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Identification and Characterization of *hupT*, a Gene Involved in Negative Regulation of Hydrogen Oxidation in *Bradyrhizobium japonicum*

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The *Bradyrhizobium japonicum hupT* gene was sequenced, and its gene product was found to be homologous to NtrB-like histidine kinases. A *hupT* mutant expresses higher levels of hydrogenase activity than the wild-type strain under hydrogenase-inducing conditions (i.e., microaerobiosis plus hydrogen, or symbiosis), whereas in noninduced *hupT* cells, *hupSL* expression is derepressed but does not lead to hydrogenase activity. We conclude that HupT is involved in the repression of HupSL synthesis at the transcriptional level but that enzymatic activation requires inducing conditions.

A remarkable feature of hydrogen oxidation in *Bradyrhizobium japonicum* is that induction of the hydrogen uptake system is triggered when the bacterium shifts to either of two extremely different lifestyles. Hydrogenase activity in Hup^+ strains develops during chemoautotrophic growth in a microaerobic, hydrogen-containing environment and when the bacterium differentiates into a symbiotic, nitrogen-fixing bacteroid (reviewed in reference 20). Although the presence of the substrate hydrogen appears to be the common denominator in both lifestyles, it has become increasingly apparent that the regulatory networks that govern the formation of the uptake hydrogenase are as complex as the different lifestyles. The structural genes for the *B. japonicum* hydrogenase enzyme are encoded by the *hupSL* genes (28), and more than 20 other *hup*-related genes with various functions in structure, assembly, processing, maturation, nickel metabolism, and regulation have been identified (3, 10–12, 23, 30, 32). In free-living bradyrhizobia, hydrogenase expression is induced by hydrogen, low oxygen concentrations, and trace amounts of nickel (14, 15, 29) and *hupSL* expression requires RpoN, integration host factor (2), and the DNA-binding transcriptional activator HoxA (31). In bacteroids, HoxA is not essential for transcription and it has been proposed that nitrogenase and hydrogenase are coregulated through $FixK_2$ (5). The pathway through which these inducing signals are perceived and transduced has not yet been elucidated in detail. A multicomponent system including a pseudohydrogenase (HupUV/HoxBC) and a repressor protein (HupT/HoxJ) has been proposed to be involved in hydrogen sensing and signal transduction in *Rhodobacter capsulatus* (6, 8) and in *Alcaligenes hydrogenophilus* (16, 17). The genes encoding the pseudohydrogenase (HupUV) in *B. japonicum* have been previously identified (3). Here, we present the nucleotide sequence and mutant analysis of a regulatory gene, *hupT*, showing that it has a repressor function in the regulatory cascade leading to *hup* gene expression under both free-living and symbiotic conditions. In addition, we studied the expression of the gene encoding the transcriptional regulator HoxA and demonstrated that *hoxA* is expressed under aerobic conditions and that expression is positively autoregulated under hydrogenase-inducing conditions (microaerobiosis plus hydrogen).

Bacterial strains, plasmids, and growth of cells. *B. japonicum* CB1809 (CSIRO, St. Lucia, Australia) is routinely grown at 30°C in PSY medium (26). To measure hydrogenase activity under free-living conditions, cells were grown in minimal salts medium (described in reference 12), supplemented with 0.05% sodium gluconate and 0.05% sodium glutamate, under a gas atmosphere of 5% H_2-5% CO_2-1% O_2-89% N_2 for 48 h. Then the cells were lysed, and equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for Western blotting (1). To obtain soybean bacteroids, surface-sterilized soybean seeds (cultivar Williams) were inoculated with *B. japonicum* cultures grown on YMB (33) and plants were grown in Leonard jar-type assemblies under bacteriologically controlled conditions in a growth chamber (18 h of daylight [25°C]-6 h of darkness [18°C] regime) as described previously (19). After 24 days, bacteroids were isolated as previously described (18) and hydrogenase activities of bacteroid suspensions were determined amperometrically (27).

The *hupT* mutant FAJ1010 was constructed by replacing an internal 900-bp *Not*I fragment of the *B. japonicum* CB1809 genome with a tetracycline resistance gene cassette (Fig. 1). To this end, *hupT* was cloned as a 4.8-kbp *Bam*HI fragment in the conditionally lethal suicide vector pJQ200mp18 (24). After *Not*I digestion, the ends were blunted with the Klenow fragment of DNA polymerase and a 2-kb tetracycline resistance gene cartridge, isolated from *Sma*I-digested pHP45-TcR (9), was ligated into the coding sequence of *hupT*. This construct, pFAJ1029, was mobilized to CB1809, and selection on PSY medium with 5% sucrose and 100 μg of tetracycline/ml forced replacement of the wild-type *hupT* copy with the truncated allele. The structure of the mutated region in the *hupT* mutant, FAJ1010, was confirmed by Southern hybridization (data not shown). To measure *hupSL* expression, a promoterless *gusA* gene was inserted into the *hupS* coding sequence in the wild-type and FAJ1010 strains as follows: the *hupS* promoter region, encompassing the entire *hupS* gene and the 5′ part of *hupL* on a 3-kb *Bam*HI fragment, was blunted with the Klenow

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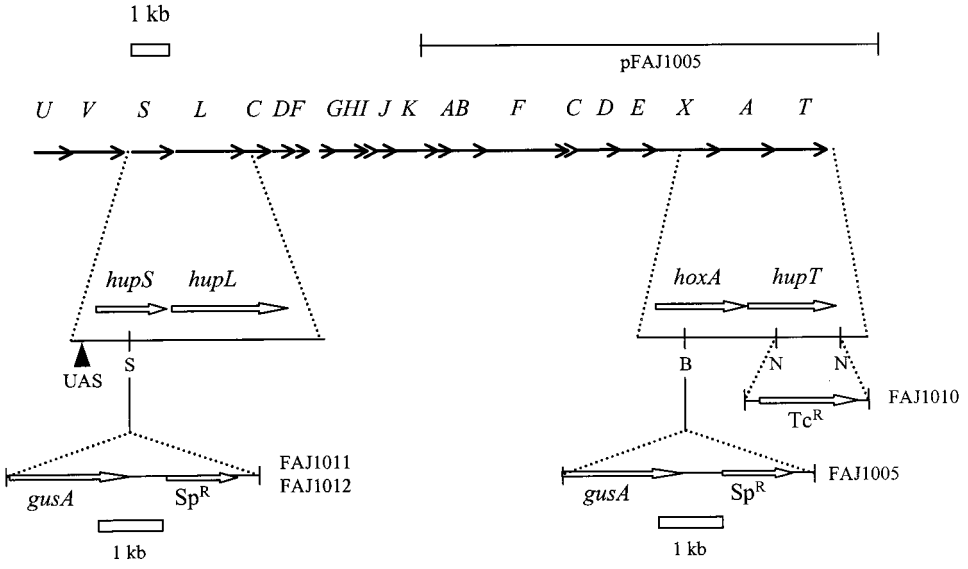

FIG. 1. Physical and genetic map of the *hup* region of *B. japonicum*. Horizontal arrows indicate the positions and sizes of the *B. japonicum hup* (*U* to *K* and *T*), *hyp* (*A* to *E*), and *hox* (*X* and *A*) genes. The horizontal line at the top of the figure represents the DNA region present in cosmid pFAJ1005. Shown in details are the regions relevant to the construction of the reporter and mutant strains, including the relevant restriction sites (S = *Sac*I, B = *Bam*HI, and N = *Not*I), as well as the locations of the tetracycline interposon (Tc^R) in mutant FAJ1010 and of the promoterless *gusA*-spectinomycin cassettes (Sp^R) used to construct genomic reporter fusions in *hupS* (present in strains FAJ1011 and FAJ1012) and *hoxA* (strain FAJ1005). UAS, HoxA-binding site.

fragment of DNA polymerase and ligated in *Sma*I-digested pJQ200UC1.

A 4-kb *Sac*I fragment from pWM5 (21), containing a promoterless *gusA* cassette preceded by a ribosome binding site and a spectinomycin resistance gene, was cloned in the *Sac*I site of *hupS*, thus putting the *gusA* gene under the transcriptional control of the *hupS* promoter region. This construct, called pFAJ1050, was mobilized to *B. japonicum*, and replacement of the wild-type *hupS* copy on the chromosome was forced by selecting for double homologous recombination as described above. The resulting reporter strains, FAJ1011 (CB1809 *hupS*::*gusA*) and FAJ1012 (FAJ1010 *hupS*::*gusA*), allow the measurement of *hupS* expression in the presence and in the absence of HupT, respectively (Fig. 1). The structures of the mutated regions in FAJ1011 and FAJ1012 were confirmed as correct by means of Southern hybridization (data not shown).

To induce cells to undergo *hupSL* transcription, cells were grown in HUM medium (4), washed twice in modified minimal salts medium (as described above), and diluted in this medium to an optical density of 0.1 at 600 nm. One-milliliter aliquots of these cell suspensions were transferred to 80-ml test tubes at 30°C (time zero) and cultured under air (aerobic growth conditions) or under a defined gas atmosphere (1% O_2-99% N_2 for microaerobic growth conditions, 5% H_2-5% CO_2-1% O_2-89% N_2 for inducing conditions). The tubes used for growth under a defined atmosphere were tightly capped with rubber stoppers and flushed twice a day with the gas mixture. β-Glucuronidase activities of the cell suspensions were quantified after 48 h of growth by using the substrate *p*-nitrophenyl-β-D-glucuronide (13) and expressed in Miller units (22).

A chromosomal insertion of a promoterless *gusA* cassette in *hoxA* was obtained by insertion of the same 4-kb *gusA*-Sp^r cassette described above into the blunted *Bam*HI site of pFAJ1020 (31) followed by mobilization to *B. japonicum* CB1809 and forced double homologous recombination. The structure of the mutated region was confirmed by Southern hybridization (data not shown), and the resulting *hoxA* mutant was called FAJ1005 (Fig. 1). An intact *hoxA* gene was supplied in *trans* by introduction of cosmid pFAJ1005 (Fig. 1) (32).

Sequencing of the *B. japonicum hupT* gene. Double-stranded DNA sequencing was carried out on DNA located downstream of the previously identified *hoxA* gene (31, 32), on both strands on overlapping fragments subcloned in pUC19, with an automated sequencer (A.L.F.; Pharmacia Biotech). Nucleotide sequences were compiled and analyzed with PC/GENE software (IntelliGenetics, Inc., Mountain View, Calif.). Analysis of codon usage (25) showed a 1,368-bp open reading frame with a codon profile characteristic for *B. japonicum* and partially overlapping the 3′ end of the *hoxA* gene, which suggests translational coupling (see below). The deduced amino acid sequence consists of 455 amino acid residues and encodes a putative protein with a predicted molecular mass of 50,059 Da. When the amino acid sequence was compared with sequences present in databases, the highest degrees of similarity were found to be with the *hoxJ* gene of *A. hydrogenophilus* and *A. eutrophus* (38.7 and 38.5% identity, respectively) (16, 17) and the *hupT* gene of *R. capsulatus* (36% identity) (8). These gene products are homologous to NtrB-like sensor histidine kinases, and in the central and C-terminal regions, functional domains for autophosphorylation, kinase or phosphatase activity, and nucleotide binding are highly conserved. The N-terminal parts of the proteins are much less conserved and are probably involved in sensing a signal. In *R. capsulatus*, the *hupT* gene product, together with the *hupU* and *hupV* gene products, is part of a repression system of hydrogen uptake gene expression (6). In *A. hydrogenophilus*, HoxJ is proposed to be involved in signal transduction from the hydrogen-sensing HoxBC complex to HoxA (17).

***hupT* mutant analysis.** In FAJ1010, HupT is truncated at amino acid 163 and thus lacks the conserved central and C-terminal domains containing the typical histidine kinase motifs. To study the function of *hupT* in *B. japonicum*, we measured hydrogenase activity amperometrically both in free-living and in symbiotic *B. japonicum* CB1809 (wild-type) and FAJ1010 (*hupT* mutant) cells. Table 1 shows that hydrogenase

TABLE 1. Hydrogenase activity in *B. japonicum* wild-type and *hupT* mutant strains grown under free-living and symbiotic conditions

Strain	Genotype	Hydrogenase activity in[a]:		
		Vegetative cells		Bacteroids
		Aerobic growth	Hydrogenase-induced growth	
CB1809	Wild type	<100	10,454	14,724
FAJ1010	*hupT*::Tc^r	<100	25,163	27,163

[a] Measured by the amperometric method and expressed in nanomoles of H_2 consumed per hour per milligram of protein. Values are means of two replicates.

TABLE 2. Effect of *hupT* on *hupSL* expression in vegetative *B. japonicum* cells under aerobic, microaerobic, or hydrogenase-inducing growth conditions

Strain	Genotype	β-Glucuronidase activity[a]		
		Aerobic growth	Microaerobic growth	Hydrogenase-induced growth
FAJ1011	*hupS*::*gusA*	4 ± 3	0	225 ± 34
FAJ1012	*hupS*::*gusA hupT*::Tc^r	35 ± 5	42 ± 2	445 ± 127

[a] Numbers are average values (in Miller units) of five replicates, assayed in duplicate, ± standard deviations.

activity in the *hupT* mutant is approximately twofold higher than that in the wild type, both in free-living induced cells and in bacteroids. In free-living cells grown aerobically in the presence or absence of hydrogen or microaerobically in the absence of hydrogen, no hydrogenase activity could be detected (Table 1), indicating that the simultaneous presence of low oxygen concentrations and hydrogen is required to obtain an active hydrogenase enzyme. When equal amounts of crude protein of free-living induced cells were probed for the presence of HupL protein by means of Western blotting with anti-HupL serum, extracts of the *hupT* mutant clearly gave a stronger signal than wild-type cells, indicating that relatively more hydrogenase protein is formed in the absence of HupT (Fig. 2, lanes d and e). A similar increase in HupL levels was observed in bacteroids of the HupT mutant (Fig. 2, compare lanes f and g). These results are consistent with the observed increase in hydrogenase activity associated with the absence of HupT (Table 1). In protein extracts prepared from aerobically grown cells, no signal was present for wild-type cells but a clear signal could be observed for the *hupT* mutant (Fig. 2, compare lanes a and b). Since the *hupT* mutant grown aerobically had no hydrogenase activity, the observed immunoreactive band presumably corresponds to inactive (i.e., unprocessed) HupL protein. In fact, the band observed in aerobic cells had a slower mobility (Fig. 2, compare lanes b and c).

To study the effect of HupT on expression of the hydrogenase structural genes under free-living conditions, a promoterless *gusA* gene was inserted in the *hupS* coding sequence in the wild type and the *hupT* mutant, as described above. β-Glucuronidase activities of the reporter strains were measured after 48 h of growth under aerobic, microaerobic, or inducing conditions (Table 2). In the wild-type FAJ1011 background, *hupSL* expression is blocked under aerobic and microaerobic conditions and is induced under conditions of microaerobiosis plus hydrogen (inducing conditions). When *hupT* is knocked out (in FAJ1012), *hupSL* expression is derepressed (although at low levels) under aerobic and microaerobic conditions. This low level of expression does not increase over time (data not shown) and is consistent with the presence of HupL protein in these cells (Fig. 2). In induced cells, expression is highly increased as a function of time and is twice the expression level seen in wild-type induced cells after 48 h (Table 2). These results suggest that HupT somehow represses *hupSL* expression under both noninducing and inducing conditions, since the level of *hupSL* expression is consistently higher in the absence of HupT. The effect of HupT on hydrogenase activity is observed only in induced cells (free-living as well as symbiotic).

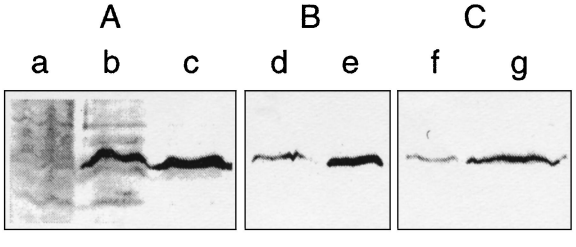

FIG. 2. Immunodetection of HupL in cell extracts. Western blots containing crude cellular extracts were incubated with HupL antiserum at a dilution of 1:1,000. Cell extracts were obtained from wild-type CB1809 (lanes a, d, and f) or *hupT* mutant strain (lanes b, c, e, and g) aerobically grown vegetative cells (panel A, lanes a and b), induced vegetative cells (panel A, lane c, and panel B), or soybean bacteroids (panel C).

We conclude from these results that HupT blocks transcription of the *hupSL* promoter under conditions in which the expression of a hydrogenase enzyme would be futile (i.e., under aerobic conditions or in the absence of the substrate hydrogen) and somehow modulates the level of expression under inducing conditions. Under these conditions, HupT might be inactivated by a process dependent on HupUV, as has been proposed for *A. eutrophus* (17). The observed increase in *hupSL* expression in the absence of HupT may indicate that the inactivation of HupT is not complete under inducing conditions.

The mechanism by which HupT affects transcription of the *hupSL* promoter in free-living cells is likely mediated by HoxA, since it has been shown that under these conditions HoxA is essential for the induction of *hupSL* expression and binds to a defined region of this promoter (31). Therefore, we propose that in free-living *B. japonicum* cells, HupT interacts with HoxA, modifying its transcription-inducing activity. HoxA is homologous to transcriptional activators of the NtrC family, whose transcriptional activities are dependent on phosphorylation by partner histidine kinases. Although the phosphorylated form of an NtrC-like regulator usually has transcriptional activation activity, in the case of *A. eutrophus* it has been proposed that HoxA is transcriptionally active in the nonphosphorylated form (17). HupT autophosphorylation has been demonstrated in *A. eutrophus* (17) and *R. capsulatus* (7). The structural similarities of *B. japonicum* HupT and these homologous proteins, together with the observed effect of HupT on *hupSL* expression, suggest that in *B. japonicum* HupT also modulates (represses) the transcriptional activity of HoxA via phosphorylation. Taking into account these observations, we hypothesize that the active form of *B. japonicum* HoxA is the nonphosphorylated form.

Expression of *hoxA* is positively autoregulated. In aerobically grown wild-type cells, there is no *hupSL* transcription and, consequently, no hydrogenase activity. This lack of *hupSL* transcription could be due to either the absence of *hoxA* transcription under aerobic conditions or the presence of inactive HoxA. To discriminate between these two possibilities, we studied the expression pattern of *hoxA* under aerobic and inducing conditions. The similar genetic organizations of *hypD*, *hypE*, *hoxX*,

TABLE 3. *hoxA* expression in vegetative *B. japonicum* cells grown under aerobic or hydrogenase-inducing conditions in the presence of HoxA

Strain	Genotype	β-Glucuronidase activity[a]	
		Aerobic growth	Hydrogenase-induced growth
FAJ1005(pFAJ1005)	Wild type	17 ± 1	171 ± 8
FAJ1005	*hoxA*	33 ± 2	31 ± 1

[a] Numbers are average values (in Miller units) of five replicates, assayed in duplicate, ± standard deviations.

and *hoxA* suggest that the genes are translationally coupled (32). Moreover, no obvious consensus promoter sequences could be identified upstream of *hoxA* or *hoxX*, except for one putative RpoN-dependent promoter 100 bp upstream of *hoxX* (32). Since a reporter plasmid containing 400 bp of DNA upstream of *hoxX* coupled to a promoterless *gusA* gene failed to show any change in expression level under inducing conditions compared to the level observed during aerobiosis (results not shown), it was concluded that this sequence does not correspond to a functional promoter. To monitor *hoxA* expression driven from any promoter further upstream, we constructed the reporter strain FAJ1005, in which the chromosomal *hoxA* coding sequence is interrupted by a promoterless *gusA* gene. An intact *hoxA* gene was supplied in *trans* on cosmid pFAJ1005, in which the insert encompasses the *hypABFCDE*, *hoxXA*, and *hupT* genes (Fig. 1). Expression was measured under aerobic and inducing conditions, as shown in Table 3. Under aerobic conditions, *hoxA* is expressed at a low basal level in the cells, presumably from a housekeeping promoter. Expression is induced 10-fold when cells are shifted to hydrogenase-inducing conditions, and this expression is under positive autoregulatory control since no induction can be seen in the absence of a functional *hoxA* gene. These data strongly suggest that under aerobic conditions, HoxA is present in the cells, although at a low level.

In this view, the observed lack of *hupSL* transcription in wild-type cells under conditions of aerobiosis indicates that HoxA is present in the inactive form and is unable to activate transcription. Since HoxA is the central transcriptional regulator of hydrogen oxidation in free-living cells, the above-presented data suggest that, at least for this type of cells, HupT modulates the transcriptional activity of HoxA, most probably through phosphorylation. Analogous to the situation in *A. eutrophus*, we hypothesize that under noninducing conditions, HupT represses *hupSL* transcription by keeping the HoxA pool in a phosphorylated state, for when *hupT* is knocked out, *hupSL* is expressed. In the absence of HupT, the available HoxA would be nonphosphorylated, leading to *hupSL* transcription and translation. It is clear that levels of transcription of *hupSL* and the content of HupL protein are still lower in aerobically grown cells than in induced cells of the *hupT* mutant. Full activation of inactive HoxA still seems to require the perception of inducing conditions by a not-yet-elucidated mechanism in which HupUV should play a major role.

Further research must focus on the identification of the additional factors required to obtain an active hydrogenase.

Nucleotide sequence accession number. The nucleotide sequence of the *hupT* gene has been deposited in the GenBank database (accession no. AF132935).

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