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Sequence Analysis and Interposon Mutagenesis of the *hupT* Gene, Which Encodes a Sensor Protein Involved in Repression of Hydrogenase Synthesis in *Rhodobacter capsulatus*

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The *hupT* gene, which represses hydrogenase gene expression in the purple photosynthetic bacterium *Rhodobacter capsulatus*, has been identified and sequenced. The nucleotide sequence of *hupT* and of the contiguous downstream open reading frame, *hupU*, is reported. The HupT protein of 456 amino acids (48,414 Da) has sequence similarity with the FixL, DctB, NtrB, and ArcB proteins and is predicted to be a soluble sensor kinase. Insertional inactivation of the *hupT* gene led to deregulation of transcriptional control, so that the hydrogenase structural operon *hupSLC* became overexpressed in cells grown anaerobically or aerobically. The HupT$^-$ mutants were complemented in *trans* by a plasmid containing an intact copy of the *hupT* gene. The *hupU* open reading frame, capable of encoding a protein of 84,879 Da, shared identity with [NiFe]hydrogenase subunits; the strongest similarity was observed with the periplasmic hydrogenase of *Desulfovibrio baculatus*.

The purple nonsulfur bacterium *Rhodobacter capsulatus* presents a remarkable metabolic versatility; it can grow photo- or chemoheterotrophically with a wide range of organic compounds as carbon and nitrogen sources, can fix CO_2 in the light or in the dark with H_2 as an electron source and therefore grow lithoautotrophically, and can also use accessory electron acceptors, such as dimethyl sulfoxide or trimethylamine-*N*-oxide, in a fermentative type of metabolism (reviewed in reference 49). Such a metabolic adaptability involves families of signal transduction proteins which often function in pairs and belong to the so-called two-component regulatory systems (44, 45). Indeed, the synthesis of hydrogenase, which in *R. capsulatus* functions physiologically as an H_2 uptake enzyme and enables the cells to grow anaerobically in the light on CO_2-H_2 or aerobically in the dark on H_2-O_2-CO_2, has been shown to depend upon the product of the $hupR_1$ gene, the amino acid sequence of which shares significant similarity with those of several transcriptional regulators of the NtrC subfamily (36). The $hupR_1$ gene mapped at the *hup* locus (6) on the genetic map of the *R. capsulatus* chromosome (53) and was found to be intercalated between the *hypAB* and *hypCDE* genes on the physical map of the *hup* locus (7).

In this paper, we describe the identification of two genes, *hupT* and *hupU*, belonging to the same cluster of *hup* and *hyp* genes (7) located in the upstream region of the hydrogenase structural operon *hupSLC* and transcribed from the same DNA strand. The deduced product of *hupT* shows significant sequence similarity to the sensor of bacterial two-component stimulus-response systems (34, 44). Inactivation of *hupT* by interposon mutagenesis demonstrated that its product plays a role in the control of hydrogenase synthesis in *R. capsulatus*.

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MATERIALS AND METHODS

Bacterial strains and cultures. The bacterial strains and plasmids used in this work are listed in Table 1. Strains of *R. capsulatus* were grown in mineral RCV medium (51) supplemented with 30 mM DL-malate as a C source and 7 mM ammonium sulfate (MN medium) as an N source (51). Strains were grown at 30°C either in the light (about 2,500 lx) or in darkness as previously described (5). Complex YPS medium consisted of 0.3% Bacto Peptone, 0.3% yeast extract, 2 mM $MgSO_4$, and 2 mM $CaCl_2$. *Escherichia coli* strains were grown aerobically in Luria-Bertani medium at 37°C (38). Antibiotics were added at the following concentrations (milligrams per liter): 100 (ampicillin), 10 (chloramphenicol), 25 (kanamycin), 100 (streptomycin), 10 (tetracycline), and 50 (trimethoprim) for *E. coli* and 10 (kanamycin) and 1 (tetracycline) for *R. capsulatus*.

Enzyme assays. Hydrogenase activity was assayed in whole cells as previously described (8) with 0.15 mM methylene blue as the electron acceptor. β-Galactosidase was assayed according to Miller (30) with *o*-nitrophenyl-β-D-galactopyranoside as the substrate. Whole cells in culture medium were made permeable with 1 drop of 0.1% sodium dodecyl sulfate and 2 drops of chloroform; the reaction was stopped with 0.5 ml of 1 M sodium carbonate (pH 10) and, after sedimentation of the cell debris, the A_{420} was read with a Uvikon spectrophotometer (Kontron). The reported values for enzyme activities are the results of at least two independent experiments performed in duplicate or triplicate. The protein concentration was estimated by use of the empirical relationship $A_{660}/5$ = milligrams of protein per milliliter (29).

DNA preparations and bacterial mating. Plasmid DNA was prepared by the alkaline method (19) and, for large-scale preparations, purified on cesium chloride gradients (38). Total *R. capsulatus* DNA was isolated as described previously (16). Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer GmbH (Mannheim, Germany). DNA restriction fragments were isolated from agarose gels with DEAE-cellulose paper (Whatman DE81) as described previously (14). DNA digestions and ligations were performed

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype	Source or reference
Strains		
R. capsulatus		
B10	Wild type	27
BSE7	Kan^r *hup*(Con)	This work
BSE8	Kan^r *hup*(Con)	This work
E. coli		
DH5α	ϕ80d*lacZ*ΔM15 F$^-$ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ$^-$ *recA1 gyrA96 relA1* Δ(*argF-lacZYA*)U169	Bethesda Research Laboratories
S17-1	Tp^r Sm^r *hsdR pro recA* RP4-2-Tc::Mu-Km::Tn*7* in chromosome	40
Plasmids		
pRK290	Tc^r; broad-host-range vector	13
pRK2013	Km^r; mobilizes pRK290	13
pPHU234	Tc^r; broad-host-range *lacZ* fusion vector	18
pPHU231	Tc^r; derivative of pRK290	17a
pAC142	Tc^r; pPHU234 with 0.73-kb *Hin*dIII insert	7
pAC145	Tc^r; pPHU234 with 6.7-kb *Hin*dIII insert	This work
pAC205	Tc^r; pPHU231 with 1.7-kb *Eco*RI-*Hin*dIII insert	This work
pUC19	Ap^r	56
pEC0	Ap^r; pUC19 with 6.7-kb *Hin*dIII insert	This work
pSE1	Ap^r Tc^r; pSUP202 with 1.77-kb *Eco*RI insert	This work
pSE7, pSE8	Ap^r Km^r Tc^r; pSE1 with 1.4-kb *Sma*I insert in opposite directions	This work
pSUP202	Ap^r Cm^r Tc^r; broad-host-range mobilizable vector	40
pUC4KIXX	Km^r Ap^r	Pharmacia LKB

according to the instructions of the manufacturer. After ligation, the hybrid DNA was introduced into *E. coli* cells by transformation. Competent *E. coli* cells were prepared as described previously (32). Transformed *E. coli* cells were selected on Luria-Bertani plates in the presence of the appropriate antibiotics. For bacterial conjugation experiments, cells of *E. coli* S17-1 (donor strain) at the exponential phase of growth and *R. capsulatus* recipient cells, both in liquid cultures, were spotted onto YPS plates and incubated overnight at 30°C. *R. capsulatus* transconjugants were selected on RCV plates in the presence of the appropriate antibiotics. Triparental conjugation was performed as described earlier (4) with the system of Ditta et al. (13).

Nucleotide and protein sequence studies. DNA sequencing was performed on both strands by the dideoxy chain termination method (39) with the Sequenase version 2.0 kit from United States Biochemical Corp. Specific oligonucleotide primers were synthesized with an Applied Biosystems synthesizer (model 381A). DNA sequence analyses were performed with Lasergene programs from DNASTAR Inc. The protein sequences were deduced by use of a table of codon frequencies (41) established from sequenced genes of members of the family *Rhodospirillaceae*. Protein alignment was done by use of the algorithm of Needleman and Wunsch (33).

Interposon mutagenesis of the *hupT* gene. The 6.7-kb *Hin*dIII-*Hin*dIII restriction fragment from B10 genomic DNA, containing the three genes, *hupT*, *hupU*, and *hypF*, upstream from the structural *hupSL* genes (see Fig. 1), was cloned into pUC19, yielding plasmid pEC0. The 1.77-kb *Eco*RI-*Eco*RI restriction fragment was obtained by use of the *Eco*RI site of the polylinker. It contained *hupT*, which was cloned in the *Eco*RI site of the chloramphenicol resistance gene in pSUP202 (a suicide plasmid mobilizable into *R. capsulatus* cells), yielding plasmid pSE1. The kanamycin resistance (Kan^r) gene cartridge was isolated from pUC4KIXX by *Sma*I digestion and then was inserted by blunt-end ligation into plasmid pSE1, previously cut with *Xho*I and treated with the Klenow fragment of DNA polymerase I. The orientation of the Kan^r gene cartridge was determined by restriction analysis, and two derived plasmids, pSE7 and pSE8, having the cartridge in opposite orientations, were selected.

The two mobilizable plasmids, pSE7 and pSE8, were intro-

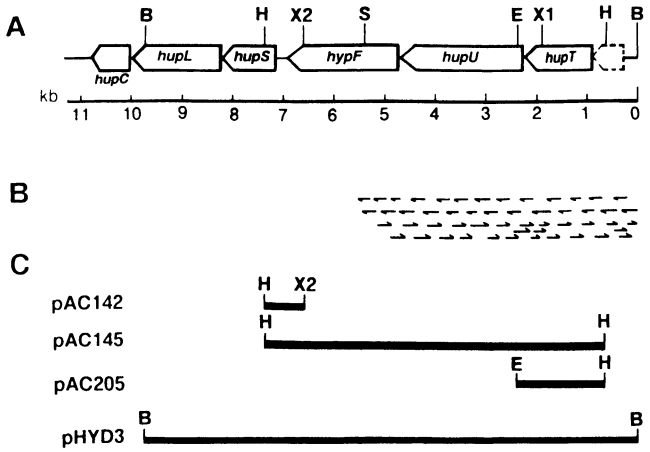

FIG. 1. Locations of the *hupT* and *hupU* ORFs in relation to the structural *hupSLC* genes. (A) Genetic and physical map. (B) Sequencing strategy. The positions of the primers and the extent of sequencing progression are shown by the positions and lengths of the arrows, which overlap by at least 30 nucleotides. (C) Locations and sizes of the DNA fragments used in complementation experiments with HupT$^-$ mutants. For plasmid pAC142 (9) and plasmid pAC145, the DNA fragments shown are fused in frame to the *lacZ* reporter gene. The third gene of the structural operon, earlier termed *hupM* (7), has been renamed *hupC* (50). B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X1, *Xho*I; X2, *Xho*II.

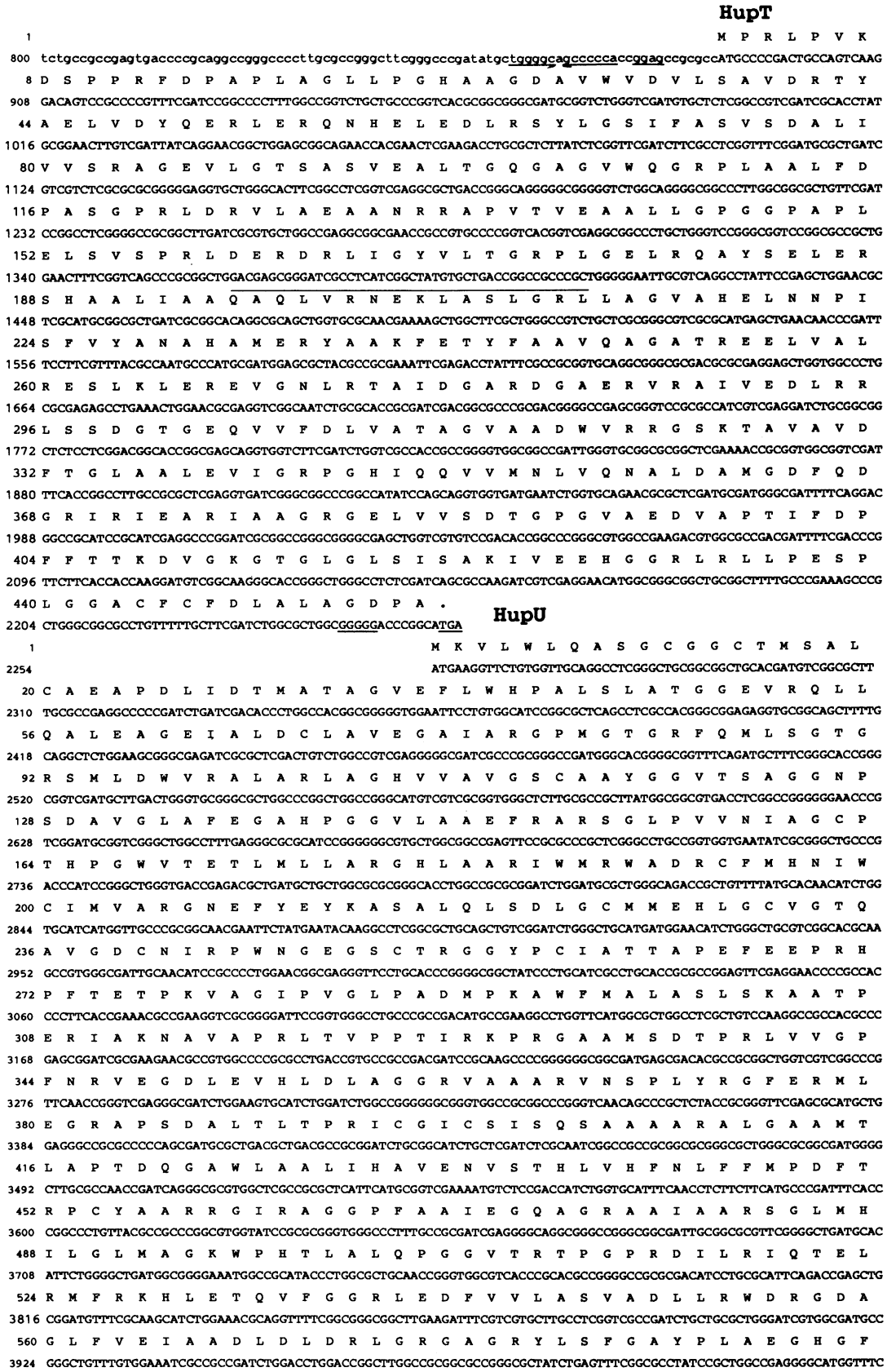

FIG. 2

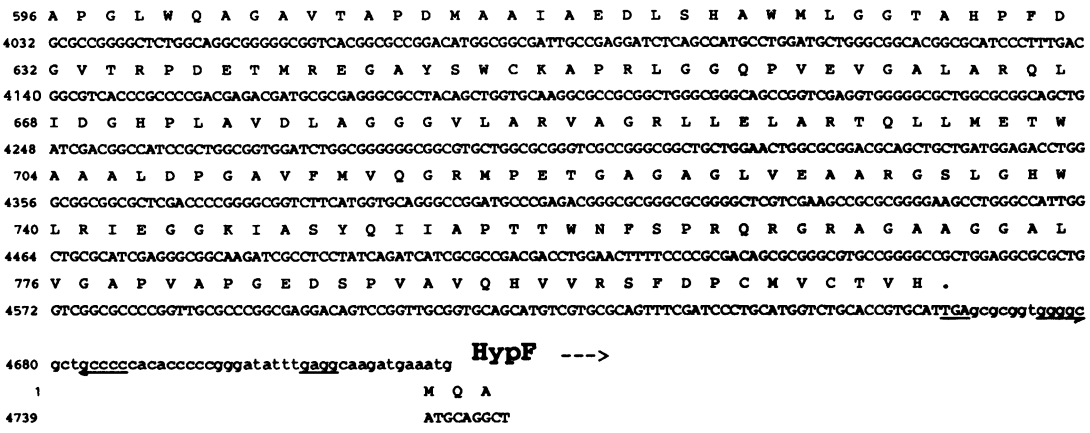

FIG. 2. Nucleotide and deduced amino acid sequences of the chromosomal *hupT* and *hupU* genes. The putative ribosome-binding sites and the TGA stop codons are underlined. Inverted repeats are marked by inverted arrows. The putative Q-linker sequence identified as described elsewhere (54) is overlined. The GenBank accession number is LO 2348.

duced by transformation into mobilizing strain *E. coli* S17-1 and then transferred by biparental conjugation into wild-type *R. capsulatus* B10. To select the double recombinants, in which the inactivated gene on the plasmid had been exchanged with the chromosomal wild-type gene, transconjugant cells were grown in the presence of kanamycin and tested for their sensitivity to tetracycline (since the Tc^r gene was borne on the plasmid, tetracycline sensitivity would indicate loss of the plasmid). Thus, the BSE7 and BSE8 mutants obtained from pSE7 and pSE8, respectively, had the Kan^r cartridge in opposite orientations in *hupT*. The constructions were checked by Southern blot analyses; restricted genomic DNA (1 μg) electrophoresed in a 0.7% agarose gel was transferred to a Hybond-N nylon membrane (Amersham) and bound to the membrane by UV cross-linking (5 min). DNA probes were labeled with digoxigenin-11-dUTP (Boehringer) and hybridized overnight at 42°C in the presence of 50% formamide.

RESULTS

Nucleotide sequence of the *hupT* and *hupU* genes. Previous work had indicated that the upstream region of the hydrogenase structural operon *hupSLC* contained hydrogenase determinants. Indeed, Hup^- mutant RS13 was recently shown to be complemented by the *hypF* gene contiguous to and upstream from *hupS* (7).

Nucleotide sequencing was carried out further upstream on the *Bam*HI-*Bam*HI insert of plasmid pBC2 (6). Upstream from *hypF*, three complete open reading frames (ORFs) were identified on the basis of the known codon preference of previously sequenced genes from *R. capsulatus*. These ORFs were found to be located on the same strand as those already identified in the hydrogenase gene cluster of pBC2 (7), with appropriately located in-frame ATG codons at the 5′ end and TGA stop codons at the 3′ end. Figure 1 shows the positions of the *hupT* and the *hupU* ORFs relative to the *hupSLC* structural operon and to the *hypF* gene, previously sequenced (7). The third ORF is shown as broken lines. The deduced product shows no significant similarity with other proteins in the data bases, and it has not yet been assigned to a function.

The nucleotide and deduced amino acid sequences of the *hupT* and *hupU* ORFs are presented in Fig. 2. A potential Shine-Dalgarno sequence is located 8 bp upstream of the putative ATG initiation codon of the *hupT* gene, positioned between nucleotides 887 and 2257. The stop codon of *hupT* was found to overlap with the start codon of the next ORF, *hupU*, in an ATGA motif with a −1 frameshift; such an arrangement is a good indication that the two genes are part of an operon and that they are translationally coupled. The positions of the two ORFs in the nucleotide sequence and the sizes of their predicted products are given in Table 2.

Sequence similarities of HupT to regulatory sensor proteins. The alignment program AALIGN revealed similarity between the C-terminal region of HupT and a domain of about 200 amino acids (aa) in the C-terminal region of several sensor proteins (34, 44) (Fig. 3). These sensor proteins are autophosphorylatable; in support of such a capacity is the presence of a putative nucleotide binding site in the deduced HupT amino acid sequence (G-rich region in domain III; Fig. 3B). HupT (456 aa) was found to be most similar to FixL (27% identical residues in a 415-aa overlap), a hemoprotein that binds O_2, has autokinase activity, and is able to transfer its phosphoryl group to the response regulator FixJ in *Rhizobium meliloti* (11, 12, 15), and to *Rhizobium leguminosarum* DctB (37), a sensor protein involved in C_4-dicarboxylate transport (27% identical residues in a 412-aa overlap). HupT shared identity with *R. capsulatus* DctS (657 aa), a sensor kinase controlling the synthesis of the high-affinity C_4-dicarboxylate transport system (17) (29% identical residues in a 196-aa overlap), and with *Klebsiella pneumoniae* NtrB (349 aa) (25), the sensor protein of the general nitrogen regulation system *ntr* (27% identical residues in a 196-aa overlap). These four putative sensor kinases and VirA (24) (21% identical residues with HupT) share the sequence (V/L)AHE(L/I)(N/Q)P, which includes the phosphorylatable histidine residue in domain I (Fig. 3B). The degrees of identity were 21% (in a 323-aa overlap) with $NifR_2$ (NtrB) (356 aa) of *R. capsulatus* (21) and 28% (in a 111-aa overlap) with the sensor-regulator protein ArcB (778 aa) of *E.*

TABLE 2. Predicted products of the *hupT* and *hupU* genes

Gene	Position in the sequence (nucleotides)	No. of aa	Calculated mol wt	Theoretical pI
hupT	887–2257	456	48,414	5.1
hupU	2254–4674	806	84,921	7.3

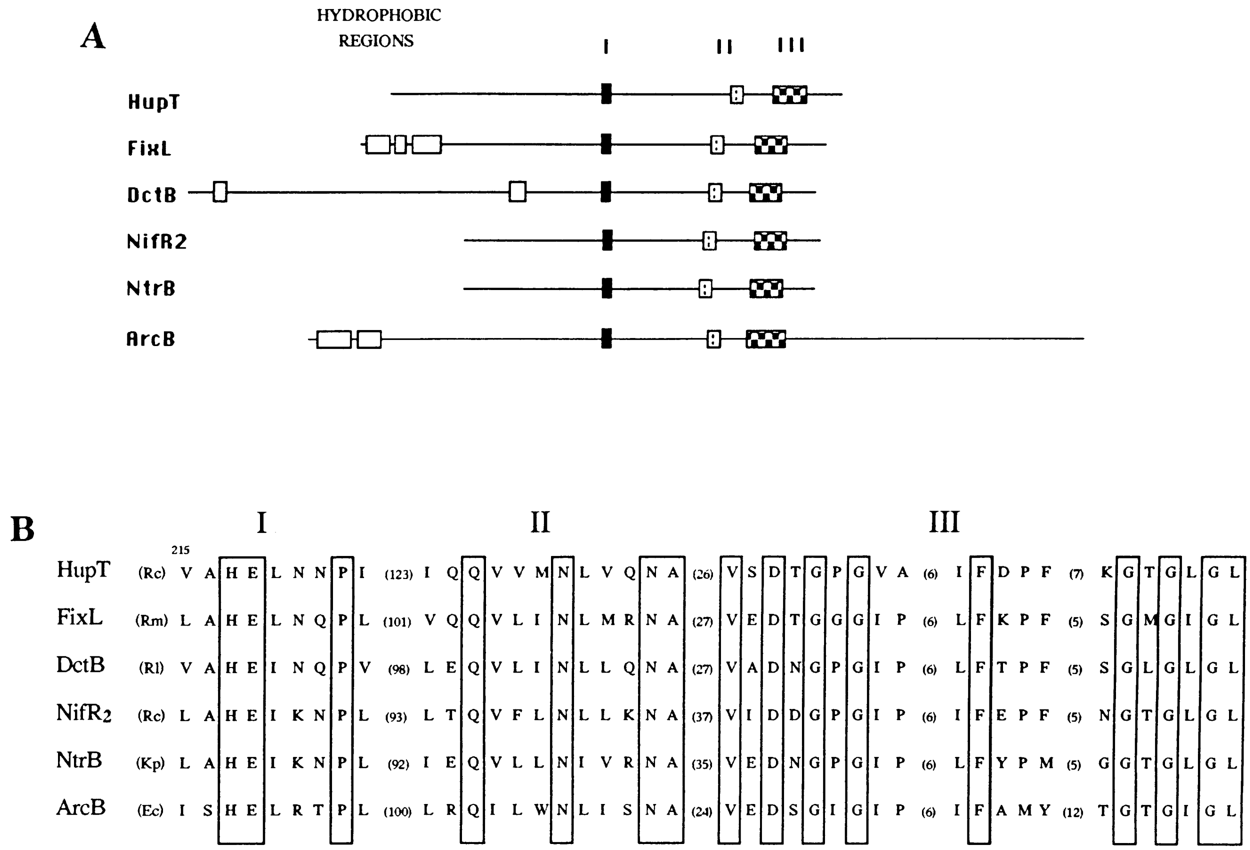

FIG. 3. (A) Domain organization of the expected amino acid sequences of HupT from *R. capsulatus* (Rc), FixL from *R. meliloti* (Rm) (11), DctB from *R. leguminosarum* (Rl) (37), $NifR_2$ (NtrB) from *R. capsulatus* (Rc) (21), NtrB from *K. pneumoniae* (Kp) (25), and ArcB from *E. coli* (Ec) (19). The sequences corresponding to regions I, II, and III are shown in panel B. HupT, $NifR_2$, and NtrB lack the hydrophobic putative membrane-spanning sequences indicated by open boxes near the N terminus (adapted from reference 44). (B) Alignment of identical amino acids in the conserved regions of HupT, starting from position 215, and the other sensor proteins. In parentheses are the number of residues between the domains in each sequence. Identical residues are boxed.

coli, which represses the *arc* (for aerobic respiration control) modulon under anaerobiosis (20). The conserved histidine, asparagine, and glycine residues characteristic of the sensor kinase family (44) are boxed in Fig. 3B. A hydrophobicity plot (22) revealed no hydrophobic sequences in the N-terminal region of the HupT protein which could anchor the protein to the membrane. HupT is therefore expected to be a soluble protein like NtrB. Among the six sensor proteins compared in Fig. 3A, only FixL, DctB, and ArcB appear to contain hydrophobic helices characteristic of membrane-spanning proteins. HupT also shared identity with recognized sensor proteins PhoR (26) (23%), CpxA (52) (22%), EnvZ (10) (23%), PhoM (1) (20%), and CheA (43) (21%) but no significant identity with NarX (42) or with the products of the hydrogenase regulatory genes *hoxX* from *Bradyrhizobium japonicum* (28% identity in a 39-aa overlap) (48) and the *hydH* gene from *E. coli* (46) and *Salmonella typhimurium* (3).

A Q-linker interdomain sequence shown by Wootton and Drummond (54) to occur at the boundaries of functionally distinct domains in bacterial regulatory and sensory transduction proteins, typified by the nitrogen regulatory proteins NtrB and NtrC, could also be identified at the N-terminal boundary of the conserved C-terminal domain of HupT. Such interdomain sequences are 15 to 25 residues long, contain glutamine residues—hence, their name Q-linker—and are also relatively rich in arginine, glutamate, serine, and proline, with hydrophobic amino acids spaced with a periodicity of ca. 4 or 5 residues in the C-terminal 15 residues of these sequences. Wootton and Drummond (54) have established a sequence pattern discriminator for Q-linkers which is a weight matrix based on the residue frequencies in the alignment of several examples of Q-linkers. Using this matrix, we identified a putative Q-linker sequence, from glutamine 196 to leucine 211, in the HupT sequence (overlined in Fig. 2). It is indeed beyond the Q-linker that the deduced NtrB and HupT proteins share identical amino acids.

Structural features of the HupU protein. The *hupU* ORF is capable of encoding a protein of 84.9 kDa. The deduced HupU protein shares amino acid sequence similarities with both the small and the large subunits of *R. capsulatus* hydrogenase (23), and the *hupU* gene can be regarded as an in-frame fusion of the *hupSL* genes. In particular, 10 of the 13 Cys residues of the mature small subunit (HupS) are conserved, but the signal peptide at the N terminus of HupS is lacking in HupU (HupU and HupS share 19% identity in a 298-aa overlap). At its C terminus, HupU shares marked similarity with the C terminus of HupL (43% identity between Pro-720 and His-806 of HupU). The *hupU* gene product shares even greater similarity with the periplasmic [NiFeSe]hydrogenase from *Desulfovibrio baculatus* (28) (28% identity in a 285-aa overlap with the small subunit and 26% in a 509-aa overlap with the large subunit) (Fig. 4). Cys-800 of HupU can be aligned with the SeCys of *D.*

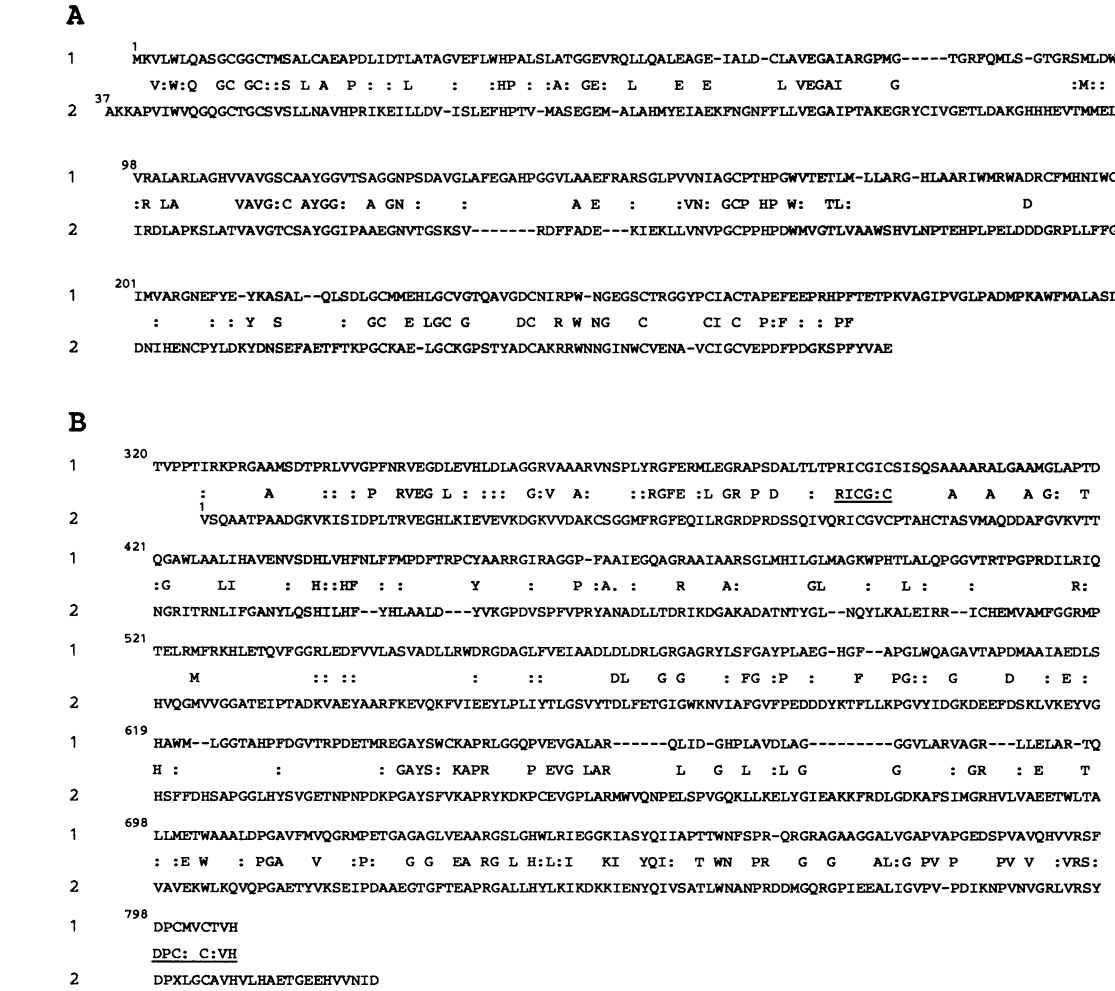

FIG. 4. Amino acid sequence alignment between the deduced product of the *hupU* ORF (line 1) and the small (A) and large (B) subunits of *D. baculatus* [NiFeSe]hydrogenase (line 2). The proposed sequences involved in nickel binding in [NiFe]hydrogenases (35, 55) are underlined in panel B. Colons indicate conservative subsitutions.

baculatus hydrogenase (marked X in Fig. 4), which has been shown to coordinate the active-site nickel atom (cf. reference 35). The fact that the regions of sequence similarities between HupU and the amino and carboxyl termini of the large subunit contain the amino acids supposed to supply ligands to the active-site nickel in [NiFe]hydrogenases (35) (underlined in Fig. 4) supports the contention that HupU is capable of binding nickel.

Insertional inactivation of the *hupT* gene. To ascertain that the *hupT* gene was indeed involved in hydrogenase synthesis, the chromosomal *hupT* gene was insertionally inactivated by a Kan^r gene cartridge (see Materials and Methods). Two HupT$^-$ mutants, BSE8 and BSE7, were selected for further studies. Plasmid loss in the mutants was checked by Southern blotting with suicide vector pSUP202 as a probe (Fig. 5A). The sizes of the restricted fragments, probed with the Kan^r gene cartridge (Fig. 5B), allowed us to confirm the orientation of the inserted cassette in each mutant (Fig. 6).

HupT repression activity. Colbeau and Vignais (9) had shown that hydrogenase synthesis in wild-type strain B10 varied according to growth conditions and that these variations could be monitored by measuring the activity of β-galactosidase expressed from a plasmid-borne *hupS::lacZ* fusion. The same type of experiments repeated with *hupT* mutants BSE7 and BSE8 are reported in Table 3, with B10 as a control. Two plasmids carrying *lacZ* fusions, pAC142 and pAC145 (Fig. 1), were introduced by conjugation into all the strains to monitor β-galactosidase activities in parallel to hydrogenase activities. The two HupT$^-$ mutants exhibited exceptionally high β-galactosidase activities in cells harboring pAC142 (Table 3). The very high β-galactosidase activities reported in Table 3 were considered to result essentially from increased transcription. Such an assumption was confirmed by the data obtained with plasmid pAC145, which carries the three intact genes, *hupT*, *hupU*, and *hypF*, and their promoter. The set of these three genes restored the wild-type, repressed phenotype in the two mutants grown either in the presence or in the absence of O_2.

In summary, the above-mentioned results clearly demonstrate that HupT plays a role in the repression of the transcription of hydrogenase structural genes.

DISCUSSION

Sequence comparisons have indicated that the product of the *hupT* gene belongs to the superfamily of sensor proteins, which usually function in tandem with a response regulator in

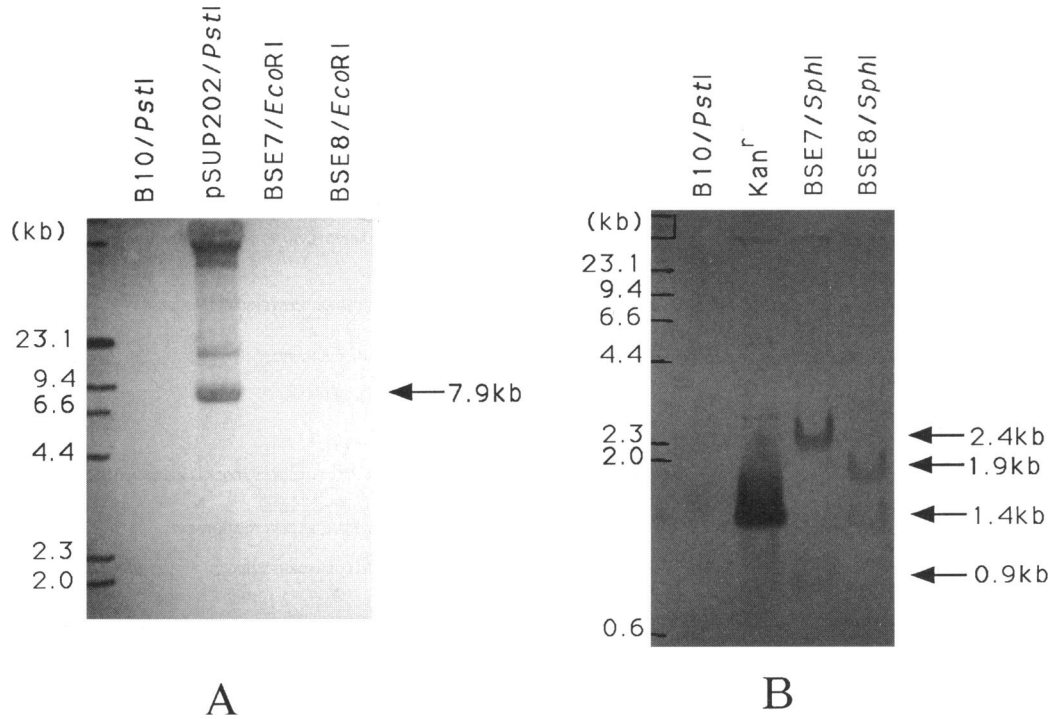

FIG. 5. Southern blotting of genomic DNA from HupT$^-$ mutants BSE7 and BSE8 and from isogenic wild-type strain B10, digested with the restriction enzymes indicated on the top of the figure. The 7.9-kb suicide vector pSUP202 (A) and the 1.4-kb Kan^r gene cartridge (B) were used as controls. The blots were probed with pSUP202 (A) or with the Kan^r gene cartridge (B). The sizes of the markers are shown on the left side of each blot, and the sizes of the hybridizing bands are shown on the right.

two-component regulatory systems. HupT is expected to be a kinase molecule capable of autophosphorylation. Indeed, a putative nucleotide binding site has been identified in the deduced amino acid sequence; it remains to be proven that HupT can autophosphorylate in response to a signal still to be identified.

Inactivation of the gene resulted in HupT$^-$ mutants exhibiting exceptionally high hydrogenase activities, even in the presence of O_2 or ammonia. This result confirms that *R. capsulatus* hydrogenase is not very sensitive to O_2, in contrast to the hydrogenases from strict anaerobes. The introduction of a plasmid-borne intact *hupT* gene (plasmid pAC205) into the HupT$^-$ mutants lowered hydrogenase activity to the wild-type level, an indication that the function of *hupT* is to participate

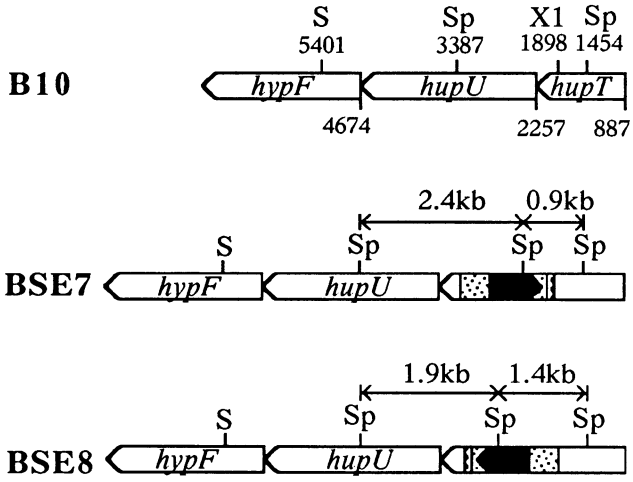

FIG. 6. Location and orientation of the Kan^r gene cartridge (stippled areas) within the *hupT* gene in the selected mutants. S, *Sal*I; Sp, *Sph*I; X1, *Xho*I.

TABLE 3. Hydrogenase and β-galactosidase activities in HupT$^-$ strains and in complemented strains compared with wild-type strain B10

Plasmid	O_2	Activity[a] in strain:					
		B10		BSE7		BSE8	
		H_2ase	βgal	H_2ase	βgal	H_2ase	βgal
None	−	3.7		37.3		64.1	
	+	15.1		46.3		118.0	
pAC142	−	1.7	0.2	20.7	5.2	39.7	4.3
	+	13.0	1.8	27.7	11.5	112.5	19.0
pAC145	−	0	0.1	1.7	0.2	2.7	0.1
	+	13.5	1.9	13.4	1.7	9.1	1.7
pAC205	−	6.1		8.3		5.8	
	+	19.9		19.4		17.7	

[a] Cells were grown overnight in MN medium at 30°C, either anaerobically (−) in the light or aerobically (+) in darkness. Hydrogenase (H_2ase) and β-galactosidase (βgal) were assayed directly in aliquots from MN cultures at an A_{660} of ca. 1.5 as described in Materials and Methods. The data were obtained in separate experiments run in duplicate. Values varied by less than 20% between different experiments. The specific hydrogenase activity is given in micromoles of methylene blue reduced per hour per milligram of protein, and that of β-galactosidase is given in micromoles of *o*-nitrophenol formed per minute per milligram of protein.

in the repression of hydrogenase expression, in particular in the presence of O_2. Components of signal transduction systems responding to O_2 have already been identified in other microorganisms. HupT shares sequence similarities with some of them, e.g., *R. meliloti* FixL which, in response to oxygen, can activate by transphosphorylation the response regulator FixJ (11); however, FixL is a hemoprotein which binds O_2, and HupT does not share significant similarity with the fragment of FixL shown to bind heme and to bind oxygen (31). Another well-known sensor, the product of the *arcB* gene, a sensor-regulator protein which represses the *arc* modulon (20), also shares identity with the HupT C-terminal domain. In this study, we showed that inactivation of the *hupT* gene in the chromosome by insertion of an antibiotic cassette resulted in a Hup^+ phenotype. On the other hand, the introduction of additional copies of the intact *hupT* gene into the $HupT^-$ mutants gave rise to a hydrogenase-deficient phenotype. These results demonstrate that *hupT* controls hydrogenase expression negatively in *R. capsulatus*; the $HupT^-$ mutants are therefore Hup^c mutants, in which hydrogenase is expressed constitutively.

The product of *hupU* presents the distinctive feature of sharing similarities with both the small and the large subunits of *R. capsulatus* hydrogenase. Similar cases have already been reported in the literature, in which a gene encoding a polypeptide homologous to an αβ FeS protein is located in the vicinity of the structural genes encoding the two subunits and in which gene products share a significant degree of identity with regions of each subunit. One such example is the *nifE* and *nifN* genes from *Azotobacter vinelandii*, which play a role in iron-molybdenum cofactor biosynthesis: the *nifEN* genes are located immediately downstream from the nitrogenase structural gene cluster *nifHDK*, and their products show considerable homology with the *nifD* (MoFe protein α subunit) and *nifK* (MoFe protein β subunit) gene products, respectively (2). Another example is the hyd_γ (or *hydC*) gene from *Desulfovibrio vulgaris* (Hildenborough), which is located downstream from the structural genes for the α and β subunits of the periplasmic hydrogenase but is transcribed in the opposite direction and which could encode a polypeptide homologous to that hydrogenase (47). The authors suggested that the hyd_γ gene product may be involved in the activation of hydrogenase and serve as a helper protein in the insertion of the FeS cluster in the catalytic center or may be an alternative hydrogenase. In the two above-mentioned reports, it was concluded that the homologous genes (*nifEN* and *nifDK*; hyd_γ and *hyd*αβ) may have an ancestral relationship. In the case of the *hupU* gene from *R. capsulatus*, it is striking and probably significant in terms of evolution that the degree of similarity of the *hupU* gene product is even higher with *D. baculatus* than with *R. capsulatus* hydrogenases.

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