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The *hupTUV* Operon Is Involved in Negative Control of Hydrogenase Synthesis in *Rhodobacter capsulatus*

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The *hupT*, *hupU*, and *hupV* genes, which are located upstream from the *hupSLC* and *hypF* genes in the chromosome of *Rhodobacter capsulatus*, form the *hupTUV* operon expressed from the *hupT* promoter. The *hupU* and *hupV* genes, previously thought to belong to a single open reading frame, encode HupU, of 34.5 kDa (332 amino acids), and HupV, of 50.4 kDa (476 amino acids), which are ≥50% identical to the homologous *Bradyrhizobium japonicum* HupU and HupV proteins and *Rhodobacter sphaeroides* HupU1 and HupU2 proteins, respectively; they also have 20 and 29% similarity with the small subunit (HupS) and the large subunit (HupL), respectively, of *R. capsulatus* [NiFe]hydrogenase. HupU lacks the signal peptide of HupS and HupV lacks the C-terminal sequence of HupL, which are cleaved during hydrogenase processing. Inactivation of *hupV* by insertional mutagenesis or of *hupUV* by in-frame deletion led to HupV$^-$ and Hup(UV)$^-$ mutants derepressed for hydrogenase synthesis, particularly in the presence of oxygen. These mutants were complemented in *trans* by plasmid-borne *hupTUV* but not by *hupT* or by *hupUV*, except when expressed from the inducible *fru* promoter. Complementation of the HupV$^-$ and Hup(UV)$^-$ mutants brought about a decrease in hydrogenase activity up to 10-fold, to the level of the wild-type strain B10, indicating that HupU and HupV participate in negative regulation of hydrogenase expression in concert with HupT, a sensor histidine kinase involved in the repression process. Plasmid-borne gene fusions used to monitor *hupTUV* expression indicated that the operon is expressed at a low level (50- to 100-fold lower than *hupS*).

The purple nonsulfur photosynthetic bacterium *Rhodobacter capsulatus* possesses a membrane-bound hydrogenase which, under physiological conditions, functions in H_2 uptake (35). This enzyme, which enables *R. capsulatus* cells to grow autotrophically with H_2 as the source of electrons (20), is also synthesized under heterotrophic growth conditions, in particular in H_2-evolving cultures in which hydrogenase synthesis is stimulated by H_2 (8).

A cluster of genes necessary for the synthesis of the [NiFe] hydrogenase of *R. capsulatus* has been isolated from the chromosome of *R. capsulatus* B10 and sequenced (6). Several genes, located in the cluster of *hup* and *hyp* genes, have been shown to be involved in the regulation of hydrogenase expression. Two of them, *hupT* and *hupR*, encode proteins which are members of the superfamily of proteins forming two-component signal transducing systems. The HupT protein, a histidine kinase sensor, participates in the repression of hydrogenase activity under heterotrophic growth conditions, in the absence of H_2 (11). The HupR protein (formerly called HupR1) belongs to the NtrC subfamily of response regulators; a mutation in the putative nucleotide-binding site of HupR leads to a lower level of expression of the hydrogenase structural *hupSLC* genes (29).

Downstream from the *hupT* gene and upstream from *hypF* are located two open reading frames (ORFs), termed *hupU* and *hupV*, previously described as a single ORF, *hupU* (11). We have corrected the *hupU* and *hupV* gene sequences, the products of which have sequence similarities with the small (HupS) and the large (HupL) subunits, respectively, of [NiFe] hydrogenases. Inactivation of *hupV* or of both the *hupU* and *hupV* genes led to derepression of hydrogenase gene expression. We demonstrate here that the *hupU* and *hupV* genes belong to the same operon as *hupT*, that they are expressed from the *hupT* promoter, and that their products participate with HupT in the repression of hydrogenase synthesis in *R. capsulatus.*

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MATERIALS AND METHODS

Bacterial strains and cultures. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* strains were grown aerobically in Luria-Bertani medium at 37°C (31). *R. capsulatus* strains were grown in minimal salts medium (RCV) (38) supplemented with 30 mM sodium DL-malate as a C source and either 7 mM L-glutamate (MG medium) or 7 mM ammonium sulfate (MN medium) as an N source. Autotrophic growth was carried out in medium without carbon (RCV with 7 mM ammonium sulfate) in jars with the GasPak system (BBL, Beckton Dickinson & Co. Cockeysville, Md.) providing gas-phase H_2 and CO_2. Strains were grown at 30°C either in the light (about 2,500 lx) or in darkness as previously described (5). Antibiotics were added at the following concentrations (in milligrams per liter): 100 (ampicillin), 10 (gentamicin), 25 (kanamycin), and 10 (tetracycline) for *E. coli* and 5 (gentamicin), 10 (kanamycin), and 1 (tetracycline) for *R. capsulatus.*

DNA preparation and bacterial mating. Standard recombinant DNA techniques and PCR amplification were performed as described by Sambrook et al. (31). Biparental conjugation was performed as described previously (11). Triparental conjugation (4) was performed with the system of Ditta et al. (9).

Nucleotide and protein sequence studies. DNA sequencing was performed on both strands by the dideoxy chain termination method (32) with the Sequenase version 2.0 kit from United States Biochemical Corp (Cleveland, Ohio). Specific oligonucleotide primers were synthesized with an Applied Biosystems synthesizer (model 381A). The DNA sequence analyses were performed with the Lasergene programs from DNASTAR Inc. (Madison, Wis.). The protein sequences were deduced by using a table of codon frequencies (34) established from sequenced genes in the family *Rhodospirillaceae*. Protein alignment was done by the Clustal method (DNASTAR Inc.).

Overexpression of the *hupU* and *hupV* genes in *E. coli.* The 0.65-kb fragment corresponding to the 5′ region of the *hupU* gene was amplified by PCR, and an

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype and/or genotype	Source or reference
Strains		
R. capsulatus		
B10	Wild type	23
BSE14	Kan^r *hup*(Con)	This work
BSE15	Kan^r *hup*(Con)	This work
BSE16	Δ*hupUV hup*(Con)	This work
E. coli		
BL21(DE3)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
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	33
Plasmids		
pRK290	Tc^r; broad-host-range vector	9
pRK2013	Km^r; mobilizes pRK290	9
pPHU231	Tc^r; derivative of pRK290	16
pPHU234	Tc^r; broad-host-range *lacZ* fusion vector	17
pPHU236	Tc^r; broad-host-range *lacZ* fusion vector	17
pAC142	Tc^r; pPHU234 with 0.73-kb *Hin*dIII-*Xho*II insert	8
pAC145	Tc^r; pPHU234 with 6.7-kb *Hin*dIII insert	11
pAC205	Tc^r; pPHU231 with 1.7-kb *Eco*RI-*Hin*dIII insert	11
pAC206	Tc^r; pPHU231 with 4.8-kb *Sal*I-*Hin*dIII insert	This work
pCR-Script SK(+)	Ap^r	Stratagene
pET-15b	Ap^r	Novagen
pUC18	Ap^r	40
pUC18Δ*SpH*	Ap^r; pUC18 with *Sph*I and *Hin*dIII sites deleted	This work
pEC0	Ap^r; pUC19 with 6.7-kb *Hin*dIII insert	11
pEC3	Tc^r; pPHU235 with 3.5-kb *Sal*I-*Xho*I insert	6
pFRK-I	Ap^r Ble^r Gm^r Km^r; *fruP* fusion vector	10
pFRGal4	Gm^r Tc^r; *fruP*::*lacZ*	10
pKES1	Ap^r; pCR-Script SK(+) with 0.65-kb PCR insert	This work
pKES3	Ap^r; pKES1 with 3.1-kb *Pst*I insert	This work
pSE11	Ap^r; pUC18Δ*SpH* with 3.1-kb *Pst*I insert	This work
pSE12, pSE13	Ap^r Km^r; pSE11 with 1.4-kb *Sma*I insert in opposite directions	This work
pSE14	Cm^r Km^r Tc^r; pSUP202 with 4.5-kb *Pst*I insert from pSE12	This work
pSE15	Cm^r Km^r Tc^r; pSUP202 with 4.5-kb *Pst*I insert from pSE13	This work
pSE36	Ap^r; pUC18 with 669-bp *Avi*II insert	This work
pSE37	Ap^r; pUC18 with 516-bp *Eco*RI insert	This work
pSE38	Ap^r; pSE37 with 0.68-kb *Bam*HI-*Kpn*I insert from pSE36	This work
pSE39	Ap^r Tc^r; pSUP202 with 1.2-kb *Eco*RI-*Hin*dIII insert from pSE38	This work
pSE42	Tc^r; pPHU234 with 626-bp *Sty*I-*Eco*RI insert	This work
pSE50	Ap^r; pET-15b with 3.2-kb *Nde*I-*Sal*I insert from pKES3	This work
pSE51	Tc^r; pPHU234 with 2.7-kb *Hin*dIII insert from pEC0	This work
pSE52	Tc^r; pPHU234 with 2.5-kb *Apa*I-*Hin*dIII insert from pSE51	This work
pSE53	Tc^r; pPHU234 with 1.8-kb *Eco*47III-*Hin*dIII insert from pSE51	This work
pSE59	Ap^r Gm^r; replacement of the Ble^r Km^r cassette of pFRK-I by a 3.2-kb *Acc*I-*Nde*I insert from pKES3	This work
pSE60	Gm^r Tc^r; pPHU234 with 6.2-kb *Hin*dIII insert from pSE59	This work
pSE61	Tc^r; pPHU236 with 0.9-kb *Eco*RI-*Eco*47III insert from pEC0	This work
pUC4KIXX	Km^r Ap^r	Pharmacia LKB
pSUP202	Ap^r Cm^r Tc^r; mobilizable vector	33

*Nde*I restriction site was created at the 5′ end of the gene. The two oligonucleotides used as primers were ES15 (5′-ATGCATATGAAGGTTCTGTGG-3′) and ES4 (5′-ATCCGACAGCTGCAGCGCCGA-3′). The PCR product was cloned in the phagemid pCR-Script SK(+) (Stratagene, La Jolla, Calif.), yielding pKES1, and sequenced. The 3.1-kb *Pst*I fragment from pEC0, comprising the 3′ region of *hupU* and the integral *hupV* gene, was cloned in the *Pst*I site of pKES1, yielding pKES3. The 3.2-kb *Nde*I-*Sal*I from pKES3 was cloned in plasmid pET-15b (Novagen, Madison, Wis.) cut with *Nde*I and *Xho*I, yielding plasmid pSE50. Plasmid pSE50 was introduced in strain BL21(DE3); cells were grown at 37°C to an optical density at 600 nm of 0.6, and then overexpression of *hupUV* was triggered by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

Interposon mutagenesis of the *hupV* gene. The 3.1-kb *Pst*I-*Pst*I restriction fragment from pEC0, containing the 3′ region of the *hupU* gene, the integral *hupV* gene, and the 5′ region of the *hypF* gene, was cloned into pUC18Δ*SpH*, yielding the plasmid pSE11. Plasmid pUC18Δ*SpH* is a derivative of pUC18 in which the *Sph*I and *Hin*dIII restriction sites have been eliminated after hydrolysis with the *Sph*I and *Hin*dIII enzymes; the plasmid was treated with T4 DNA polymerase before religation. The kanamycin resistance (Kan^r) gene cartridge, isolated from pUC4KIXX by *Sma*I digestion, was inserted in both orientations by blunt-end ligation into plasmid pSE11 cut with *Sph*I and treated with T4 DNA polymerase. Two derived plasmids, pSE12 and pSE13, having the Kan^r cartridge in opposite orientations within *hupV* were selected. Since the Kan^r gene cartridge has a *Pst*I restriction site, partial hydrolyses were performed to obtain, from pSE12 and pSE13, the 4.5-kb *Pst*I-*Pst*I fragment containing *hupV* with the cartridge (0.05 U of *Pst*I per μg of DNA; 30 and 45 min for pSE12 and 45 and 60 min for pSE13). These DNA fragments, obtained from pSE12 and pSE13, were cloned in the *Pst*I site of pSUP202 (a suicide plasmid mobilizable into *R. capsulatus* cells), yielding pSE14 and pSE15, respectively. These two mobilizable plasmids were introduced into the mobilizing strain *E. coli* S17.1 by transformation and then transferred to the wild-type *R. capsulatus* strain B10 by biparental conjugation. The BSE14 and BSE15 mutants, obtained with pSE14 and pSE15, respectively, were selected by their capacity to grow in the presence of kanamycin and their sensitivity to tetracycline. Each mutant has the Kan^r gene cartridge in either orientation in *hupV*. The absence of plasmid and the orientation of the cartridge in the mutants were checked by Southern blot analyses with pSUP202

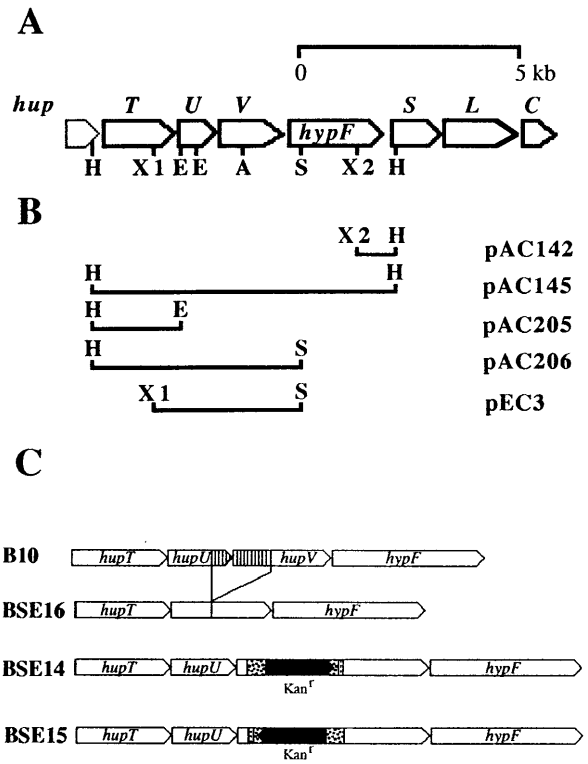

FIG. 1. Locations of the *hupU* and *hupV* ORFs in relation to the hydrogenase structural *hupSLC* genes. (A and B) Genetic map (A) and locations of the DNA fragments used in complementation experiments with the HupV$^-$ and Hup (UV)$^-$ mutants (B). For plasmids pAC142 and pAC145, the indicated DNA fragments were fused in frame to the *lacZ* reporter gene. A, *Avi*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X1, *Xho*I; X2, *Xho*II. (C) Locations and orientations of the Kan^r gene cartridge (black area) within the *hupV* gene in the BSE14 and BSE15 mutants and of the deleted fragment (hatched area) in the *hupU* and *hupV* genes.

and the Kan^r gene cartridge as the respective probes labeled with digoxigenin-11-dUTP as described by Elsen et al. (11).

Deletion mutagenesis of the *hupUV* genes. A 669-bp *Avi*II-*Avi*II fragment of pEC0 containing the 3′ half of the *hupV* gene was cloned in the *Sma*I site of pUC18, yielding pSE36. A 516-bp *Eco*RI-*Eco*RI fragment of pEC0 carrying the 5′ end of the *hupU* gene was inserted in the *Eco*RI site of pUC18, to give plasmid pSE37. By using the polylinker sites, the *hupU* gene borne on pSE36 was obtained by *Bam*HI-*Kpn*I hydrolysis and cloned in pSE37 hydrolyzed with the same enzymes. The obtained plasmid, pSE38, then carries the 5′ half of *hupU* fused in frame with the 3′ half of *hupV*. Partial hydrolysis (*Eco*RI at 0.5 U/μg of DNA and *Hin*dIII at 1 U/μg of DNA for 40 min) released the 1.2-kb *Eco*RI-*Hin*dIII fragment, which was treated with the Klenow fragment of DNA polymerase I and cloned into plasmid pSUP202 cut with *Eco*RI and treated with the Klenow fragment of DNA polymerase I, yielding plasmid pSE39. pSE39 was first introduced by transformation into S17.1 and then transferred into the two HupV$^-$ mutants, BSE14 and BSE15. Recombinants for each HupV$^-$ mutant, resulting from a single recombination of pSE39 in the chromosome, were first selected in the presence of kanamycin and tetracycline. These simple recombinants were grown in liquid MG medium in anaerobiosis, without an antibiotic, to allow the loss of the integrated plasmid. After about 120 bacterial generations, the bacteria were plated on MG medium and screened for Kan^s and Tc^s phenotypes. Only one Hup(UV)$^-$ mutant, BSE16, was obtained; it was derived from the HupV$^-$ mutant BSE15. The absences of the plasmid, of the Kan^r gene cartridge, and of the deleted fragment were checked by Southern blot analyses with probes labeled with digoxigenin-11-dUTP as described by Elsen et al. (11).

Expression of the *hupU* and *hupV* genes in *R. capsulatus*. Plasmid pKES3, described above, was cut with *Acc*I, treated with the Klenow fragment of DNA polymerase I, and then cut with *Nde*I. A fragment of 3.2 kb was obtained and purified; it carried the *hupU* and *hupV* ORFs. The Km^r/Ble^r cassette of plasmid pFRK-I was excised with *Eco*RI, treated with the Klenow fragment of DNA polymerase I, and then cut with *Nde*I; it was replaced by the 3.2-kb *Acc*I-*Nde*I fragment from pKES3, leading to plasmid pSE59. The fragment of 6.2 kb containing *hupU* and *hupV* under the control of *fruP* was excised from pSE59 by cutting with *Hin*dIII and cloned into plasmid pPHU234, which was first cut with *Hin*dIII and dephosphorylated with calf alkaline phosphatase. The obtained plasmid, termed pSE60, was introduced by triparental conjugation into the wild-type B10 and mutant strains of *R. capsulatus*.

Construction of translational fusions. pEC0 was hydrolyzed with *Sty*I, treated with the Klenow fragment of DNA polymerase I, and then hydrolyzed with *Eco*RI. We obtained a 626-bp fragment, containing the promoter region and the 5′ region of the *hupT* gene, which was cloned in the broad-host-range *lacZ* fusion vector pPHU234 cut with *Sca*I and *Eco*RI. The new plasmid, pSE42, carried a translational fusion between the first 108 codons of *hupT* and the *lacZ* gene (lacking the first 7 codons). Plasmid pSE51 was constructed by inserting the 2.7-kb *Hin*dIII fragment of pEC0 in plasmid pPHU234 cut with *Sca*I. Plasmid pSE51 was cut with *Apa*I, treated with T4 DNA polymerase, and then cut with *Hin*dIII. The obtained 2.5-kb fragment was cloned in plasmid pPHU234 cut with *Sca*I and *Hin*dIII, yielding plasmid pSE52. In the same manner, plasmid pSE53 was constructed by insertion of the 1.8-kb *Eco*47III-*Hin*dIII fragment from pSE51 in plasmid pPHU234 cut with *Sca*I and *Hin*dIII. Plasmid pSE61 was derived from pPHU236 cut with *Eco*RI and *Sca*I, in which the 0.9-kb *Eco*RI-*Eco*47III fragment from pEC0 has been inserted.

Enzyme assays. Hydrogenase activity was assayed in whole cells as previously described (7) with 0.15 mM methylene blue as the electron acceptor. β-Galactosidase was assayed as described by Miller (26) with *o*-nitrophenyl-β-D-galactopyranoside as the substrate, as previously described (11).

Nucleotide sequence accession number. The sequences have been submitted to GenBank under accession number L02348, and the corrected sequences have been submitted to GenBank.

RESULTS

The *hupU* and *hupV* gene products of *R. capsulatus*. We have previously reported on the occurrence of two contiguous ORFs, initially termed *hupT* and *hupU*, located in the upstream region of the hydrogenase structural operon, *hupSLC* (11). The former *hupU* ORF has been resequenced, and several errors in the published sequence were found. It appears that the former *hupU* ORF actually contains two distinct ORFs, now termed *hupU* and *hupV* (Fig. 1). The changes which were made, in particular a C deletion at position 3233 which creates a frameshift and six insertions, led to a change in the numbering of the initial nucleotide sequence (11). The stop codon of *hupU* is found to overlap the start codon of *hupV* in an ATGA motif with a −1 frameshift; the same arrangement is found between the *hupU* and the *hupT* genes (Fig. 1). A potential Shine-Dalgarno sequence (GGGGGG) is located 5 bp upstream from the putative ATG translation initiation codon of *hupV*. The deduced products have molecular masses of 34.5 kDa (332 amino acids) for HupU and 50.4 kDa (476 amino acids) for HupV.

Experiments were carried out to overproduce the HupU and HupV proteins in *E. coli* by using the expression vector pET-15b (Novagen). Only one protein, corresponding in size to the HupU protein predicted from the corrected nucleotide sequence, was overproduced (data not shown). The absence of HupV in this experiment could be explained by poor recognition in *E. coli* of the Shine-Dalgarno sequence present upstream from the *R. capsulatus hupV* gene. Indeed, similarly, overexpression of the *hupTUV* genes cloned in the same vector led to overproduction of only the HupT protein, with HupU and HupV being undetectable (data not shown). It appears, therefore, that heterologous coexpression of the *hupU* and *hupV* genes will require the cloning of each gene under the control of a strong independent promoter.

Structural features of the HupU and HupV proteins. The HupU and HupV proteins have a high degree of identity with the HupU (58.3%) and HupV (49.2%) proteins of *Bradyrhizobium japonicum* (2) and with HupU1 (75.5%) and the first 64 amino acids of HupU2 (82.8%) of *Rhodobacter sphaeroides* (15). They also have amino acid sequence similarities with the small and the large subunits, respectively, of [NiFe]hydrogenases (20% identity between *R. capsulatus* HupS [without the signal peptide] and HupU and 29% identity between the mature HupL and the HupV proteins [22]); in particular, several

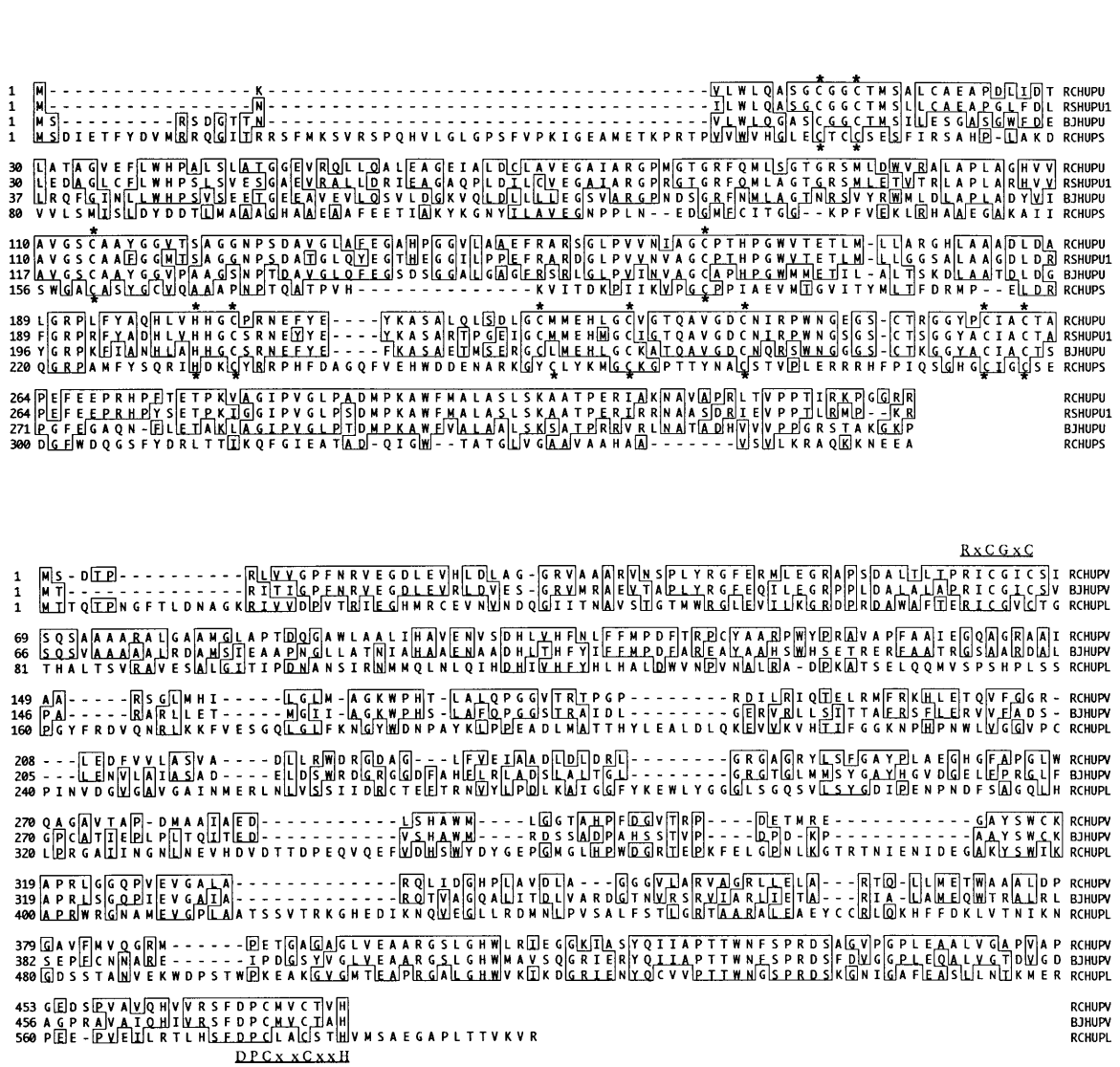

FIG. 2. Amino acid sequence alignment by the Clustal method of the deduced products of the *hupU* ORF of *R. capsulatus* (RCHUPU), the *hupU1* ORF of *R. sphaeroides* (RSHUPU1), the *hupU* ORF of *B. japonicum* (BJHUPU), and the small subunit (HupS) of *R. capsulatus* hydrogenase (RCHUPS) (A) and of the deduced products of the *hupV* ORFs of *R. capsulatus* (RCHUPV) and *B. japonicum* (BJHUPV) and the large subunit (HupL) of *R. capsulatus* hydrogenase (RCHUPL) (B). Identical amino acids are boxed. The putative residues involved in cluster Fe-S binding are noted by asterisks located above the HupU sequence and below the HupS sequence in panel A. The putative nickel-binding sites of [NiFe]hydrogenases are underlined in panel B.

important residues are conserved. The HupU proteins are rich in cysteines, with a Cys arrangement similar to that found in HupS for the formation of Fe-S clusters. All of the Cys residues of the small subunit of *Desulfovibrio gigas* hydrogenase involved in the binding of the three Fe-S clusters (37) are present in the HupU protein (Fig. 2). The alignment of the *R. capsulatus* HupU, *R. sphaeroides* HupU1, *B. japonicum* HupU, and *R. capsulatus* HupS protein sequences is shown in Fig. 2. The signal peptide present at the N terminus of HupS is lacking in the HupU proteins. The motif located at the C terminus of the small subunit of class I [NiFe]hydrogenases (39), a unique feature of membrane-bound hydrogenases suggested to play a role in anchoring the protein to the membrane (25, 39), was not found in the HupU proteins. Therefore, HupU is expected to be a soluble protein located in the cytoplasmic compartment. The HupV proteins of *R. capsulatus* and *B. japonicum* contain the putative Ni-binding site of the [NiFe] hydrogenase large subunit, at the N-terminal (RxCGxC) and C-terminal (DPCxxCxxH) ends of the protein (27, 37) (Fig. 2), indicating that HupV may be able to bind nickel. Another interesting feature of these HupV proteins is that they lack the amino acid sequence which is proteolytically removed in HupL during the maturation of the protein (24; reviewed in references 13 and 36).

Increased hydrogenase synthesis in the HupV$^-$ and Hup (UV)$^-$ mutants. In order to investigate the physiological role of the *hupV* product, the chromosomal *hupV* gene was inactivated by insertion of a Kan^r gene cartridge (see Materials and Methods). Two HupV$^-$ mutants, BSE14 and BSE15, containing the Kan^r gene cartridge in either orientation in the *hupV* gene were selected (Fig. 1C). The two mutants had quite different hydrogenase activities (see below), an indication that the cartridge could have a polar effect on the gene placed downstream. To eliminate a possible polar effect of the car-

TABLE 2. Hydrogenase (H_2ase) and β-galactosidase (βgal) activities in aerobically or anaerobically grown cells of the wild-type strain B10, the $HupV^-$ strains BSE14 and BSE15, and the $Hup(UV)^-$ strain BSE16 harboring plasmid pAC142[a]

Strain	Anaerobiosis				Aerobiosis				
	MG		MN		MG		MN		MN + H_2 (H_2ase)[b]
	H_2ase[c]	βgal[d]	H_2ase	βgal	H_2ase	βgal	H_2ase	βgal	
B10(pAC142)	42.8	4.5	1.7	0.2	5.8	1.1	13.0	1.8	32.0
BSE14(pAC142)	23.0	3.4	33.1	4.1	67.2	14.4	57.3	8.5	52.3
BSE15(pAC142)	2.5	2.3	3.4	1.8	5.0	7.0	3.7	4.7	4.1
BSE16(pAC142)	28.5	3.4	28.1	3.3	73.0	13.6	69.0	8.7	47.6

[a] Cells were grown overnight at 30°C, either anaerobically in the light or aerobically in darkness, in MN or MG medium to an optical density at 600 nm of ca 1.5.
[b] Ten percent H_2 gas was added in the air phase above the cultures, and hydrogenase activity was assayed after 3 h of induction.
[c] Specific hydrogenase activity given in micromoles of methylene blue · $hour^{-1}$ · milligram of $protein^{-1}$.
[d] Specific β-galactosidase activity given in micromoles of *o*-nitrophenol · $minute^{-1}$ · milligram of $protein^{-1}$.

tridge, another mutant without the cartridge was constructed. In the third mutant, BSE16, the *hupU* and *hupV* genes were partially deleted and an in-frame deletion allele was generated by a gene replacement strategy. BSE16 contains the 5′ end of *hupU* and the 3′ end of *hupV* fused in frame and thus is a $Hup(UV)^-$ mutant (Fig. 1C).

The $HupV^-$ mutants, BSE14 and BSE15, and the $Hup(UV)^-$ mutant, BSE16, were grown under the conditions indicated in Table 2, and their hydrogenase activities were compared with those of the wild-type strain B10. In B10, hydrogenase activity levels are high in cells grown anaerobically in the light, i.e., under conditions which favor nitrogenase synthesis and reduction of protons to H_2 by the nitrogenase enzyme. H_2 has been shown to stimulate hydrogenase biosynthesis (8).

We chose five growth conditions to study the mutants (Table 2). In anaerobic MG medium in the light and in MN medium in the presence of air plus 10% H_2, the level of hydrogenase synthesis is high, while in anaerobic MN medium in the light and in MG or MN medium under aerobiosis, where nitrogenase is repressed, the level of hydrogenase synthesis is low in the wild-type strain B10 (Table 2) (8). The introduction of plasmid pAC142 carrying the *hupS*::*lacZ* fusion previously described (8) allowed us to assess the activation or repression of *hupSLC* gene expression by the determination of β-galactosidase activity. The BSE14 mutant, which has the cartridge oriented in the direction of *hupV* transcription, exhibited high levels of hydrogenase activity under aerobiosis and in MN medium under anaerobiosis compared with the wild-type strain B10. This activity resulted from a high level of *hupS* gene expression in the $HupV^-$ mutant, as monitored with pAC142. This phenotype resembled that of the $HupT^-$ mutant, BSE8 (11). No additional increase was observed upon the addition of 10% H_2 to the culture of BSE14 in aerobic MN medium, an indication that the *hupSLC* genes were already fully expressed (Table 2).

In the BSE16 mutant, inactivation of the *hupU* and *hupV* genes led to a phenotype very similar to the phenotype of the $HupV^-$ mutant BSE14. Except in cells grown phototrophically in MG medium, in which H_2 produced by the nitrogenase stimulates hydrogenase synthesis in B10, the BSE16 mutant exhibited a much higher level of hydrogenase activity than the wild-type B10 under all tested growth conditions (Table 2). The phenotypes of the $HupV^-$ mutant, BSE14, and of the $Hup(UV)^-$ mutant, BSE16, were similar, with a remarkable correspondence between the hydrogenase and β-galactosidase activities in cells harboring pAC142 under all tested growth conditions. In both mutants, maximal derepression was observed in the presence of O_2 and absence of H_2 (Table 2).

Curiously, the second $HupV^-$ mutant, BSE15, showed a phenotype totally different from that of BSE14; hydrogenase activity remained very low under all growth conditions, and there was no real correlation between hydrogenase activity (very low) and β-galactosidase activity (relatively high) as observed in the wild-type strain B10 and in the other $HupV^-$ mutant, BSE14 (Table 2). These results remain unexplained.

In conclusion, inactivation of the *hupV* gene in mutant BSE14 or of both the *hupU* and *hupV* genes in mutant BSE16 led to derepression of hydrogenase gene expression, as has been observed in $HupT^-$ mutants (11), an indication that the products of the three genes *hupTUV* participate in repression of hydrogenase synthesis in *R. capsulatus.*

Complementation of the $HupV^-$ and $Hup(UV)^-$ mutants and restoration of repressed hydrogenase gene expression. To demonstrate that the observed changes in hydrogenase biosynthesis were due to the mutated *hupU* and *hupV* genes, complementation experiments were carried out. The DNA fragments and locations of the *hup* genes used are shown in Fig. 1. The introduction of plasmid pAC206, harboring the *hupTUV* genes, in either BSE14 or BSE16 reduced by severalfold the hydrogenase activity in the mutants; the hydrogenase levels then became comparable to those of the wild-type strain B10 under three of the conditions tested (MN anaerobiosis and MG or MN aerobiosis) (Table 3). Since plasmid pAC205 carrying only *hupT* or plasmid pEC3 with only the *hupUV* genes failed to complement either BSE14, BSE15, or BSE16 (Table 3), it appears that *hupUV* are not expressed from pEC3 but require the *hupT* promoter for expression. Thus, *hupU* and *hupV* form with *hupT* the *hupTUV* operon.

Table 3 shows that the *hupTUV* operon borne on plasmid pAC206 did not restore the wild-type phenotype in the BSE15 mutant but that *hupTUV hypF* on plasmid pAC145 did so; these results confirm that the Kan^r gene cartridge in BSE15 has a polar effect on *hypF.*

To confirm that inactivation of the *hupU* and *hupV* genes was indeed responsible for the derepression of hydrogenase activity in the BSE14 and BSE16 mutants, the *hupUV* genes were cloned under the control of the *R. capsulatus fru* promoter (*fruP*), which is inducible by fructose. The resulting plasmid, pSE60, was introduced in the three mutants. Plasmid pFRGal4, which carries a *fruP*::*lacZ* fusion, was used as a control (10). We could check that pFRGal4 had no effect on hydrogenase activity, either in the wild-type strain B10 (in which *hup* gene expression is repressed) or in the BSE16 mutant (in which the *hup* genes are overexpressed) (Table 4). In addition, this plasmid allowed us to monitor the increase in transcriptional activity of *fruP* during induction by fructose. In the absence of fructose, only a low level of β-galactosidase activity was detected. This activity was increased 30- to 50-fold

TABLE 3. Hydrogenase activities in the HupV$^-$ strains BSE14 and BSE15, in the Hup(UV)$^-$ strain BSE16, and in complemented strains compared with that in the wild-type strain B10 harboring plasmid pAC145, which carries an extra copy of the *hupTUV hypF* genes

Strain	Phenotype	Plasmid	Hydrogenase activity during[a]:			
			Anaerobiosis		Aerobiosis	
			MG	MN	MG	MN
B10	Wild type	pAC145 (*hupTUV hypF*)	42.9	9.2	8.4	11.2
BSE14	HupV$^-$	pAC205 (*hupT*)	20.1	27.8	51.5	44.7
		pEC3 (*hupUV*)	29.7	32.6	80.0	66.7
		pAC206 (*hupTUV*)	37.8	3.7	16.4	12.8
		pAC145 (*hupTUV hypF*)	41.8	2.5	17.3	8.9
BSE15	HupV$^-$	pAC205 (*hupT*)	2.6	2.2	3.4	4.9
		pEC3 (*hupUV*)	3.8	0.5	5.9	5.2
		pAC206 (*hupTUV*)	6.4	4.1	5.2	2.2
		pAC145 (*hupTUV hypF*)	39.8	10.8	13.7	7.2
BSE16	Hup(UV)$^-$	pAC205 (*hupT*)	24.3	27.3	65.5	58.8
		pEC3 (*hupUV*)	36.5	32.4	82.7	59.1
		pAC206 (*hupTUV*)	57.2	5.7	15.1	5.5
		pAC145 (*hupTUV hypF*)	57.2	5.0	18.7	7.2

[a] The unit of specific hydrogenase activity and the conditions are the same as in Table 2.

after 3 h of induction by fructose (0.1 mM), under aerobiosis (Table 4) as well as under anaerobiosis (not shown). The presence of plasmid pSE60 (*fruP*::*hupUV*) in the Hup(UV)$^-$ mutant BSE16 led to repression of hydrogenase activity to a level comparable to that of the wild-type strain B10. This effect was observed in the presence as well as in the absence of fructose; this suggests that the basal expression of *fruP* was high enough to allow complementation of the mutant by the HupU and HupV proteins even when they were synthesized at low levels. The HupV$^-$ mutant BSE14 was also complemented in *trans* by plasmid pSE60 (Table 4).

In conclusion, complementation in *trans* of the Hup(UV)$^-$ BSE16 mutant and of the HupV$^-$ BSE14 mutant, which overproduce hydrogenase, led to a fivefold decrease in hydrogenase activity in the complemented mutants, to the wild-type level. Thus, the HupU and HupV proteins participate in the repression of hydrogenase gene expression.

The *hupU* and *hupV* genes are expressed from the *hupT* promoter. The genetic organization and the complementation experiments presented above indicate that the *hupT*, *hupU*, and *hupV* genes form an operon. To localize the promoter of the *hupTUV* operon and study its expression, a set of translational fusions with *lacZ* as a reporter gene was constructed and introduced in the wild-type strain B10. The pPHU234 plasmid, which carries only the promoterless *lacZ* gene, was used as a control (Fig. 3).

When the sequence upstream from the ATG of *hupT* was eliminated, as is the case for the inserts of plasmids pSE52 and pSE53, no β-galactosidase activity was detectable. This result confirms that *hupV* is expressed from the *hupT* promoter. The level of *hupV* expression from plasmid pSE51 was constant under heterotrophic growth conditions and two times lower under autotrophic conditions. It is of note that the level of β-galactosidase synthesized from the *hupT* promoter (Fig. 3) is about 50- to 100-fold lower than that expressed from the activated *hupS* promoter (Table 2). Therefore, although the activity of the *hupT* promoter is some twofold higher in photoheterotrophy than in autotrophy, when it is practically nil, this activity is still very low.

Curiously, there were differences in *lacZ* expression depending on the position of the *lacZ* gene downstream from the *hupT* promoter. These differences may be explained by the nature of the translational fusions, leading to a difference in translational efficiency or to the formation of secondary structures in mRNA. Northern (RNA) blot analyses were done to determine the size and the number of transcripts of the *hupTUV* operon. Unfortunately, the very high instability of the transcripts did not allow us to carry out the experiments successfully.

DISCUSSION

Sequence comparisons have indicated that the products of the *hupU* and *hupV* genes have similarities with the small and the large subunits, respectively, of [NiFe]hydrogenases, in particular with the *Desulfovibrio baculatus* enzyme (11). It is expected that the two proteins form a dimer, as for hydrogenase. These proteins are probably located in the cytoplasmic compartment, since HupU lacks the HupS signal peptide which

TABLE 4. Complementation of the Hup(UV)$^-$ and HupV$^-$ mutants by expression of the *hupUV* genes from the fructose-inducible promoter *fruP*[a]

Strain	Phenotype	Plasmid	Fructose	H_2ase	βgal
B10	Wild type	pFRGal4	−	8.2	0.04
			+	12.7	1.2
		pSE60	−	8.8	
			+	8.3	
BSE16	Hup(UV)$^-$	pFRGal4	−	66.6	0.02
			+	60.2	1.1
		pSE60	−	18.7	
			+	13.9	
BSE14	HupV$^-$	pSE60	−	15.0	
			+	9.3	

[a] The cultures were done in duplicate in MN medium under aerobiosis. Fructose was added at a final concentration of 0.1 mM to one culture, while the other received no addition. Enzymatic activities were measured after 3 h of induction. The units for hydrogenase (H_2ase) and β-galactosidase (βgal) activities are as in Table 2.

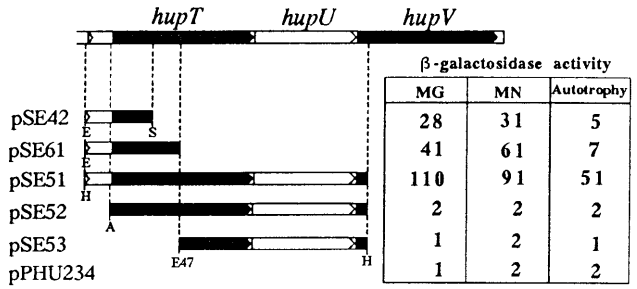

FIG. 3. β-Galactosidase activities in the wild-type strain B10 containing *lacZ* translational fusions to *hupT* and *hupV*. Cells were grown phototrophically in the absence of oxygen. The DNA fragments cloned upstream from *lacZ* in the fusion vectors are shown on the left. Specific β-galactosidase activity is in nanomoles of *o*-nitrophenol · minute^{-1} · milligram of protein^{-1}. A, *Apa*I; E, *Eco*RI; E47, *Eco*47III; H, *Hin*dIII; S, *Sty*I.

allows translocation of the protein across the membrane. HupV lacks the C-terminal extension which is proteolytically cleaved after nickel incorporation during the process of maturation of the HupL subunit (30). This C-terminal extension, present in the precursor form of the large subunit of the hydrogenase 3 of *E. coli*, has been shown recently to be necessary for Ni incorporation. It may keep the protein in a conformation required for the coordination of Ni. A mutant lacking this extension has no hydrogenase 3 activity, and the mutated enzyme lacking the C-terminal extension could not bind Ni (1). Our present aim is to obtain amounts of *R. capsulatus* HupV protein large enough to determine its metal content. Since HupU and HupV contain the potential ligands for the Fe-S clusters and for Ni, they may be expected to bind H_2 and eventually have hydrogenase activity. However, we have been unable, up to now, to detect hydrogenase activity in soluble cellular fractions.

Several genes have been found to encode proteins sharing structural motifs with the large subunit of [NiFe]hydrogenases, such as HupK in *Rhizobium leguminosarum* (28) and in *R. capsulatus* (6) and HoxV in *Alcaligenes eutrophus* (21) and in *Azotobacter vinelandii* (3, 14). These proteins show amino acid sequence conservation at the proposed Ni environment, but key residues for Ni ligation are not entirely conserved. HupK in *R. leguminosarum* has been proposed to act as a scaffold for hydrogenase Ni cofactor assembly, with the missing residues, important for ligation of Ni in the hydrogenase, permitting the cofactor to escape the scaffolding protein (18). However, HupV contains all of the cysteine residues proposed to supply the ligands to the active-site nickel (27) and indeed shown by Volbeda et al. (37) to ligand Ni in *D. gigas* [NiFe]hydrogenase. Such a structure is not compatible with a role of scaffolding protein for HupV. Furthermore, the presence of a highly active hydrogenase in the HupV$^-$ (BSE14) and Hup(UV)$^-$ (BSE16) mutants indicates that HupUV are not essential in metallocluster assembly.

We show here that HupV is involved in negative control of hydrogenase biosynthesis and that the HupU and HupV proteins act in concert with the sensor HupT. It seems likely that the HupUV dimer can be a primary sensor capable of triggering a regulatory cascade involving the histidine kinase HupT. It remains to be determined whether the stimuli detected by HupUV are H_2, O_2, nickel ions, or the three together, and it remains to be demonstrated that the HupUV proteins can interact with HupT.

Genes homologous to the *hupU* and *hupV* genes of *R. capsulatus* have been found in *B. japonicum* and in *R. sphaeroides*. In *B. japonicum*, these genes, called *hupU* and *hupV*, are located just upstream from the hydrogenase structural *hupSL* genes, with no *hypF* between *hupV* and *hupS* (2), and in *R. sphaeroides*, these recently discovered genes, termed *hupU1* and *hupU2*, have been proposed to be part of the *hupTU1U2* operon with the upstream *hupT* gene, as in *R. capsulatus* (15). Black et al. (2) showed that *B. japonicum* HupV is necessary for transcriptional activation of hydrogenase and proposed a possible nickel- or other environmental factor (i.e., oxygen or hydrogen)-sensing role for HupUV. In contrast, we have shown that in *R. capsulatus*, the HupV$^-$ or Hup(UV)$^-$ mutant expressed hydrogenase at a high level, especially in the absence of H_2. Since *B. japonicum* requires microaerophilic conditions to synthesize hydrogenase (19), there may be additional superimposed controls in response to oxygen, either in *R. capsulatus* or in *B. japonicum* or in both, which may explain the differences observed in the two bacteria.

The three proteins HupT, HupU, and HupV are encoded by three genes organized in an operon, the *hupTUV* operon, which is expressed from a promoter located upstream from *hupT*. This operon is expressed at a level comparable to that of regulatory genes, e.g., the *nifR3 ntrB ntrC* operon in *R. capsulatus* (ca. 220 nmol of *o*-nitrophenol formed per min per mg of protein) (12). The study of the *hupTUV*::*lacZ* fusions suggests that there may be regulation of the expression of the *hupTUV* operon; indeed, *hupTUV* expression is twice as high under heterotrophic growth conditions as it is during autotrophy. This is consistent with the facts that repression of hydrogenase gene transcription is higher during heterotrophy than during autotrophy and that the HupT, HupU, and HupV proteins are part of the repression system.

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