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A Genetic Region Downstream of the Hydrogenase Structural Genes of *Bradyrhizobium japonicum* That Is Required for Hydrogenase Processing

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Deletion of a 2.9-kb chromosomal *Eco*RI fragment of DNA located 2.2 kb downstream from the end of the hydrogenase structural genes resulted in the complete loss of hydrogenase activity. The normal 65- and 35-kDa hydrogenase subunits were absent in the deletion mutants. Instead, two peptides of 66.5 and 41 kDa were identified in the mutants by use of anti-hydrogenase subunit-specific antibody. A hydrogenase structural gene mutant did not synthesize either the normal hydrogenase subunits or the larger peptides. Hydrogenase activity in the deletion mutants was complemented to near wild-type levels by plasmid pCF1, containing a 6.5-kb *Bgl*II fragment, and the 65- and 35-kDa hydrogenase subunits were also recovered in the mutants containing pCF1.

Hydrogen is produced as a by-product of nitrogenase activity, and it represents a loss of energy from the N_2-fixing system. The evolved H_2 can be recycled by uptake hydrogenases present in many types of aerobic N_2-fixing bacteria, including *Bradyrhizobium japonicum*. The hydrogenase of *B. japonicum* has been purified and consists of two subunits with molecular masses of 65 and 33 to 35 kDa (1, 26). The structural genes of *B. japonicum* are encoded in a single operon (23). Cosmids pHU1 (11) and pSH22 (13), isolated by two groups, contain essential Hup determinants (including hydrogenase structural genes), which complement Hup^- strains of *B. japonicum*. In *Alcaligenes eutrophus* (6, 16), *Rhodobacter capsulatus* (3, 27), *Rhizobium leguminosarum* (12), *Escherichia coli* (19, 21), *Azotobacter vinelandii* (18, 22), and *A. chroococcum* (5), besides hydrogenase structural genes, a number of other genetic loci are somehow involved in H_2 oxidation. In *B. japonicum*, although areas downstream of the hydrogenase structural genes have been proposed to be involved in H_2 uptake activity (11), nothing is known about their organization and functions. In this study, we constructed and characterized mutations in these regions. The results suggest that the 6.4-kb downstream fragment region adjacent to the hydrogenase structural genes is involved in hydrogenase processing in *B. japonicum*.

(A preliminary report of this work was presented at the 92nd General Meeting of the American Society for Microbiology, 26 to 30 May 1992, New Orleans, La. [8a].)

Bacterial strains, growth, and membrane preparation. *B. japonicum* JH is a derivative of USDA 110 (10) and expresses hydrogenase activity under derepression conditions in the presence of Ni (7, 10, 25). *B. japonicum* JH101 and JH103 are constitutive hydrogenase [Hup(Con)] mutants isolated from strain JH (10). These strains were used as wild-type strains for site-directed mutagenesis. *B. japonicum* was grown in modified Bergersen's medium (7). Hydrogenase activity was derepressed by incubation of cells (5×10^8 cells per ml) in an H_2-CO_2 atmosphere containing low O_2 levels in a non-carbon-containing medium (7) with $MgCl_2$ omitted. DNA isolations and Southern blotting were performed as described before (7, 8). For membrane preparation (see Fig. 3), cells were grown heterotrophically or derepressed for hydrogenase activity in the presence of 5 μM nickel. The cells were harvested by centrifugation and broken by passage through a French pressure cell (8). The crude extracts were centrifuged at 12,000 × *g* for 20 min to remove unbroken cells. The supernatant solution (cell extract) was again centrifuged at 115,000 × *g* (4°C) for 3 h to pellet membranes. The membrane pellets were resuspended in 50 mM potassium buffer containing 1 mM phenylmethylsulfonyl fluoride and homogenized in an ice-cold ground-glass homogenizer. Protein samples were electrophoresed on a 10 or 12.5% sodium dodecyl sulfate-polyacrylamide gel, and protein transfer (onto a Nytran membrane) and immunoblotting were carried out as previously described (8). Plant infection and isolation of bacteroids from nodules were done as described elsewhere (14). Hydrogenase activity (H_2 uptake) was determined amperometrically with O_2 (air) as the electron acceptor (7). Protein was determined by using a bicinchoninic acid protein kit (8) or the modified Lowry method (17).

Creation of site-directed mutations and complementation of hydrogenase activity in the mutants. To investigate the property of a region downstream of the hydrogenase structural genes of *B. japonicum*, we constructed site-directed mutations at this locus in both wild-type JH and Hup(Con) strains (JH101 and JH103) (10). For creation of site-directed mutations, a 6.4-kb *Bgl*II fragment located adjacent to the hydrogenase structural genes on pSH22 (Fig. 1) was isolated and cloned into the *Bgl*II site of *Eco*RI-deleted pRK290 (broad-host-range vector) (4), yielding pCF1 (Fig. 1). The 2.9-kb *Eco*RI fragment (within the 6.4-kb *Bgl*II fragment) of pCF1 was replaced by a 1.2-kb *Eco*RI fragment of the kanamycin resistance gene from pUC4-KISS (2), yielding pFM1, and the 2.9-kb *Eco*RI fragment of pCF1 was cloned into the *Eco*RI site of pRK290, yielding pCF2-2 (Fig. 1). The 4.7-kb *Bgl*II fragment (containing the Kan^r gene) of pFM1 was isolated and cloned into the *Bgl*II site of suicide vector pSUP202M (which was derived from pSUP202 [24] by inserting a *Bgl*II linker into the *Pst*I site of pSUP202), resulting in pSMK1. pSMK1 conferred kanamycin and tetracycline resistance. pSMK1 was transformed into a mobilizing strain of *E. coli*, S17-1 (24), and conjugated to *B. japonicum* JH and Hup(Con) strains. Recombinant mutants

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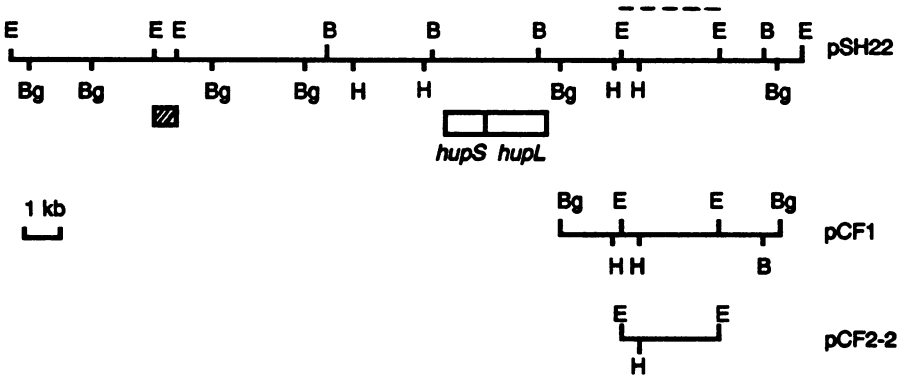

FIG. 1. Restriction map of cosmid pSH22 and its subclones. *hupS* and *hupL* indicate the hydrogenase structural genes for the small and large subunits, respectively. The hatched box indicates a region involved in Ni metabolism (7). Restriction enzymes: E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII. The broken line above pSH22 indicates the region of DNA that was deleted in the chromosome of the mutants.

resulting from marker rescue of pSMK1 in the genome were selected and confirmed as previously described (7).

As Hup(Con) strains (JH101 and JH103) express hydrogenase activity constitutively, the site-directed mutations created from these Hup(Con) strains facilitated the determination of hydrogenase activity during heterotrophic growth (without the need for derepression of hydrogenase). Typical results of hydrogenase assays are shown in Table 1. Both site-directed mutants JH101Km4 (created from JH101) and JH103Km33 (created from JH103) were almost completely devoid of O_2-dependent hydrogenase activity (Table 1), as well as methylene blue-dependent activity (data not shown). Low but certainly significant activity was detected in the mutant strains harboring plasmid pCF1 (Table 1).

Added nickel is required to obtain hydrogenase activity in derepressed cultures of wild-type JH (25). Hydrogenase activities as a function of nickel concentration were determined in parent strains JH and JH103, mutant strains, and mutant strains containing plasmid pCF1 (Fig. 2). Mutant strain JHKm4, created from strain JH, showed no hydrogenase activity after derepression (Fig. 2A), even with 5 μM Ni^{2+} added. Under derepression conditions, the hydrogenase activity of JHKm4 was completely complemented (expression of the hydrogenase phenotype) to wild-type (strain JH) levels by pCF1, which contains the 6.4-kb *Bgl*II DNA fragment (Fig. 2A). In contrast to the results obtained under heterotrophic growth conditions (used for Table 1 experiments), pCF1 complemented the hydrogenase activity of JH103Km33 to about 75 to 80% the parental levels under derepression conditions (Fig. 2B). Therefore, it seems that the degree of complementation is dependent on the growth and incubation conditions used.

TABLE 1. Hydrogenase activity of heterotrophically growing cells of Hup(Con) JH101, JH103, and mutants in the presence of 5.0 μM $NiCl_2$[a]

Strain	Hydrogenase activity (nmol of H_2/h/10^8 cells)	% of wild-type activity
JH101	205	100
JH101Km4	0.7	0.3
JH101Km4(pCF1)	22	11
JH103	184	100
JH103Km33	1.8	1
JH103Km33(pCF1)	27	15

[a] Hydrogenase activity was determined amperometrically with O_2 (air) as the electron acceptor. Data are means of duplicates. Under hydrogenase derepression conditions, pCF1 complemented the hydrogenase activity of the mutants to 75 to 80% the wild-type levels (data not shown here, but see Fig. 2B).

Immunoblotting experiments. To determine whether the loss of hydrogenase activity in the site-directed mutants was due to the absence of hydrogenase peptides per se or to the absence of a gene product necessary for hydrogenase activity, we performed Western blotting (immunoblotting) with membranes (Fig. 3A) and soluble fractions (Fig. 3B) of strains JH and JH103 and the mutants. Neither JHKm4 nor JH103Km33 contained the 65- and 35-kDa hydrogenase subunits (Fig. 3A, lanes 2, 5, and 8). Instead, two other peptides larger (66.5 and 41 kDa) than the normal 65- and 35-kDa hydrogenase subunits were identified in the deletion mutants by use of anti-hydrogenase subunit-specific antibody (20, 26). The results indicate that the loss of hydrogenase activity in the mutants is due to the loss of the 65- and 35-kDa hydrogenase subunits. A Tn*5*-induced hydrogenase structural gene insertion mutant (strain JH47) (15) did not contain either the larger peptides (66.5 and 41 kDa) or the mature (65- and 35-kDa) hydrogenase subunits (data not shown). Therefore, these large peptides (66.5 and 41 kDa) must represent unprocessed forms of the hydrogenase polypeptides. The 66.5-kDa (unprocessed) peptide was not very apparent in wild-type membranes (Fig. 3A, lanes 1, 4, and 7) but was apparent in wild-type soluble fractions (Fig. 3B). Both of the mature (65- and 35-kDa) hydrogenase subunits were synthesized in the deletion mutants (JHKm4 and JH103Km33) containing plasmid pCF1 (Fig. 3A, lanes 3, 6, 9), although some of the unprocessed forms were more visible in the mutants containing pCF1 (for both membranes and soluble fractions) than in the wild type. Under heterotrophic growth conditions, double mutant JH103Km33 containing pCF1 synthesized significant levels of the 65- and 35-kDa hydrogenase subunits (Fig. 3A, lane 9), even though its hydrogenase activity was much lower than that in the wild type (Table 1) or that expressed under derepression conditions (Fig. 2B).

For the unprocessed small subunit, there is a putative 46-amino-acid leader peptide (as determined from the nucleotide sequence); this peptide is not contained in the mature small subunit of hydrogenase protein (as determined from the N-terminal amino acid sequence) (23). The size that we observed for the unprocessed small subunit (41 kDa) agrees well with the deduced amino acid sequence of this small

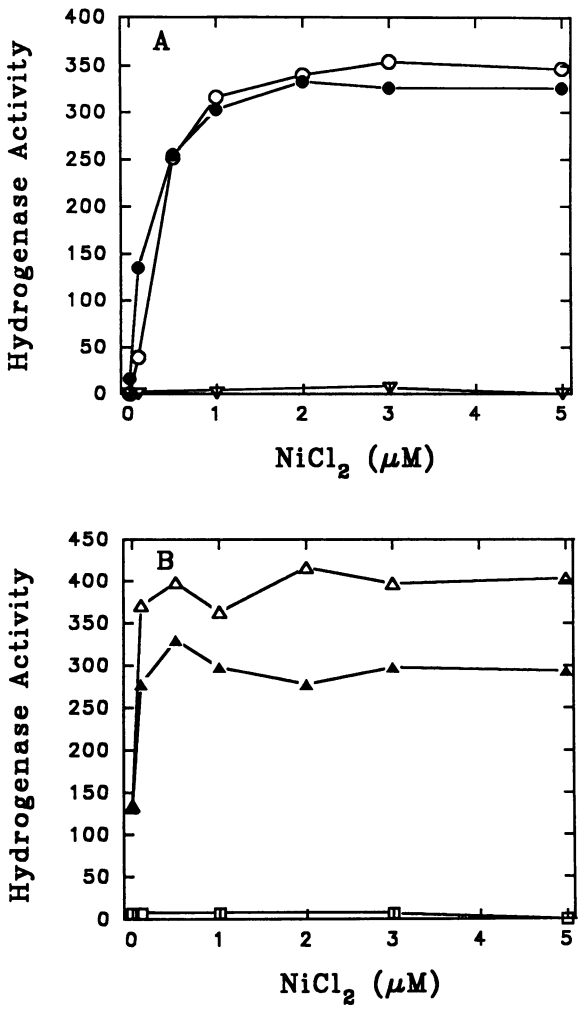

FIG. 2. Derepression of hydrogenase activity in the presence of different amounts of nickel. (A) Hydrogenase activity (nanomoles of H_2 oxidized per hour per 10^8 cells) in JH (○), JHKm4 (▽), and JHKm4(pCF1) (●). (B) Hydrogenase activity (nanomoles of H_2 oxidizied per hour per 10^8 cells) in JH103 (△), JH103Km33 (□), and JH103Km33(pCF1) (▲).

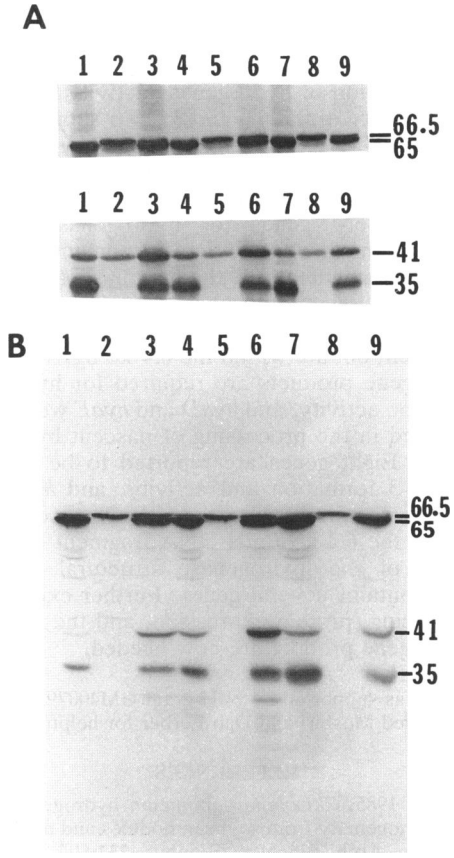

FIG. 3. (A) Immunoblot analysis of membranes from different strains. (Top) Probed with anti-65-kDa subunit-specific antibody. (Bottom) Probed with anti-35-kDa subunit-specific antibody. Ten micrograms of protein was loaded in each lane. Lanes: 1, JH (wild type); 2, JHKm4; 3, JHKm4(pCF1); 4 and 7, JH103; 5 and 8, JH103Km33; 6 and 9, JH103Km33(pCF1). Lane 1 to 6 contained samples from derepressed cells; lanes 7 to 9 contained samples from heterotrophically growing cells. (B) Immunoblot analysis of soluble fractions from different strains. Anti-65 and 35-kDa subunit-specific antisera were added together for this immunoblot. Twenty micrograms of protein was loaded in each lane. Lanes are as in panel A.

subunit, including the putative signal peptide. It is likely that the 41-kDa peptide is processed into the 35-kDa protein by the removal of the putative leader peptide (46 amino acids). Processing of a 40-kDa precursor to a 35-kDa subunit by the removal of the N terminus (46 amino acids) has also been proposed for the *E. coli* hydrogenase 1 small subunit, as determined by an analysis of a mutant containing a high-copy-number plasmid with six hydrogenase-related genes (19). As determined from the N-terminal amino acid sequence (23), there is no leader peptide for the large subunit (65 kDa) of *B. japonicum* hydrogenase. It is possible that the 66.5-kDa peptide is processed into the mature 65-kDa hydrogenase subunit at the carboxy terminus or that some other proteolytic cleavage or modification occurs. It was recently reported that an unprocessed form of the large subunit of *A. vinelandii* hydrogenase was processed into the mature form at the C-terminal portion of the protein (9). Processing of the small subunit was not reported for this bacterium. The processing of two subunits of membrane-bound hydrogenase was also reported for *A. eutrophus* H16 (16). It is interesting to note that the unprocessed small subunit (41 kDa) of hydrogenase was only present in the membranes of the *B. japonicum* mutants, indicating that this unprocessed small peptide is more hydrophobic than the mature form (35 kDa). Indeed, the unprocessed peptides (66.5 and 41 kDa) could not be solubilized from the membranes by the same solubilization buffer (data not shown) as that used for the solubilization and purification of the mature hydrogenase subunits (65 and 35 kDa) (the unprocessed peptides remained in the membrane debris after detergent solubilization). Purified *B. japonicum* hydrogenase contains only the mature subunits (20, 26). Perhaps the processing of the hydrogenase protein of *B. japonicum* occurs after its association with the membrane. In contrast, for *E. coli* hydrogenase 1, the unprocessed hydrogenase was present predominantly in the soluble fractions (19). Under symbiotic conditions, bacteroids of both JHKm4 and JH103Km33 did not contain significant hydrogenase activity or the 65- and 35-kDa hydrogenase subunits. Instead, the two unprocessed forms of the hydrogenase subunits were present (data not shown). The 65- and 35-kDa hydrogenase subunits (and

significant hydrogenase activity) were positively identified in bacteroids of wild-type JH and JH103 as controls (data not shown).

Complementation of hydrogenase activity and the corresponding recovery of the 65- and 35-kDa subunits of hydrogenase protein in JHKm4 and JH103Km33 by pCF1 indicate that the 6.4-kb *Bgl*II fragment probably has its own promoter or subpromoter (which acts as a transcriptional unit) and contains accessory genes for hydrogenase processing. pCF2-2, containing the 2.9-kb *Eco*RI fragment of DNA (Fig. 1), did not complement the hydrogenase activity of the mutants (data not shown), probably meaning that the genes required for hydrogenase processing extend over the 2.9-kb *Eco*RI fragment but are within the 6.4-kb *Bgl*II fragment. In *E. coli*, six gene products are required for hydrogenase 1 production and activity, and *hyaD* and *hyaE* were suggested to be involved in the processing of nascent hydrogenase 1 protein (19). Eight genes are reported to be required for hydrogenase 3 formation and activity, and *hycH* (one of eight genes) is required for hydrogenase 3 processing (21). It is likely that the 6.4-kb *Bgl*II DNA fragment of the region downstream of the hydrogenase structural genes of *B. japonicum* contains several genes. Further experiments on gene sequencing, protein expression, and the characterization of each gene product are now needed.

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