on subclones. pVK102, pVK101, and pRK415 have been described (20, 24).

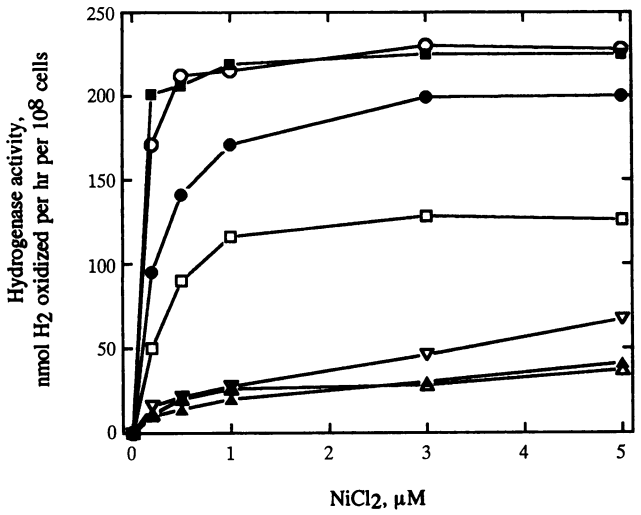

FIG. 5. Derepression of hydrogenase activity from strains JH (○), JHK7 (▽), JHK70 (●), JHK7(pJF25) (△), JHK7(pJF26) (□), JHK7(pJF27) (▲), and JHK70(pJF23) (■).

pletely restored hydrogenase activity of the mutant strain JHK7 to the wild-type level (Fig. 2); this result indicates that the products of the *hupNOP* genes are sufficient to permit incorporation of nickel into the enzyme.

To investigate the roles of each gene product, deletion clones were constructed and conjugated into the mutant strain JHK7 (in which the *hupNOP* operon was deleted) to address trans complementation. pJF27, containing only *hupP* (Fig. 4), did not complement hydrogenase activity of the mutant (Fig. 5). Similarly, pJF25, containing *hupO* and *hupP* but without *hupN* (Fig. 4), did not complement the activity of the mutant (Fig. 5). Activity of mutant strain JHK7 was restored to 30–55% of the wild-type level by pJF26, which has only a functional *hupN* gene (Figs. 4 and 5). Hydrogenase activity of a different mutant strain, JHK70, with a chromosomal deletion in *hupP* but with the entire *hupN* and *hupO* genes was higher than that of the pJF26-complemented JHK7 but still lower than the wild type; this was especially evident at low concentrations of nickel (<0.5 μM). Strain JHK70, lacking *hupP*, was fully complemented to wild-type levels of activity by pJF23 (Fig. 5). The mutant and complementation results indicate that all three genes (*hupNOP*) are important for nickel-dependent activity but that *hupN* plays the most significant role.

DISCUSSION

We have subcloned a 4.5-kb fragment of *B. japonicum* DNA which complemented the previously created nickel metabolism locus-deleted mutant JHK7. Nucleotide sequence analysis of the 4.5-kb *Bgl* II fragment revealed four ORFs (ORF1 and *hupNOP*). The *hupN*, *hupO*, and *hupP* genes are most likely organized as an operon preceded by a putative σ^{54}-type promoter. pJF23, containing *hupNOP* (excluding ORF1), fully complemented the hydrogenase activity of the mutant strain JHK7, indicating that the products of *hupNOP* play a role in nickel metabolism. Further complementation studies indicated that the products of *hupN* and *hupO* (especially HupN) probably play the more crucial roles in this nickel metabolism.

HupN was predicted to be a transmembrane protein with six membrane-spanning segments. Most notably, HupN showed considerable identity (56%) to the previously described HoxN (high-affinity nickel-specific transport protein) of *A. eutrophus*, indicating that some common domains exist in *B. japonicum* HupN and *A. eutrophus* HoxN. This is not surprising, since both proteins interact with nickel. There are seven histidines present in HupN. Four of those are conserved in *B. japonicum* HupN and *A. eutrophus* HoxN. The conserved histidines may act as nickel ligands in both proteins. Conserved histidines in large subunits of hydrogenases from diverse hydrogenase-containing bacteria and in ureases from jack bean and *K. areogenes* have been proposed to be nickel ligands (25, 26). Histidine-rich regions are also proposed to be involved as metal-binding sites for other proteins, such as UreE in *K. aerogenes* (27) and copper-containing hemocyanin (28). Recently, Lee *et al.* (6) purified the UreE protein and demonstrated that it binds to and subsequently provides nickel for urease activity. Neither HupO nor HupP shows significant homology to any of the proteins in the Swiss-Prot database. However, the motifs Cys-Arg-Arg-Cys and His-Ala-His exist in HupO, and these regions could be directly involved in liganding nickel.

Analysis of mutants in these genes and use of various plasmid constructs to complement mutants were useful to conclude that HupN, HupO, and HupP all play roles in nickel metabolism for hydrogenase. A deletion clone, pJF26, harboring just the *hupN* gene complemented hydrogenase activity of the mutant strain JHK7 to 30–55% of the wild-type level, whereas pJF25, containing intact *hupOP* but not *hupN*, was unable to restore appreciable activity to the *hupNOP* deletion strain (JHK7). Similarly, the plasmid pJF27, carrying a deletion in *hupNO*, could not complement strain JHK7. Although the specific deletion of *hupP* showed less effect on hydrogenase activity in response to nickel levels than the deletion of *hupN* and *hupNO*, a role for the HupP protein in maximal expression of hydrogenase activity cannot be eliminated, particularly at very low nickel levels (e.g., 0.1–0.5 μM; see Figs. 2 and 5).

Recently the product of the *hypB* gene from *E. coli* was reported to be required for insertion of nickel into *E. coli* hydrogenase (5). The results suggested that HypB catalyzes donation of nickel to the hydrogenase apoprotein. GTP hydrolysis is proposed to be utilized to reverse the interaction between either HypB or another nickel-binding protein and hydrogenase after the nickel has been incorporated (5). Very recently, we have identified a *hypB* gene homologous to the *E. coli hypB* located ≈5.5 kb downstream from the end of the hydrogenase structural genes of *B. japonicum* (29). *B. japonicum* HypB has an extremely histidine-rich region (24 histidines in a 39-aa stretch) and GTP-binding domains (29). Recently, we have expressed and purified *B. japonicum* HypB protein by chromatography on a nickel-affinity column (data not shown). We propose that HypB binds to and accumulates nickel in a GTP-requiring reaction for its subsequent mobilization into the enzyme. It is possible that HypB and products of *hupNOP* may work together or individually in sequential steps of mobilization of nickel into the hydrogenase enzyme.

We thank Roxann Ingersoll, Tim Raeke, and Elizabeth Nanthakumar (at the Core Facility of the Johns Hopkins University) for assistance with DNA sequence determination. This work was supported by Department of Energy Grant DE-FG02-89ER14011.

1. Hausinger, R. F. (1987) *Microbiol. Rev.* **51**, 22–42.
2. Eberz, G., Eitinger, T. & Friedrich, B. (1989) *J. Bacteriol.* **171**, 1340–1345.
3. Eitinger, T. & Friedrich, B. (1991) *J. Biol. Chem.* **266**, 3222–3227.
4. Navarro, C., Wu, L.-F. & Mandrand-Berthelot, M.-A. (1993) *Mol. Microbiol.* **9**, 1181–1191.
5. Maier, T., Jacobi, A., Sauter, M. & Böck, A. (1993) *J. Bacteriol.* **175**, 630–635.
6. Lee, M. H., Pankratz, H. S., Wang, S., Scott, R. A., Finnegan, M. G., Johnson, M. K., Ippolito, J. A., Christianson, D. W. & Hausinger, R. P. (1993) *Protein Sci.* **2**, 1042–1052.
7. Stults, L., O'Hara, E. & Maier, R. J. (1984) *J. Bacteriol.* **159**, 153–158.

8. Kim, H. & Maier, R. J. (1990) *J. Biol. Chem.* **265**, 18729–18732.
9. Fu, C. & Maier, R. J. (1991) *Appl. Environ. Microbiol.* **57**, 3511–3516.
10. Fu, C. & Maier, R. J. (1991) *Appl. Environ. Microbiol.* **57**, 3502–3510.
11. Fu, C. & Maier, R. J. (1992) *Arch. Microbiol.* **157**, 493–498.
12. Graham, L. A., Stults, L. W. & Maier, R. J. (1984) *Arch. Microbiol.* **140**, 243–246.
13. Fu, C. & Maier, R. J. (1993) *FEMS Microbiol. Lett.* **109**, 33–38.
14. Simon, R., Priefer, U. & Pühler, A. (1983) *Biotechnology* **1**, 784–791.
15. Hom, S. S. M., Graham, L. A. & Maier, R. J. (1985) *J. Bacteriol.* **161**, 882–887.
16. Russell, P., Schell, M. G., Nelson, K. K., Halverson, L. J., Sirotkin, K. M. & Stacey, G. (1985) *J. Bacteriol.* **164**, 1301–1308.
17. O'Brian, M. R. & Maier, R. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3219–3223.
18. Bishop, P. E., Guevarra, J. G., Engelke, J. A. & Evans, H. J. (1976) *Plant Physiol.* **57**, 542–546.
19. Eskew, L. E., Welch, R. M. & Cary, E. E. (1984) *Plant Physiol.* **76**, 103–105.
20. Knauf, V. C. & Nester, E. W. (1982) *Plasmid* **8**, 45–54.
21. Thöny, B. & Hennecke, H. (1989) *FEMS Microbiol. Rev.* **63**, 341–358.
22. Klein, P., Kanehisa, M. & DeLisi, C. (1985) *Biochim. Biophys. Acta* **815**, 468–476.
23. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
24. Keen, N. T., Kobayashi, D. & Trollinger, D. (1988) *Gene* **70**, 191–197.
25. Przybyla, A. E., Robbins, J., Menon, N. & Peck, H. D., Jr. (1992) *FEMS Microbiol. Rev.* **88**, 109–136.
26. Wu, L.-F. (1992) *Res. Microbiol.* **143**, 347–351.
27. Mulrooney, S. B. & Hausinger, R. P. (1990) *J. Bacteriol.* **172**, 5837–5843.
28. Gaykema, W. P. J., Hol, W. G. J., Vereijken, J. M., Soeter, N. M., Bak, H. J. & Beintema, J. J. (1984) *Nature (London)* **309**, 23–29.
29. Fu, C. & Maier, R. J. (1994) *Biochim. Biophys. Acta* **1184**, 135–138.