APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1994, p. 1768–1774
0099-2240/94/$04.00+0

Vol. 60, No. 6

# Increased Nitrogenase-Dependent $H_2$ Photoproduction by *hup* Mutants of *Rhodospirillum rubrum*

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Received 3 December 1993/Accepted 15 March 1994

**Transposon Tn*5* mutagenesis was used to isolate mutants of *Rhodospirillum rubrum* which lack uptake hydrogenase (Hup) activity. Three Tn*5* insertions mapped at different positions within the same 13-kb *Eco*RI fragment (fragment E1). Hybridization experiments revealed homology to the structural hydrogenase genes *hupSLM* from *Rhodobacter capsulatus* and *hupSL* from *Bradyrhizobium japonicum* in a 3.8-kb *Eco*RI-*Cla*I subfragment of fragment E1. It is suggested that this region contains at least some of the structural genes encoding the nickel-dependent uptake hydrogenase of *R. rubrum*. At a distance of about 4.5 kb from the fragment homologous to *hupSLM*, a region with homology to a DNA fragment carrying *hypDE* and *hoxXA* from *B. japonicum* was identified. Stable insertion and deletion mutations were generated in vitro and introduced into *R. rubrum* by homogenotization. In comparison with the wild type, the resulting *hup* mutants showed increased nitrogenase-dependent $H_2$ photoproduction. However, a mutation in a structural *hup* gene did not result in maximum $H_2$ production rates, indicating that the capacity to recycle $H_2$ was not completely lost. Highest $H_2$ production rates were obtained with a mutant carrying an insertion in a nonstructural *hup*-specific sequence and with a deletion mutant affected in both structural and nonstructural *hup* genes. Thus, besides the known Hup activity, a second, previously unknown Hup activity seems to be involved in $H_2$ recycling. A single regulatory or accessory gene might be responsible for both enzymes. In contrast to the nickel-dependent uptake hydrogenase, the second Hup activity seems to be resistant to the metal chelator EDTA.**

In purple nonsulfur bacteria, $H_2$ photoproduction from organic carbon sources is catalyzed by the nitrogenase complex and $H_2$ consumption is mediated by a hydrogen uptake (Hup) hydrogenase. During photoheterotrophic growth with a limiting supply of bound nitrogen, the Hup hydrogenase is expressed along with the $N_2$ fixation system. It has been suggested that the hydrogenase recycles the $H_2$ produced by the nitrogenase and thereby decreases the rate and yield of $H_2$ photoproduction (for a review, see reference 34).

Biochemical and physiological investigations revealed that the membrane-bound uptake hydrogenases from the purple bacteria *Rhodobacter capsulatus* (28), *Rhodospirillum rubrum* (22), *Chromatium vinosum* (31), and *Thiocapsa roseopersicina* (29) belong to the family of NiFe-hydrogenases. Studies on the molecular biology of $H_2$ oxidation in photosynthetic bacteria have been confined almost exclusively to *Rhodobacter capsulatus*. In this organism a 25-kb DNA region comprising 18 genes required for hydrogenase formation has been identified and completely sequenced. This region contains the structural *hup* genes, i.e., *hupS*, *hupL* and *hupM* (recently renamed *hupC* [35]), encoding hydrogenase subunits, as well as regulatory and accessory genes related to the $H_2$ oxidation system of *Rhodobacter capsulatus* (7). According to a recent classification of at least 30 hydrogenases so far sequenced, the membrane-bound NiFe-hydrogenases from, e.g., *Rhodobacter capsulatus*, *Bradyrhizobium japonicum*, *Rhizobium leguminosarum*, *Azotobacter vinelandii*, and *Escherichia coli* belong to one group (class I) of closely related hydrogenases (for reviews, see references 35 and 38).

An approach to improve nitrogenase-dependent $H_2$ photoproduction is the inactivation of the putative competing $H_2$ uptake activity. Therefore, we expanded the molecular analysis of $H_2$ oxidation systems in photosynthetic bacteria to *R. rubrum*, an organism which has been successfully used in biotechnological studies on $H_2$ photoproduction (24, 36, 39). In this report we describe the isolation and partial genetic and physiological characterization of *R. rubrum hup* mutants. Our results indicate that the $H_2$ oxidation system of *R. rubrum* is more complex than previously assumed and that very possibly at least two distinct hydrogenases are involved in $H_2$ recycling.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Strains and plasmids used in this study are listed in Table 1 and described in the text. A spontaneous streptomycin-resistant mutant of *R. rubrum* (strain K100) was used for all conjugation experiments and as the wild-type control in comparative studies. *E. coli* strains were grown at 37°C either in LB medium (26) or on PA plates (17.5 g of Penassay broth [Difco] per liter, 1.2% agar). Stock cultures of *R. rubrum* were cultivated photosynthetically at 28°C in malate-ammonium sulfate minimal medium (RCVB medium) (37). Antibiotic resistance was selected by using PY medium (20) solidified with 1.2% agar. Plates were incubated at 30°C aerobically in the dark or anaerobically in the light by using a GasPak jar (BBL Microbiology Systems Becton Dickinson). Nitrogenase derepression NIT medium is minimal medium with 7 mM L-glutamate substituting for $(NH_4)_2SO_4$ and 50 mM L-lactate instead of malate. For derepression of uptake hydrogenase, HG medium (22) with 16 mM L-lactate and 9 mM $(NH_4)_2SO_4$ was used. Nitrogenase-mediated $H_2$ photoproduction of cells growing in NIT medium was analyzed as described previously (19). Precultures were grown in the same media as experimental cultures. Antibiotics were used at the following concentrations (in micrograms per milliliter): for

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *R. rubrum* strains | | |
| S1 | Wild type | ATCC 11170 |
| K100 | Spontaneous $Sm^r$ mutant of *R. rubrum* S1 | 19 |
| K15 | $Hup^-$, insertion mutant, $Sm^r$ $Km^r$ | This work |
| K30 | $Hup^-$, insertion mutant, $Sm^r$ $Km^r$ | This work |
| K4A | $Hup^-$, deletion mutant, $Sm^r$ $Km^r$ | This work |
| *E. coli* strains | | |
| JM83 | Host for pUCplasmids | 33 |
| S17-1 | RP4-2 (Tc::Mu) (Km::Tn*7*) integrated in the chromosome | 30 |
| Plasmids | | |
| pUC8 | $Ap^r$, $lacZ_\alpha$ | 33 |
| pACYC184 | $Tc^r$ $Cm^r$ | 5 |
| pACYC184::Tn*5* | pACYC184 (Cm::$Tn5_\chi$), $Km^r$ $Tc^r$ | 21 |
| pWKR102A | *mob*, $Gm^r$ $Cm^r$ | 8 |
| pSUP1011 | pSUP101::Tn*5*, $Cm^r$ $Km^r$ | 30 |
| pAC76 | 3.5-kb *Hin*dIII fragment carrying *hupSLM* from *Rhodobacter capsulatus* cloned into pRK292, $Tc^r$ | 6 |
| pHA2 | 1.3-kb *Eco*RI-*Hin*dIII fragment from pAC76 cloned into pUC18, $Ap^r$ | H. Almon (Bonn) |
| pHA3 | 2.3-kb *Hin*dIII-*Sph*I fragment from pAC76 cloned into pUC18, $Ap^r$ | H. Almon (Bonn) |
| pHU1 | pLAFR1 carrying 25-kb *B. japonicum* DNA, $Tc^r$ | 12 |
| pMKH1.1 | 5.9-kb *Hin*dIII fragment from pHU1 cloned into pACYC184, $Cm^r$ | This work |
| pMKE1.1 | 5-kb *Eco*RI fragment from pHU1 cloned into pACYC184, $Tc^r$ | This work |
| pMK15 | 13-kb *Eco*RI fragment from *R. rubrum* carrying *hup*::Tn*5* insertion 15 cloned into pUC8, $Ap^r$ $Km^r$ | This work |
| pMK30 | 13-kb *Eco*RI fragment from *R. rubrum* carrying *hup*::Tn*5* insertion 30 cloned into pUC8, $Ap^r$ $Km^r$ | This work |
| pMK90 | 2.4-kb *Eco*RI fragment from *R. rubrum* carrying *hup*::Tn*5* insertion 90 cloned into pUC8, $Ap^r$ $Km^r$ | This work |
| pMK15X | pMK15 carrying a nontransposable derivative of Tn*5*, $Ap^r$ $Km^r$ | This work |
| pMK30X | pMK30 carrying a nontransposable derivative of Tn*5*, $Ap^r$ $Km^r$ | This work |
| pMK4A | *hup* deletion derivative originating from pMK15 and pMK30, $Ap^r$ $Km^r$ | This work |
| pMK15m | *Eco*RI fragment from pMK15X cloned into pWKR102A, $Gm^r$ $Km^r$ | This work |
| pMK30m | *Eco*RI fragment from pMK30X cloned into pWKR102A, $Gm^r$ $Km^r$ | This work |
| pMK4Am | *Eco*RI fragment from pMK4A cloned into pWKR102A, $Gm^r$ $Km^r$ | This work |

*E. coli*, ampicillin, 150; chloramphenicol, 50; gentamicin, 10; kanamycin, 25; tetracycline, 5; and for *R. rubrum*, gentamicin, 4; kanamycin, 50; streptomycin, 200.

**Bacterial matings.** Equal volumes (0.5 to 1 ml) of aerobically grown log phase *E. coli* donor cells and photoheterotrophically grown late-log-phase *R. rubrum* recipient cells were mixed, collected in a microcentrifuge tube, and resuspended in 100 μl of the supernatant. The cell mixture was spread on a membrane filter (pore size, 0.45 μm) placed on the surface of a PY plate. After 12 to 15 h of incubation at 30°C under aerobic conditions in the dark, cells were washed from the filter with 1 ml of RCV medium (without a C and N source), diluted, and plated on selective PY plates.

**Transposon Tn*5* mutagenesis and Hup screening.** The suicide vector pSUP1011 (30) was used for random Tn*5* mutagenesis of *R. rubrum* K100. $Km^r$ transconjugants were obtained after *E. coli* S17-1-mediated conjugation of pSUP1011 into *R. rubrum* K100. The donor was counterselected with streptomycin (200 μg/ml). For the Hup screening procedure, microtiter plates were used as growth and test vessels. For derepression of the uptake hydrogenase, Tn*5* insertion mutants were cultivated photoheterotrophically for two subsequent passages in HG medium supplemented with 10 μM $NiCl_2$. Under these conditions Hup activity in *R. rubrum* reached its maximum (22). To distinguish $Hup^+$ and $Hup^-$ phenotypes, we examined $H_2$-dependent methylene blue (MB) reduction. Portions (0.1 ml) of the cultures were mixed with an equal volume of MB solution (0.2 mM MB, 300 mM NaF, 8 mM EDTA, 100 mM $K_2HPO_4$ [pH 7]). Microtiter plates were transferred to a glass chamber, which was first flushed with $N_2$ to remove air and subsequently flushed with $H_2$. $Hup^+$ cultures decolorized the dye within 15 to 30 min, whereas $Hup^-$ cultures remained blue up to 90 to 120 min. Sodium fluoride included in this assay efficiently inhibited unspecific MB reduction. In addition, NaF and EDTA accelerate $H_2$-dependent MB reduction (25).

**DNA isolation and standard genetic techniques.** Large amounts of *E. coli* plasmid DNA were isolated by the lysozyme-sarcosyl method and purified through isopycnic centrifugation in CsCl-ethidium bromide gradients (2). Total DNA of *R. rubrum* was isolated in the same way, except that shearing of DNA with a syringe was omitted. Small amounts of *E. coli* plasmid DNA were isolated by a rapid-boiling method (13). A Geneclean Kit (Bio 101 Inc., La Jolla, Calif.) was used for the purification of DNA fragments separated on agarose gels. Agarose gel electrophoresis, restriction mapping, transformations, and cloning procedures were performed by standard techniques (26). Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories. Digestion and ligation conditions were as recommended by the manufacturer of the enzymes. For Southern hybridization, DNA fragments were separated in vertical 1% agarose gels, denatured, and transferred to nitrocellulose filters (Millipore). Filters were hybridized to digoxigenin-labeled probes. DNA labeling, hybridization, and detection were performed according to the standard protocol of Boehringer, Mannheim, Germany, for the digoxigenin DNA labeling and detection kit.

**Enzyme assays and quantitative determinations.** The up-

take hydrogenase activity of intact cells was measured manometrically with phenanzine methosulfate as an artificial electron acceptor, and nitrogenase activity was determined by the acetylene reduction technique as described previously (19). Specific enzyme activities are expressed in Units per milligram of protein (1 U = 1 μmol $min^{-1}$). Bacterial growth was monitored by measuring the optical density of cultures at 660 nm (*R. rubrum*) and 580 nm (*E. coli*) or measuring the cell protein by the method of Lowry et al. (23). L-Lactate and L-glutamate were determined enzymatically by using test kits from Boehringer.

## RESULTS

**Isolation of *R. rubrum hup* mutants by random Tn*5* mutagenesis.** Kanamycin-resistant ($Km^r$) transconjugants from matings between *E. coli* S17-1(pSUP1011) and *R. rubrum* K100 were isolated with a frequency of approximately $10^{-5}$ per donor. The occurrence of auxotrophic mutants as well as mutants with altered carotenoid synthesis (blue or green colonies) indicated random transposition of Tn*5* into the chromosome of *R. rubrum*. As described in Materials and Methods, a specific test system to screen for the lack of $H_2$-dependent MB reduction ($Hup^-$ phenotype) had to be adapted for *R. rubrum*. By using this method we were able to isolate four Tn*5*-induced $Hup^-$ mutants. The $Hup^-$ phenotype was confirmed by manometric measurements of $H_2$ uptake activities. Residual uptake hydrogenase activities were less than 5% of the wild-type activity (100% activity was in the range of 0.4 to 0.6 U mg of $protein^{-1}$).

**Identification of *R. rubrum hup* genes by hybridization to *B. japonicum* and *Rhodobacter capsulatus hup* genes.** Homologies between the *R. rubrum* genome and *hup* structural genes from *B. japonicum* and *Rhodobacter capsulatus* were identified by Southern hybridization experiments with subcloned fragments from cosmid pHU1 (12) and plasmid pAC76 (6) as probes. Probes P1 to P4 used in this study are outlined in Fig. 1A. As an example, the result of two hybridization experiments with probes P1 and P3 is given in Fig. 1B. A single 13-kb *Eco*RI fragment of *R. rubrum* total DNA strongly hybridized to the *hup* probe P1 (Fig. 1A), which carries the *hupSL* genes and adjacent regions from *B. japonicum*. As shown in Fig. 1B, three Tn*5* insertions (insertions 15, 83, and 30 in Fig. 1B) resulting in a $Hup^-$ phenotype were localized in this 13-kb *Eco*RI fragment (called E1). The same hybridization signals were obtained with a *hupLM* probe from *Rhodobacter capsulatus* (*hup* probe P4 in Fig. 1A). A *hupS(L)* probe from *Rhodobacter capsulatus* (probe P3 in Fig. 1A) also hybridized to the 13-kb *Eco*RI fragment. In addition, homology of a 3.3-kb *Eco*RI fragment from *R. rubrum* with probe P3 was observed (Fig. 1B).

**Cloning of *hup*::Tn*5*-containing DNA fragments and mapping of Tn*5* insertion sites.** Total DNA of Tn*5*-induced $Hup^-$ mutants of *R. rubrum* was isolated by CsCl-ethidium bromide centrifugation, digested with *Eco*RI, and ligated into pUC8. Since Tn*5* does not contain *Eco*RI sites (17), recombinant plasmids harboring *hup*::Tn*5*-containing *Eco*RI fragments could be selected by vector-encoded $Ap^r$ and Tn*5*-encoded $Km^r$. The 13-kb *Eco*RI fragment (E1) homologous to the structural genes *hupSLM* was isolated from Tn*5* mutants 15, 30, and 83. As shown in Fig. 2, the mutations mapped at different positions within this fragment. By Southern hybridization analysis of the cloned *hup*::Tn*5* *Eco*RI fragments, homology to the uptake hydrogenase structural genes from *B. japonicum* and *Rhodobacter capsulatus* (*hup* probes P1, P3, and P4 in Fig. 1A) was found in a 3.8-kb *Eco*RI-*Cla*I subfragment

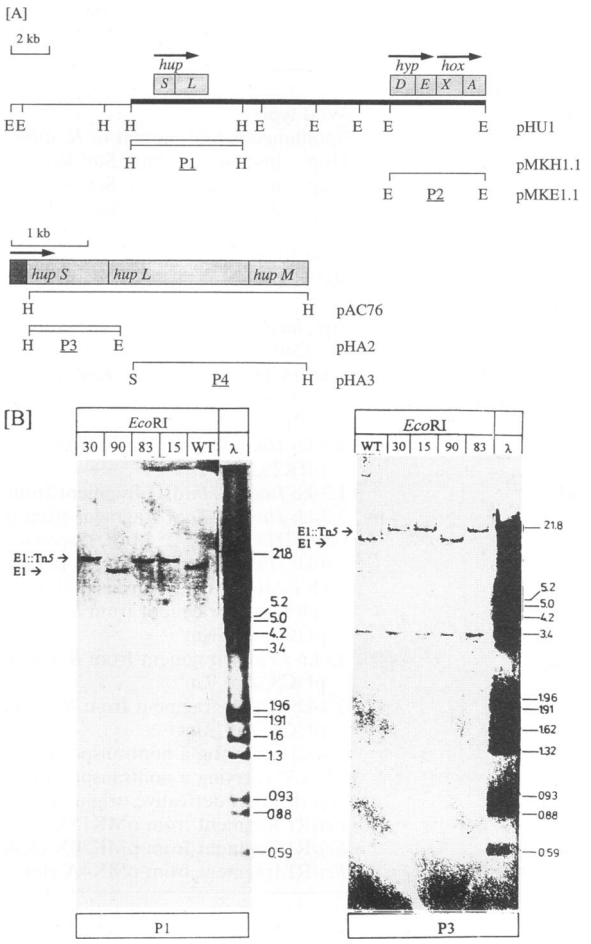


FIG. 1. Identification of *R. rubrum* DNA fragments homologous to the *hup* structural genes from *B. japonicum* and *Rhodobacter capsulatus*. (A) Physical maps of DNA inserts from cosmid pHU1, plasmid pAC76, and subclones. *B. japonicum hup* sequences reside in a 15.5-kb stretch of pHU1 (bold line) (12, 27). *hupS* and *hupL* indicate the hydrogenase structural genes for the small and large subunit, respectively. Genes involved in the regulation of hydrogenase (*hypD*, *hypE*, *hoxX*, and *hoxA*) have been identified by DNA sequence analysis 4.5 kb downstream of the structural genes (32). The 3.5-kb *Hin*dIII fragment of pAC76 contains the uptake hydrogenase structural operon (*hupSLM*) from *Rhodobacter capsulatus* except the 3′ part of *hupS* encoding the putative leader peptide of HupS (dark box) (6). Subclones of pHU1 and pAC76 carrying fragments P1 to P4 were used as probes in Southern hybridization experiments. The arrows indicate the direction of transcription. Restriction enzymes: E, *Eco*RI; H, *Hin*dIII; S, *Sph*I. (B) *Eco*RI-digested total DNA from the wild type (WT) of *R. rubrum* and Tn*5*-induced $Hup^-$ mutants was hybridized with the *B. japonicum hupSL* probe P1 (left) and the *Rhodobacter capsulatus hupS(L)* probe P3 (right). Arrows indicate the hybridizing 13-kb *Eco*RI fragment from the wild type (E1) and the corresponding fragment from Tn*5* insertion mutants (E1::Tn*5*). Sizes of λ DNA fragments (*Eco*RI-*Hin*dIII digestion) are given in kilobases. Both *hup* probes and λ DNA were labeled with digoxigenin.

(called *hup* region A in Fig. 2) of fragment E1. Tn*5* mutation 15 mapped in this fragment. A 4.9-kb *Eco*RI fragment from pHU1 (probe P2 in Fig. 1A), which contains regulatory (*hoxXA*) and accessory (*hypDE*) genes required for hydrogenase formation in *B. japonicum* (32), hybridized to a second region (called *hup* region B in Fig. 2) of fragment E1. Tn*5*

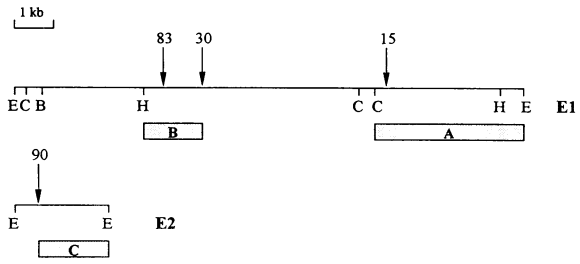


FIG. 2. Physical maps of *R. rubrum* DNA fragments containing *hup* genes. Restriction maps are given for the enzymes *Bam*HI (B), *Cla*I (C), *Eco*RI (E), and *Hin*dIII (H). Vertical arrows indicate Tn*5* insertion sites in different Hup$^-$ mutants. Bars below the physical maps indicate regions homologous to *Rhodobacter capsulatus* and *B. japonicum hup* genes. The *hup* probes used in Southern hybridization experiments are outlined in Fig. 1A. The uptake hydrogenase structural genes (probes P1, P3, and P4) hybridized to *hup* region A, which was localized in fragment E1. Homology to regulatory/accessory *hup* genes from *B. japonicum* (probe P2) was found in fragments E1 (*hup* region B) and E2 (*hup* region C).

mutations 30 and 83 mapped within *hup* region B, which is localized about 4.5 kb from *hup* region A.

A further Tn*5* mutation exhibiting a Hup$^-$ phenotype (mutation 90 in Fig. 2) was mapped in a 2.4-kb *Eco*RI fragment (called E2). The cloned fragment E2 also hybridized to the 4.9-kb *Eco*RI fragment from pHU1 (probe P2 in Fig. 1A) and represents a *hup*-specific sequence from *R. rubrum* called *hup* region C.

**Construction of stable *hup* mutants of *R. rubrum*.** Although Tn*5* exhibits relatively high stability once established in a genome (3), reversion of Tn*5*-induced mutations by precise excision of the transposon or further transposition events cannot be excluded. To construct truncated, nontransposable Tn*5* derivatives, an internal 2.3-kb *Xho*I fragment of Tn*5* containing genes essential for transposition (16) was deleted from the cloned Tn*5* insertions 15 and 30, yielding plasmids pMK15X and pMK30X, respectively (Fig. 3a and b). As shown in Fig. 3c, a 4.5-kb fragment of chromosomal *R. rubrum* DNA located between Tn*5* insertions 15 and 30 was deleted by fusing plasmids pMK15X and pMK30X. The resulting *Eco*RI fragments carrying either insertions of nontransposable Tn*5* derivatives or the 4.5-kb deletion were subsequently cloned into the suicide vector pWKR102A, transformed into *E. coli* S17-1, and mated into *R. rubrum* K100. Since pWKR102A does not replicate in *R. rubrum*, homogenotization (double-crossover recombination) of the corresponding mutations was selected by Km$^r$ and test for loss of vector-encoded Gm$^r$. Double-crossover events were verified by Southern hybridization experiments (data not shown). According to this strategy, two stable *R. rubrum* mutant strains, designated K15 and K30, carrying an insertion in a region homologous to the structural *hup* genes (*hup* region A in Fig. 2) or in a fragment hybridizing to nonstructural *hup*-specific sequences from *B. japonicum* (*hup* region B in Fig. 2), respectively, were isolated. In addition, a 4.5-kb fragment located between Tn*5* insertions 15 and 30 (Fig. 3c) was deleted, resulting in mutant strain K4A. As revealed by manometric measurements of $H_2$ uptake activities and $H_2$-dependent MB reduction assays, all *hup* mutants exhibited less than 5% of wild-type Hup activity. Photoheterotrophic growth of *hup* mutants in RCVB medium, as well as in hydrogenase and nitrogenase derepression media, was similar to that of the wild type (data not shown).

**Comparative physiological studies on $H_2$ photoproduction by wild-type and *hup* mutants of *R. rubrum*.** $H_2$ photoproduction of batch cultures grown in lactate-glutamate medium was analyzed. $H_2$ production by the wild type has been shown to decline markedly in cultures older than 100 h and to disappear after 120 h. This is paralleled by a twofold increase in uptake hydrogenase activity (19). In contrast, stationary-phase cultures of *hup* mutants continued to produce $H_2$. Interestingly, two different phenotypes were observed for *hup* mutants as follows (Fig. 4; Table 2). (i) Stationary-phase cultures of mutant K15 liberated $H_2$ with a relatively low rate of 10 ml of $H_2$ $h^{-1}$ g (dry weight)$^{-1}$ over a period of 28 days. The yield of

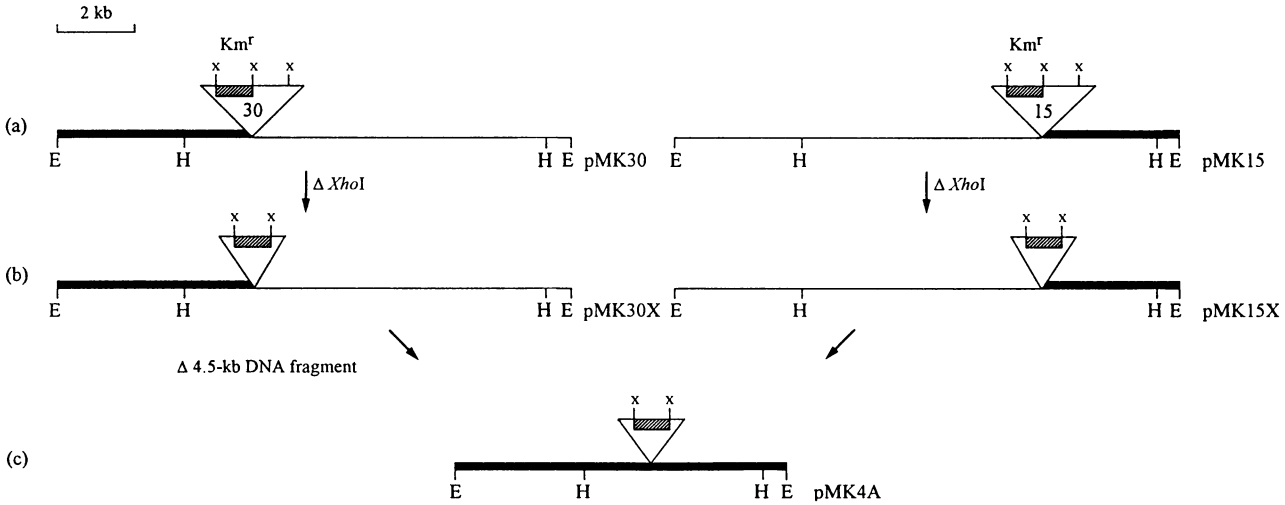


FIG. 3. In vitro construction of stable insertion and deletion mutations in *R. rubrum hup* DNA. (a) *Eco*RI fragments isolated from Tn*5* mutations 30 and 15 (plasmids pMK30 and pMK15, respectively). Tn*5*, represented by triangles, is not drawn to scale. Only the *Xho*I sites and the location of the Km$^r$ gene (hatched box) within Tn*5* are indicated. (b) Truncated Tn*5* derivatives were constructed by deleting one *Xho*I fragment of Tn*5*, which carries genes essential for transposition. (c) Fusion of the left and right *Eco*RI-*Xho*I fragments (bold lines) from pMK30X and pMK15X flanking the Tn*5*-derived Km$^r$ gene resulted in the deletion derivative pMK4A. A 4.5-kb fragment of *R. rubrum* DNA located between both Tn*5* insertions was deleted in this plasmid. Abbreviations: E, *Eco*RI; H, *Hin*dIII; X, *Xho*I.

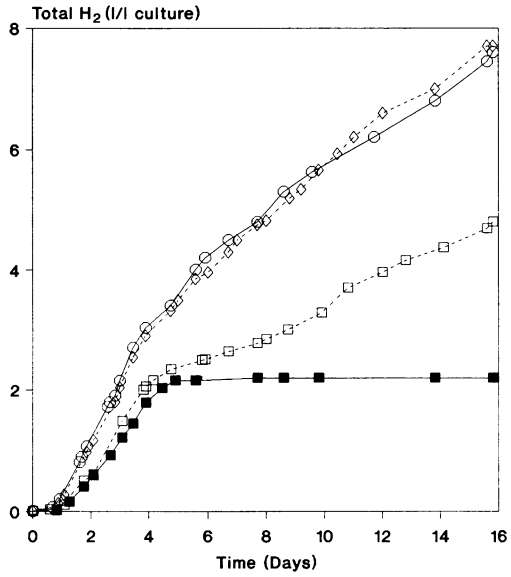


FIG. 4. $H_2$ accumulation in cultures of the wild type and *hup* mutants of *R. rubrum*. The total amounts of $H_2$ (expressed in liters per liter of culture) liberated during batch cultivation in lactate-glutamate medium over a period of 16 days are shown. Within this period the insertion mutant K30 and the deletion mutant K4A reached the same $H_2$ yields. Mutant K15 continued to produce $H_2$ up to 28 days (data not shown). Symbols: ■, $H_2$ production by the wild type; □, $H_2$ production by K15; ○, $H_2$ production by K30; ◇, $H_2$ production by K4A.

$H_2$ from lactate was 73% ($H_2$ yield from the wild type was 52%). During the exponential and early stationary growth phases, $H_2$ liberation by mutant K15 was comparable to that by the wild type. (ii) Mutants K30 and K4A, respectively, produced $H_2$ at significantly higher rates than K15 did. $H_2$ production rates by stationary-phase cultures were about 22 ml of $H_2$ $h^{-1}$ g (dry weight)$^{-1}$ and thus were twice the rate observed with mutant K15. In addition, during the first 5 days these mutants exhibited significantly increased $H_2$ production rates (Table 2) and produced considerably larger amounts of $H_2$ than did the wild type and mutant K15 (Fig. 4). The high $H_2$ production rates observed with mutants K30 and K4A were paralleled by increased $H_2$ yields (Table 2). It is notable that $H_2$ production by *hup* mutants continued at low rates even when lactate was exhausted, so that the total amount of $H_2$ collected exceeded the theoretical maximum based on lactate consumption. A possible explanation for this observation is that glutamate, besides supplying nitrogen for growth, was converted into $H_2$. Therefore, the theoretical amount of $H_2$ related to glutamate dissimilation was subtracted from the measured volume of total $H_2$ produced. By using this corrected value, an approximate lactate-related $H_2$ yield was calculated (Table 2; see also Table 3).

In a previous communication we reported that the metal chelator EDTA stimulated $H_2$ photoproduction of *R. rubrum* by (i) inhibition of the uptake hydrogenase as a result of a decreased Ni availability and (ii) increasing nitrogenase activity as a result of mobilization of iron (19). To further characterize the $H_2$ oxidation system operating in nitrogenase-derepressed cells of *R. rubrum*, we analyzed the effect of EDTA on $H_2$ photoproduction by *hup* mutants. As reported for the wild type, the addition of 0.5 mM EDTA to lactate-glutamate medium significantly increased nitrogenase activities of all *hup* mutants (up to 120 mU mg of protein$^{-1}$) (Table 3). EDTA-supplemented cultures of the wild type and mutant K15 showed exactly the same kinetics of $H_2$ production and produced a total amount of 6.8 liters of $H_2$ per liter of culture, corresponding to an $H_2$ yield of 73% (Table 3). However, in comparison with the wild type and mutant K15, EDTA-containing cultures of mutants K30 and K4A exhibited higher $H_2$ production rates and yielded the highest levels of $H_2$ (8.2 to 8.3 liters of $H_2$ per liter) in long-term experiments (Table 3).

## DISCUSSION

The Tn*5* mutagenesis system adapted here for *R. rubrum* was shown to be effective for the isolation of mutants impaired in their ability to oxidize $H_2$. The Hup$^-$ phenotypes described in this study are characterized by the lack of $H_2$-dependent reduction of MB and phenanzine methosulfate. By mapping the Tn*5* insertions, two *Eco*RI fragments of 13 kb (called E1) and 2.4 kb (called E2) carrying *R. rubrum hup* genes were identified. On the basis of hybridization experiments, three *hup* regions (regions A, B, and C in Fig. 2) homologous to genes involved in $H_2$ metabolism of *Rhodobacter capsulatus* and *B. japonicum* were localized on these two fragments.

Homology to the hydrogenase structural genes *hupSLM* from *Rhodobacter capsulatus* and *hupSL* from *B. japonicum* found in *hup* region A of *R. rubrum* correlated well with the Tn*5* insertion in position 15 (Fig. 2). Cauvin et al. (4) have shown that a *hupM* mutation in *Rhodobacter capsulatus* resulted in a physiologically inactive hydrogenase (Aut$^-$ phenotype) but did not affect $H_2$-dependent MB reduction. Since

TABLE 2. $H_2$ photoproduction with the wild type and *hup* mutants of *R. rubrum*[a]

| Strain | Phenotype | $H_2$ production rate (ml of $H_2$/h/g (dry wt) after: | | Total $H_2$ produced in 16 days (liters/liter)[b] | $H_2$ yield (%)[c] | Maximum nitrogenase activity (mU/mg of protein)[d] |
|---|---|---|---|---|---|---|
| | | 2–5 days | >5 days | | | |
| K100 | Wild type | 25 ± 4 | 0 | 2.2 ± 0.1 | 52 | 46 |
| K15 | Hup | 25 ± 3 | 10 ± 2 | 4.5 ± 0.1 | 73[e] | 49 |
| K30 | Hup | 39 ± 2 | 22 ± 2 | 7.3 ± 0.1 | 82 | 60 |
| K4A | Hup | 41 ± 3 | 22 ± 2 | 7.3 ± 0.1 | 82 | 60 |

[a] Batch cultures were grown in NIT medium containing 50 mM L-lactate and 7 mM L-glutamate. Data are means of duplicate determinations.
[b] Total $H_2$ expressed in liters per liter of culture. $H_2$ was collected in a calibrated cylinder filled with a $CO_2$ trap (4% NaOH, 20% NaCl). Gas samples were analyzed by gas chromatography (19).
[c] Yield of $H_2$ from L-lactate consumed as a percentage of the theoretical maximum (values were corrected for L-glutamate dissimilation [see text]).
[d] Specific nitrogenase activities of whole cells, expressed as nanomoles of $C_2H_4$ per minute per milligram of protein, were determined on the basis of acetylene reduction.
[e] The total amount of $H_2$ produced over a period of 28 days was 6.7 liters/liter.

TABLE 3. $H_2$ photoproduction in the presence of 0.5 mM EDTA[a]

| Strain | Phenotype | Stationary-phase $H_2$ production rates (ml of $H_2$/h/g [dry wt]) | Total $H_2$ produced in 15 days (liters/liter)[b] | $H_2$ yield (%)[c] | Maximum nitrogenase activity (mU/mg of protein)[d] |
|---|---|---|---|---|---|
| K100 | Wild type | 30 ± 2 | 6.8 | 73.5 | 120 |
| K15 | $Hup^-$ | 30 ± 2 | 6.8 | 73.5 | 110 |
| K30 | $Hup^-$ | 40 ± 2 | 8.2 | 93 | 120 |
| K4A | $Hup^-$ | 42 ± 3 | 8.3 | 95.3 | 120 |

[a] Batch cultures were grown in NIT medium supplemented with 0.5 mM EDTA.
[b] See Table 2, footnote *b*.
[c] See Table 2, footnote *c*.
[d] See Table 2, footnote *d*.

mutant K15 of *R. rubrum* showed no discernible $H_2$-dependent MB reduction, it is likely that Tn*5* insertion 15 is located in *hupS* or *hupL* but not in *hupM*. In addition to the 13-kb *Eco*RI fragment containing the structural *hup* genes of *R. rubrum*, a 3.3-kb *Eco*RI fragment hybridized to the *Rhodobacter capsulatus hupS(L)* probe P3 (Fig. 1). Since it has been shown that proteins of the Ni-dependent carbon monoxide oxidation system in *R. rubrum* contain cysteine and histidine motifs similar to those conserved in Ni-containing hydrogenases (18), it cannot be excluded that sequences not specific for the $H_2$ oxidation system are located on this fragment.

At a distance of about 4.5 kb from *hup* region A of *R. rubrum*, we identified *hup* region B by homology to a 4.9-kb *Eco*RI fragment from pHU1 (Fig. 2). Recently, this fragment and an adjacent region of *B. japonicum* were sequenced and the genes *hypD*, *hypE*, *hoxX*, and *hoxA* were identified by homology to genes involved in $H_2$ metabolism by *E. coli*, *Rhodobacter capsulatus*, *Alcaligenes eutrophus*, and *Azotobacter vinelandii* (32). No distinct functions have yet been assigned to the *hypD* and *hypE* gene products, which are thought to be somehow involved in hydrogenase processing (14). The *hoxA* gene product belongs to the NtrC family of transcriptional regulators, and it has been speculated that HoxX and HoxA may represent a two-component regulatory system (11). The *hoxA* counterpart in *Rhodobacter capsulatus* (*hupR*$_1$) is localized 5.3 kb downstream of the *hupSLM* operon and is also closely linked to *hyp* genes (7). Since the aforementioned genes show a high degree of conservation in different species, it is assumed that the *hup* region B of *R. rubrum* might contain analogous regulatory or accessory genes, or both, required for $H_2$ uptake activity. This assumption is supported by the characteristics of the corresponding *hup* mutants discussed below.

The use of cloned *hup*::Tn*5* DNA allowed us to construct stable *hup* mutations (Fig. 3), which were used to replace wild-type *hup* regions by homogenotization. Considering our hybridization results, we suggest that the insertion mutations are localized in one of the structural *hup* genes and in a regulatory or accessory gene in mutants K15 and K30, respectively, whereas the deletion mutant K4A is devoid of both *hup* gene regions. The phenotypic characteristics of these *R. rubrum hup* mutants revealed that the $H_2$ oxidation system of *R. rubrum* efficiently recycles the $H_2$ produced by the nitrogenase. However, importantly, mutant K15 exhibited lower $H_2$ production rates than mutants K30 and K4A, indicating that a mutation in a structural *hup* gene did not completely abolish the capacity to oxidize $H_2$ (Table 2; Fig. 4). Thus, besides the known uptake hydrogenase (now called hydrogenase 1), at least one more hydrogenase (hydrogenase 2) seems to be involved in $H_2$ recycling. Comparison of $H_2$ accumulation in cultures of wild-type *R. rubrum* and the two types of *hup* mutants described in this study (K15 versus K30 and K4A [Fig. 4]) demonstrated that a physiologically active form of hydrogenase 1 is formed only under conditions of substrate limitation in the late stationary growth phase. In agreement with results of earlier studies (34), it is therefore very likely that $H_2$ oxidation by hydrogenase 1 is coupled to the photoreduction of $CO_2$. In contrast to hydrogenase 1, hydrogenase 2 recycles $H_2$ even when the organic substrate is present at high levels. The physiological function of this enzyme remains to be elucidated.

The highest average $H_2$ yield from lactate (82%) was reached with mutants K30 and K4A, indicating that both mutants are devoid of hydrogenase 1 and hydrogenase 2. Since the deletion mutant K4A and the insertion mutant K30 exhibited the same phenotype, it is reasonable to suggest that a gene located in *R. rubrum hup* region B is responsible for both hydrogenases. While definitive assignation of a pleiotropic *hup* gene in *R. rubrum* awaits further characterization, it is notable that in *Alcaligenes eutrophus* the expression of two distinct hydrogenases which differ in cellular location, subunit composition, and cofactor content is regulated by the *hoxA* gene product (9). Furthermore, *hypD* and *hypE* are required for the formation of all three hydrogenase isoenzymes in *E. coli* (14).

Additional support for the existence of two distinct hydrogenases in *R. rubrum* is provided by results of experiments on $H_2$ production by EDTA-supplemented cultures (Table 3). In the presence of EDTA, $H_2$ production by mutant K15 was comparable to that by the wild type. This confirms our previous observation that EDTA completely inhibits synthesis of the Ni-dependent uptake hydrogenase in *R. rubrum* (22). The increased $H_2$ production by EDTA-supplemented cultures of mutants K30 and K4A may indicate that hydrogenase 2 does not depend on nickel. Whether this enzyme belongs to the group of Fe hydrogenases remains to be investigated. These assumptions predict hydrogenase 2 activity in nitrogenase-derepressed cells of mutant K15. However, we failed to detect MB- and phenazine methosulfate-linked Hup activity in this mutant. This may be due to a poor affinity of the enzyme to the electron acceptors used or to a high oxygen sensitivity which has been reported for Fe hydrogenases isolated from strictly anaerobic bacteria (1, 10).

During this work on *R. rubrum*, a *hupL* mutant of *Rhodobacter capsulatus* was isolated by interposon mutagenesis (15). In agreement with our results on $H_2$ production by *R. rubrum*, it was shown for *Rhodobacter capsulatus* that by using *hup* mutants the efficiency of $H_2$ production could be significantly increased. However, the $H_2$ yield reported for the *Rhodobacter capsulatus hupL* mutant (15) was in the range of that obtained with mutants K30 and K4A of *R. rubrum*. This may indicate that in contrast to *R. rubrum*, *Rhodobacter capsulatus* does not possess an additional $H_2$ oxidation system.

## ACKNOWLEDGMENTS

We thank P. M. Vignais and H. Almon for providing plasmids. M. Kern thanks W. Klipp for help and hospitality extended to her.

This work was supported by a grant of the Bundesministerium für Forschung und Technologie, Bonn, Germany, as part of the research project Biological Hydrogen Formation.

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