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# Involvement of *hyp* Gene Products in Maturation of the $H_2$-Sensing [NiFe] Hydrogenase of *Ralstonia eutropha*

THORSTEN BUHRKE,[1] BORIS BLEIJLEVENS,[2] SIMON P. J. ALBRACHT,[2] AND BÄRBEL FRIEDRICH[1]*

*Institut für Biologie, Humboldt-Universität zu Berlin, 10115 Berlin, Germany,[1] and Swammerdam Institute for Life Sciences, Biochemistry, University of Amsterdam, NL-1018 TV Amsterdam, The Netherlands[2]*

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**The biosynthesis of [NiFe] hydrogenases is a complex process that requires the function of the Hyp proteins HypA, HypB, HypC, HypD, HypE, HypF, and HypX for assembly of the $H_2$-activating [NiFe] site. In this study we examined the maturation of the regulatory hydrogenase (RH) of *Ralstonia eutropha*. The RH is a $H_2$-sensing [NiFe] hydrogenase and is required as a constituent of a signal transduction chain for the expression of two energy-linked [NiFe] hydrogenases. Here we demonstrate that the RH regulatory activity was barely affected by mutations in *hypA*, *hypB*, *hypC*, and *hypX* and was not substantially diminished in *hypD*- and *hypE*-deficient strains. The lack of HypF, however, resulted in a 90% decrease of the RH regulatory activity. Fourier transform infrared spectroscopy and the incorporation of $^{63}Ni$ into the RH from overproducing cells revealed that the assembly of the [NiFe] active site is dependent on all Hyp functions, with the exception of HypX. We conclude that the entire Hyp apparatus (HypA, HypB, HypC, HypD, HypE, and HypF) is involved in an efficient incorporation of the [NiFe] center into the RH.**

Hydrogen plays a major role in bacterial energy metabolism. Many microorganisms can generate reducing power by hydrogen oxidation, while others can release excess reducing equivalents in the form of dihydrogen. Both reactions are catalyzed by enzymes called hydrogenases. The family of [NiFe] hydrogenases is most widespread in nature (for a review, see reference 1). Crystallographic and spectroscopic analyses of hydrogenases from sulfate-reducing bacteria revealed a structure consisting of a large catalytic site-containing subunit and a small three iron sulfur cluster-containing electron-transferring subunit. The $H_2$-activating site is a bimetallic center carrying a nickel and an iron atom. The two metals are coordinated by thiolate groups provided by four cysteine residues, and the iron bears three nonprotein ligands: one CO and two $CN^-$'s (17, 30, 44).

The assembly of the [NiFe] active site is a complex process that requires at least six accessory gene products, the HypA, HypB, HypC, HypD, HypE, and HypF proteins (for a review, see reference 7). HypB is able to bind $Ni^{2+}$ ions (16, 35) and displays GTPase activity, which is required for nickel incorporation (26). HypC is considered a chaperone assisting metal center assembly (24). Recent studies showed that HypF is involved in the incorporation of CO and/or $CN^-$ and that carbamoylphosphate serves as the source of these diatomic ligands (29). The precise roles of HypA, HypD, and HypE are not yet defined. Recently, it was demonstrated that HypE and HypF of *Helicobacter pylori* interact in the yeast two-hybrid assay (33). A few organisms contain an additional open reading frame, HypX, that is necessary to obtain high level of hydrogenase activity (6, 11, 34). The last step in the maturation of [NiFe] hydrogenases is catalyzed by a specific endopeptidase which cleaves off a short peptide from the C terminus of the large subunit prior to oligomerization of the polypeptides (15, 40).

The facultative lithoautotrophic proteobacterium *Ralstonia eutropha* H16 possesses two energy-linked [NiFe] hydrogenases, a membrane-bound hydrogenase (MBH) coupled to the respiratory chain via a *b*-type cytochrome (3, 36) and a cytoplasmic hydrogenase (SH) that displays $NAD^+$-reducing activity (37, 42). The SH and MBH structural genes are clustered on megaplasmid pHG1 of *R. eutropha* in two distinct operons, together with MBH- and SH-specific accessory genes (38). A complete set of *hyp* genes (*hypA1B1F1CDEX*) is associated with the MBH operon (10). Three of the *hyp* genes form a second copy (*hypA2B2F2*) downstream of the SH genes (45). Mutations in any of the *hyp* genes have a pleiotropic effect on the SH and MBH, leading to a substantial decrease or a complete loss of enzymatic activity due to a failure to assemble the [NiFe] active site (6, 10, 45). The duplicated *hyp* gene products compensate for each other physiologically.

Hydrogenase gene expression in a number of *R. eutropha* strains depends on the availability of molecular hydrogen. $H_2$ is recognized by the cells via an intracytoplasmic protein complex consisting of a regulatory hydrogenase (RH) and the histidine protein kinase HoxJ. The signal is transmitted on the DNA level by the response regulator HoxA (21). The hydrogen-sensing RH shows typical features of a subclass of [NiFe] hydrogenases (18). Counterparts of this protein are present in *Rhodobacter capsulatus* (13) and *Bradyrhizobium japonicum* (5). Studies with soluble extracts (31) and purified RH from *R. eutropha* (2) showed a [NiFe] active site with electron paramagnetic resonance and Fourier transform infrared (FTIR) spectral properties resembling those of standard [NiFe] hydrogenases. The redox properties and the activity, however, dramatically differed. Although RH-like proteins show enzymatic activity in assays, such as the $H_2$-dependent reduction of redox dyes or the $D_2/H^+$ exchange, the absolute activity is ca. 2

* Corresponding author. Mailing address: Institut für Biologie, Humboldt-Universität zu Berlin, Chausseestr. 117, 10115 Berlin, Germany. Phone: (49) 30-20-93-81-01. Fax: (49) 30-20-93-81-02. E-mail: baerbel.friedrich@rz.hu-berlin.de.

TABLE 1. Strains and plasmids

| Strain or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| *R. eutropha* | | |
| H16 | Wild type, $SH^+$ $MBH^+$ $RH^+$ $HoxJ^-$ (*hoxJg*1264a) | DSM428, ATCC 17699 |
| HF470 | $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) $RH^+$ | This study |
| HF510 | $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) $RH^-$ (*hoxC*Δ) | This study |
| HF410 | *hypA1*Δ *hypA2*Δ $HoxJ^-$ | 45 |
| HF417 | *hypB1*Δ *hypB2*Δ $HoxJ^-$ | 45 |
| HF441 | *hypF1*Δ *hypF2*Δ $HoxJ^-$ | 45 |
| HF340 | *hypC*Δ $HoxJ^-$ | 10 |
| HF338 | *hypD*Δ $HoxJ^-$ | 10 |
| HF339 | *hypE*Δ $HoxJ^-$ | 10 |
| HF469 | *hypX*Δ $HoxJ^-$ | 6 |
| HF503 | *hypA1*Δ *hypA2*Δ $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) | This study |
| HF504 | *hypB1*Δ *hypB2*Δ $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) | This study |
| HF505 | *hypF1*Δ *hypF2*Δ $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) | This study |
| HF506 | *hypC*Δ $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) | This study |
| HF507 | *hypD*Δ $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) | This study |
| HF508 | *hypE*Δ $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) | This study |
| HF509 | *hypX*Δ $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) | This study |
| HF573 | $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) *nor*(*R2A2B2*)Δ::Φ(*hoxK′-lacZ*) | This study |
| HF439 | *hyp1*Δ (*hyp*[*A1B1F1CDEX*]Δ) | This study |
| HF575 | *hyp1*Δ *hyp2*Δ *(hyp[A1B1F1CDEX]*Δ) *(hyp[A2B2F2]*Δ) | This study |
| HF581 | *hyp1*Δ *hyp2*Δ $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) $RH^+$ *nor*(*R2A2B2*)Δ::Φ(*hoxK′-lacZ*) | This study |
| HF582 | *hyp1*Δ *hyp2*Δ $SH^-$ (*hoxH*Δ) $MBH^-$ (*hoxG*Δ) $RH^-$ (*hoxC*Δ) *nor(R2A2B2)*Δ::Φ(*hoxK′-lacZ*) | This study |
| *E. coli* | | |
| JM109 | F′ *traD36 lacI*$^q$ Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/e14$^-$ ($McrA^-$) Δ(*lac-proAB*) *thi gyrA96* ($Nal^r$) *endA1 hsdR17*($r_K^-$ $m_K^+$) *relA1 supE44 recA1* | 46 |
| S17-1 | $Tra^+$ *recA pro thi hsdR chr*::RP4-2 | 39 |
| Plasmids | | |
| pBluescript SK− | $Ap^r$, *lacZ′*, T7 gene 10 promoter, f1 *ori* | Stratagene Cloning Systems |
| LITMUS 28 | $Ap^r$, *lacZ′*, ColE1 *ori* | New England Biolabs |
| pLO1/pLO2 | $Km^r$, *sacB*, RP4 *oriT*, ColE1 *ori* | 22 |
| pEDY309 | RK2 *ori*, $Tc^r$, $Mob^+$, promoterless *lacZ* gene | 18 |
| pCH297 | 3.5-kb *Kpn*I fragment containing *′hypDEXhoxA′* in pBluescript SK− | 22 |
| pCH412 | 2.1-kb *Sma*I-*Pvu*II fragment containing *hypA1*Δ in pLO1 (*Pme*I cut) | 9 |
| pCH547 | 2.7-kb fragment containing a 6.3-kb in-frame deletion in *hypA1B1F1CDEX* in pLO1 | This study |
| pCH371 | 2.7-kb *Kpn*I-*Xba*I fragment containing *hypB1F1* in pTZ18R | J. Dernedde and B. Friedrich |
| pCH424 | pLO1 with a 2.2-kb *Sal*I-*Sma*I fragment containing *hoxG*Δ | 4 |
| pCH455 | 15-kb *Hin*dIII fragment of pGE15 containing the SH gene region in pBluescript KS+ | 27 |
| pCH474 | pLO1 with a 976-bp *Sac*I fragment containing *hoxH*Δ | 27 |
| pCH615 | 0.8-kb *Eco*47III fragment containing *′hoxJ* into *Pme*I-digested pLO3 | 21 |
| pCH644 | pLO1 with a 2.45-kb *Pst*I fragment containing *hoxC*Δ | 21 |
| pCH857 | 5.1-kb *Ecl*136II-*Bgl*II fragment of pCH455 cloned into LITMUS 28 (*Eco*RV-*Bgl*II cut) | This study |
| pCH858 | 4.1-kb fragment of a *Pvu*II partial digest of pCH857, religated | This study |
| pCH859 | 1.4-kb *Bgl*II fragment (Klenow treated) of pCH858 cloned into the *Pme*I site of pLO2 | This study |
| pCH872 | *nor*(*R2A2B2*)Δ::Φ(*hoxK′-lacZ*) in pLO1 | O. Lenz and B. Friedrich |
| pGE6 | 8.6-kb *Eco*RI fragment containing the *hyp1* region in pVK101 | 12 |
| pGE15 | 15.0-kb *Hin*dIII fragment of pHG1 in pVK101 | 42 |
| pGE151 | Derivative of pRK404 | 19 |
| pGE301 | Φ(*hoxK′-lacZ*), $Tc^r$ | 22 |
| pGE378 | pEDY309 with a 2.8-kb *Hin*dIII-*Xba*I fragment containing $P_{SH}$-*hoxB-hoxC* and with a 2.2-kb *Pvu*II-*Ecl*136II fragment containing $P_{lac}$-*hoxA* | 18 |
| pGE457 | 1.4-kb *Pst*I-*Eco*RV fragment of pCH371 containing *hypF1* cloned into *Pst*I-*Ecl*136II-cut pGE151 | This study |

[a] $Nal^r$, nalidixic acid resistant; $Tc^r$, tetracycline resistant; $Ap^r$, ampicillin resistant; $Km^r$, kanamycin resistant.

orders of magnitude lower than that of energy-linked [NiFe] hydrogenases (2, 43). Furthermore, unlike standard cases, the RH-type protein lacks a C-terminal extension in the large subunit; therefore, it is conceivable to exclude a proteolytic step in the maturation of this protein (18). This observation raises the question as to whether the Hyp protein-assisted metal center assembly process participates in RH activation.

We show here that HypF is almost indispensable for the synthesis of active RH. Mutations in the remaining *hyp* genes affect the regulatory capacity of the RH to a lesser extent but clearly decrease its $H_2$-oxidizing activity if the RH is expressed at an elevated level.

## MATERIALS AND METHODS

**Strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. Strains with the initials HF were derived from wild-type *R. eutropha* H16. *Escherichia coli* JM109 (46) was used for standard cloning procedures, and *E. coli* S17-1 (39) was used for conjugative plasmid transfer to *R. eutropha* strains.

A 1.4-kb *Ssp*I-*Ecl*136II fragment of pCH297 was cloned into the *Ecl*136II site of pCH412. The resulting plasmid pCH547 harbors a 6,303-bp in-frame deletion in the *hyp1* region of the MBH operon (*hyp*[*A1B1F1CDEX*]Δ; *hyp1*Δ). For construction of a deletion in the *hyp2* region of the SH operon, a pCH455-derived 5.1-kb *Ecl*136II-*Bgl*II fragment was subcloned into the *Eco*RV-*Bgl*II-cut LITMUS 28. The resulting plasmid, pCH857, was partially digested with *Pvu*II, and a 4.1-kb fragment was religated to give pCH858, which contains a 3,780-bp deletion in the *hyp2* region (*hyp*[*A2B2F2*]Δ; *hyp2*Δ). Finally, *hyp2*Δ was inserted

as a 1.4-kb Klenow-treated *Bgl*II fragment into the *Pme*I site of pLO2, yielding plasmid pCH859.

For complementation studies, *hypF1* was cloned as a 1.4-kb *Pst*I-*Eco*RV fragment derived from pCH371 into *Pst*I-*Ecl*136II-cut pGE151 to give pGE457.

**Media and growth conditions.** *E. coli* strains were grown in Luria broth (LB). *R. eutropha* strains were grown in modified LB medium containing 0.25% (wt/vol) sodium chloride (LSLB) or in mineral salts medium (38) containing 0.4% fructose (FN) or a mixture of fructose and glycerol (0.2% [wt/vol] each; FGN) as the carbon sources. Sucrose-resistant segregants of *sacB*-harboring strains were selected on LSLB plates containing 15% (wt/vol) sucrose (22). Solid media contained 1.5% (wt/vol) agar. Antibiotics were used at the following concentrations: 350 μg of kanamycin $ml^{-1}$ and 15 μg of tetracycline $ml^{-1}$ for *R. eutropha* and 25 μg of kanamycin $ml^{-1}$, 15 μg of tetracycline $ml^{-1}$, and 100 μg of ampicillin $ml^{-1}$ for *E. coli*.

**Conjugative plasmid transfer and gene replacement.** Mobilizable plasmids were transferred from *E. coli* to *R. eutropha* by using a spot mating technique (39). Gene replacement in *R. eutropha* was achieved by using an allelic exchange procedure based on the conditionally lethal *sacB* gene (22). The resulting isolates were screened for the presence of the desired mutation by PCR amplification of the respective target site (4). Deletion-carrying isolates were identified on the basis of the altered electrophoretic mobility of the amplification products. Suicide plasmids pCH424 (*hoxG*Δ), pCH474 (*hoxH*Δ), and pCH644 (*hoxC*Δ) were used for the deletion of the genes for the large subunits of the MBH, SH, and RH, respectively. The *hoxJ*a1264g exchange was achieved by using pCH615. The *hyp1* region of the MBH operon was deleted in *R. eutropha* H16 by using plasmid pCH547, yielding HF439 (*hyp*[*A1B1F1CDEX*]Δ; *hyp1*Δ). Subsequently, the *hyp2* region of the SH region was deleted in HF439 by using plasmid pCH859 to generate HF575 (*hyp*[*A1B1F1CDEX*]Δ*hyp*[*A2B2F2*]Δ; *hyp1*Δ *hyp2*Δ). Plasmid pCH872 was used for the introduction of the Φ(*hoxK′-lacZ*) gene fusion into the chromosomal *norR2A2B2* gene region of *R. eutropha* strains.

**Cell fractionation and immunoblot analysis.** *R. eutropha* cells were disrupted by two passages through a chilled French pressure cell (Amicon) at 900 $lb/in^2$. Cell debris and membranes were separated from the soluble fraction by ultracentrifugation (90,000 × *g*). Soluble proteins of *R. eutropha* extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels and subsequently transferred to Protran BA85 nitrocellulose membranes (Schleicher & Schuell) according to a standard protocol (41). The RH subunits HoxC and HoxB were detected by using anti-HoxC serum (diluted 1:1,000) and anti-HoxB serum (diluted 1:10,000), respectively, and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Dianova, Hamburg, Germany).

**In-gel activity staining.** Soluble proteins of *R. eutropha* extracts were separated by native PAGE (4 to 15%). Subsequently, the gel was incubated in $H_2$-saturated 50 mM potassium phosphate buffer (pH 7.0) containing 0.09 mM phenazine methosulfate (PMS) and 0.06 mM nitroblue tetrazolium (NBT) under an atmosphere of 100% $H_2$. Purple bands occured upon incubation at 30°C in the dark, indicating PMS-mediated reduction of NBT.

**$^{63}$Ni labeling.** Cells were grown in the presence of 120 nM $^{63}NiCl_2$ (6.38 mCi/ml; Amersham). Soluble extracts were prepared and subjected to native PAGE. Gels were dried and autoradiographed by using an SI 550 storage PhosphorImager (Molecular Dynamics) as described earlier (18).

**FTIR spectroscopy.** FTIR spectra were obtained with a Bio-Rad FTS 60A spectrometer equipped with a mercury cadmium telluride (MCT) detector. Spectra were recorded at room temperature with a resolution of 2 $cm^{-1}$. Typically, averages of 1,524 spectra were determined against proper blanks. Samples of soluble extracts (10 μl) were loaded into a gastight transmission cell ($CaF_2$, 56-μm pathlength). Samples of whole cells were prepared by drying 100-μl aliquots of a cell suspension on a $CaF_2$ window, and the dried placard was measured. The spectra were corrected for the baseline by using a spline function provided by the Bio-Rad software.

**Assays.** $H_2$-oxidizing activity was quantified by an amperometric $H_2$ uptake assay as described previously by using an $H_2$ electrode with methylene blue as the electron acceptor (31). β-Galactosidase activity was determined as described previously (47), and the activities were calculated according to the Miller method (28), except that the cell density was measured at 436 nm. The proteins of the soluble extracts were determined according to the protocol of Lowry (23).

## RESULTS

**Regulatory properties of mutants with deletions in individual *hyp* genes.** For $H_2$-responding strains of *R. eutropha*, such as HF470 (Table 1), a functional RH is absolutely necessary to express the genes for the SH and MBH. Mutants with impaired RH fail to grow on $H_2$ as an energy source (21). To test whether mutations in the various *hyp* genes affect the regulatory activity of the RH, we examined two different sets of *hyp* mutants (Table 1). The first group of mutants carried single site in-frame deletions in *hypC* (HF340), *hypD* (HF338), *hypE* (HF339), and *hypX* (HF469), and the second group of mutants was characterized by deletions in both copies of the respective *hyp* genes, i.e., *hypA1A2* (HF410), *hypB1B2* (HF417), and *hypF1F2* (HF441). We knocked out the SH and MBH in these strains by deletions of the corresponding structural genes *hoxH* and *hoxG*, respectively, in order to avoid interferences with their dominant activities in the enzyme assays. Furthermore, since the *hyp* mutants were originally constructed from the non-$H_2$-responding strain *R. eutropha* H16 (Table 1), the activity of the histidine kinase HoxJ was restored in the *hyp* mutants by site-directed mutagenesis as described previously (21). A codon conversion in *hoxJ* replaced serine at position 422 by a glyine residue.

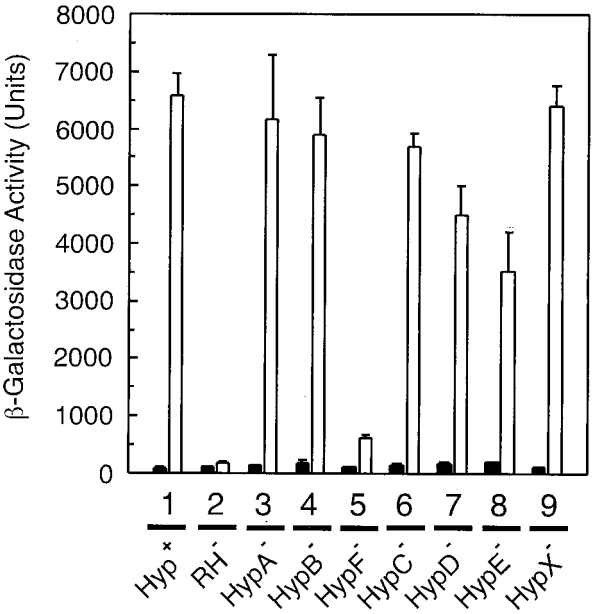


FIG. 1. RH regulatory activity in the *hyp* deletion strains. *R. eutropha* strains harboring the plasmid-based Φ(*hoxK′-lacZ*) fusion were grown in FGN medium in the absence (black bars) or in the presence (white bars) of hydrogen. Cells were harvested at an optical density at 436 nm ($OD_{436}$) of 8.0 ± 0.3, and the β-galactosidase activity was determined according to the protocol of Miller (28). Lane 1, HF470; lane 2, HF510; lane 3, HF503; lane 4, HF504; lane 5, HF505; lane 6, HF506; lane 7, HF507; lane 8, HF508; lane 9, HF509.

Since the MBH and SH genes are regulated coordinately, transcription was monitored by using the plasmid-borne MBH gene fusion Φ(*hoxK′-lacZ)* as a representative parameter. The *hoxK* gene encodes the MBH small subunit and is the first gene of the MBH operon (19). As expected, the reference strain HF470 (Fig. 1, lane 1) showed low β-galactosidase reporter activity in the absence of $H_2$ and high activity in the presence of $H_2$. The RH-negative strain failed to activate the MBH promoter under both conditions (Fig. 1, lane 2). The loss of HypA (lane 3), HypB (lane 4), HypC (lane 6), and HypX (lane 9) scarcely affected the MBH promoter activity, whereas mu-

TABLE 2. RH-mediated $H_2$-oxidizing activities in the *hyp* deletion strains

| Strain[a] | Relevant characteristic | Sp act[b] (mU/mg of protein) | | $^{63}$Ni incorporation[c] ($RH_{overpr}$[e]) |
|---|---|---|---|---|
| | | $RH_{wt}$[d] | $RH_{overpr}$[e] | |
| HF470 | $Hyp^+$ $RH^+$ | 0.34 | 58.61 | ++ |
| HF510 | $Hyp^+$ $RH^-$ | <0.05 | <0.05[f] | –[f] |
| HF503 | *hypA1*Δ*A2*Δ | 0.28 | 3.73 | + |
| HF504 | *hypB1*Δ*B2*Δ | 0.34 | 1.74 | + |
| HF505 | *hypF1*Δ*F2*Δ | <0.05 | <0.05 | – |
| HF506 | *hypC*Δ | 0.27 | 0.88 | + |
| HF507 | *hypD*Δ | 0.19 | 0.20 | – |
| HF508 | *hypE*Δ | 0.23 | 0.42 | – |
| HF509 | *hypX*Δ | 0.29 | 49.42 | ++ |

[a] All strains are $SH^-$ $MBH^-$ $HoxJ^+$.
[b] Cells were grown in FGN medium in the presence of $H_2$. The specific activities were determined amperometrically with methylene blue as an electron acceptor. The values are the mean of two independent experiments.
[c] Cells were grown in the presence of $^{63}NiCl_2$. Proteins of soluble extracts were separated by native PAGE, and the gel was subjected to autoradiography. ++, strong signal; +, weak signal; –, no signal.
[d] $RH_{wt}$, RH expressed from megaplasmid pHG1.
[e] $RH_{overpr}$, overproduced RH expressed from plasmid pGE378.
[f] Strain HF510 containing control vector pEDY309 instead of pGE378.

tations in *hypD* (lane 7) and *hypE* (lane 8) led to a moderate decrease of β-galactosidase to a level of 50 to 70%. A dramatic downregulation occurred by mutation of *hypF* (lane 5). Mutant HF505 retained only 10% of the MBH promoter activity. These results showed that HypF is a major component for the $H_2$-sensing function of the RH, whereas the other *hyp* gene products seem to play a subordinate role in the synthesis of active RH.

**Effects of *hyp* mutations on the biochemical characteristics of the RH.** To explore whether the regulatory properties of the *hyp* mutants correlate with the enzymatic activity of the RH, the mutants were cultivated in fructose-glycerol minimal medium supplied with $H_2$. Soluble extracts were prepared and $H_2$-oxidizing activity was determined amperometrically by using methylene blue as the electron acceptor. The data are summarized in Table 2. In a regular RH-producing background (column 1), the $RH^-$ and $HypF^-$ mutants were the only strains that were severely affected in their enzymatic activity. The level of activity obtained with the $HypD^-$ and $HypE^-$ strains correlated well with the diminished MBH promoter activity (Fig. 1). The wild-type-like activity profile of the remaining $Hyp^-$ mutants was in line with the regulatory data.

The pattern changed substantially in strains that produced the RH at an elevated level caused by the introduction of the *hoxBC*-harboring plasmid pGE378 (Table 2). With the exception of the *hypX* mutant, all of the *hyp*-deficient strains showed a dramatic decrease of hydrogenase activity, which directly correlated with low $^{63}$Ni incorporation by the *hyp* strains (Table 2). Western blot analysis, conducted with an antibody raised against the large HoxC subunit of the RH, confirmed the expression of RH protein in the *hyp* mutants (Fig. 2). Nevertheless, with the exception of the *hypX* mutant, the rest of the *hyp* strains exhibited a decreased band intensity pointing to less-stable RH protein.

Evidence for changes in the structure of the active site of RH mutant proteins was also obtained by FTIR spectroscopy. This method can be applied only to extracts from RH-overproducing strains due to sensitivity limits (31). Thus, extracts prepared from the pGE378-containing *hyp* mutants were analyzed for the presence of infrared bands from metal-bound CO and $CN^-$ (Fig. 3). As expected, the $Hyp^+$ control (trace A) showed a strong absorption at 1,943 $cm^{-1}$, which corresponds to one CO ligand and the two bands at 2,072 $cm^{-1}$ and 2,081 $cm^{-1}$ are indicative for the presence of two $CN^-$ as reported previously (2, 31). A similar spectrum was obtained with extracts of the *hypX* mutant (Fig. 3, trace B), a finding which is in good agreement with its wild-type-like phenotype (Fig. 1 and Table 2). No FTIR bands in the 2,150 to 1,850 $cm^{-1}$ spectral region could be detected in the spectra of the rest of the *hyp* mutants, even in those derivatives which showed residual promoter and hydrogenase activities. As an example, the spectrum of the *hypB1*Δ *hypB2*Δ mutant extract is shown (Fig. 3, trace C). Obviously, the concentration of intact RH molecules in the mutants extracts was below the detection limit of the instrument. The use of intact mutant cells for FTIR analysis to circumvent the possibility of the destruction of labile RH maturation intermediates during the extract preparation yielded the same results.

**Complete deletion of the two megaplasmid-borne *hyp* DNA regions.** The previous data indicate a graded significance of the various *hyp* gene products in the RH synthesis. If the Hyp proteins are instrumental as chaperones in a series of concerted steps, the loss of one of the seven proteins by mutation may be phenotypically suppressed and less apparent. Therefore, we completely deleted all known *hyp* genes in *R. eutropha* and raised the question of whether and to what extent introduction of the individudal *hyp* genes restored the loss of the *hyp* gene regions.

Large in-frame deletions in both the *hyp1* and *hyp2* regions yielded mutant HF575. As described above, the SH and MBH

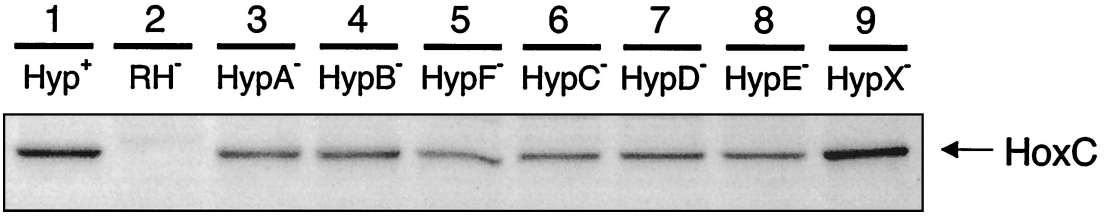


FIG. 2. RH protein stability in the *hyp* deletion strains. *R. eutropha* strains harboring plasmid pGE378 for RH overproduction were grown in FGN medium under hydrogenase derepressing conditions. The presence of the RH large subunit HoxC in soluble extracts was analyzed by the immunoblot technique. A total of 20 μg of protein was applied to each lane. Lane 1, HF470; lane 2, HF510 (containing control vector pEDY309 instead of pGE378); lane 3, HF503; lane 4, HF504; lane 5, HF505; lane 6, HF506; lane 7, HF507; lane 8, HF508; lane 9, HF509.

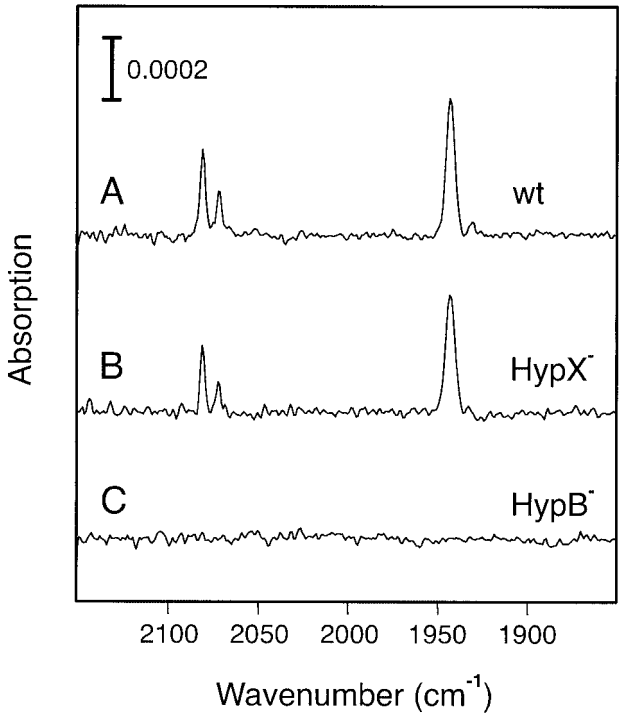


FIG. 3. FTIR spectra of soluble extracts containing the overproduced RH. Soluble extracts were prepared from *R. eutropha* strains grown in FGN medium under hydrogenase-derepressing conditions. The spectra were recorded by using the as-isolated, concentrated extracts containing the oxidized RH. Trace A, HF470(pGE378); trace B, HF509(pGE378); trace C, HF504(pGE378).

activities were also blocked by mutations in the subunit genes *hoxH* and *hoxG*, and the kinase HoxJ was reactivated by a Ser/Gly replacement. To prepare the strain for subsequent plasmid-based complementation, the Φ(*hoxK′-lacZ*) gene fusion was inserted into the NO reductase gene region *norR2A2B2* on the chromosome, a locus which is dispensable for hydrogen metabolism (32).

The resulting mutant HF581 was cultivated in fructose-glycerol minimal medium with or without $H_2$ supplementation and tested for β-galactosidase activity. Surprisingly, the mutant still exhibited 10% of the wild-type activity (Fig. 4, lane 2), which corresponds to the level of activity observed before with the $HypF^-$ strain (Fig. 1, lane 5). A knockout of the RH gene *hoxC* on the other hand completely abolished the MBH promoter activity (Fig. 4, lane 3). This result indicates that the residual expression of β-galactosidase in the *hyp*-negative strain HF581 is mediated by a small population of NiFe-containing RH molecules.

The amperometric assay was not sensitive enough to detect a low level of hydrogenase activity in the *hyp*-negative strain HF581. The in-gel hydrogenase assay with PMS as the electron acceptor is more appropriate for detecting even traces of enzymatic activity. This method initially also failed to demonstrate $H_2$-oxidizing activity in extracts of the the *hyp*-negative strain HF581 (Fig. 5A, lane 3). In the course of characterizing mutants with alterations in the SH protein, it was observed that addition of $Zn^{2+}$ to the growth medium had a stabilizing effect on the structure of the SH (C. Massanz and B. Friedrich, unpublished results). Therefore, we grew cells of the *hyp*-negative strain HF581 in minimal medium supplemented with 1 μM $ZnCl_2$. The resulting extract clearly developed hydrogenase activity (Fig. 5 A, lane 4). Immunoblot analysis showed that addition of $Zn^{2+}$ had a particularly stabilizing effect on the small subunit HoxB of the RH (Fig. 5 B, lane 4). Stabilization of the RH protein was not observed by supplementing the minimal medium with $Co^{2+}$, $Cu^{2+}$, or $Mn^{2+}$ ions (data not shown).

**Complementation of RH activity.** It was reported before that the activity of the two energy-linked hydrogenases was completely restored in mutants devoid of *hyp* gene products by introducing the respective *hyp* gene on a plasmid (10, 45). An analogous complementation experiment was conducted by using the *hyp*-negative strain HF581 as the recipient. Plasmid pGE6 harboring the complete *hyp1* region (*hypA1B1F1CDEX)* was introduced into HF581. The resulting transconjugants were able to activate the MBH promoter in the presence of $H_2$ up to 80% of the wild-type level (Fig. 4, lane 4). If the product of *hypF* is the major player in the RH cofactor insertion, introduction of *hypF1* on plasmid pGE457 should substantially complement the MBH promoter activity. In fact 30% of β-galactosidase activity were recovered in the transconjugants (Fig.

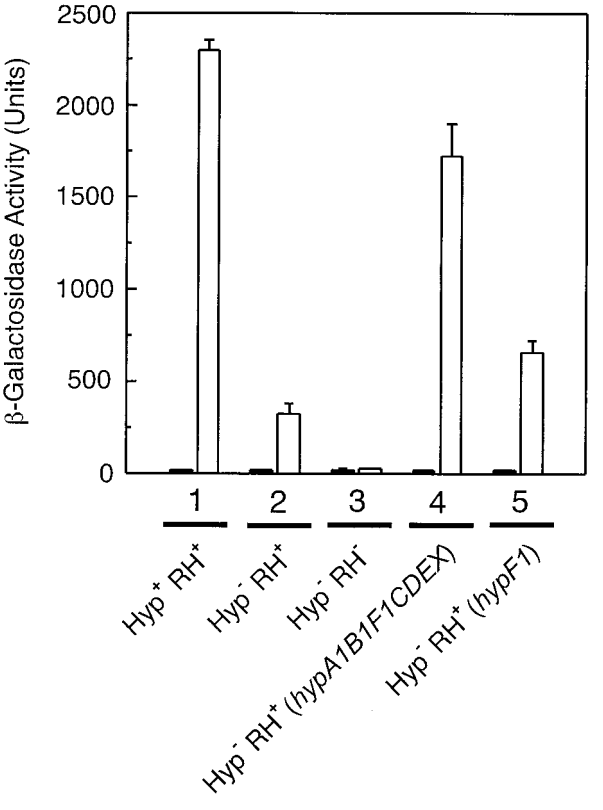


FIG. 4. RH regulatory activity in the *hyp*-negative strain HF581. *R. eutropha* strains harboring the Φ(*hoxK′-lacZ*) fusion integrated into the *norR2A2B2* gene region of the chromosome were grown in FGN medium in the absence (black bars) or in the presence (white bars) of hydrogen. Cells were harvested at an optical density at 436 nm of 8.0 ± 0.3 and the β-galactosidase activity was determined according to the protocol of Miller (28). Lane 1, HF573(pGE151); lane 2, HF581(pGE151); lane 3, HF582(pGE151); lane 4, HF581(pGE6); lane 5, HF581(pGE457).

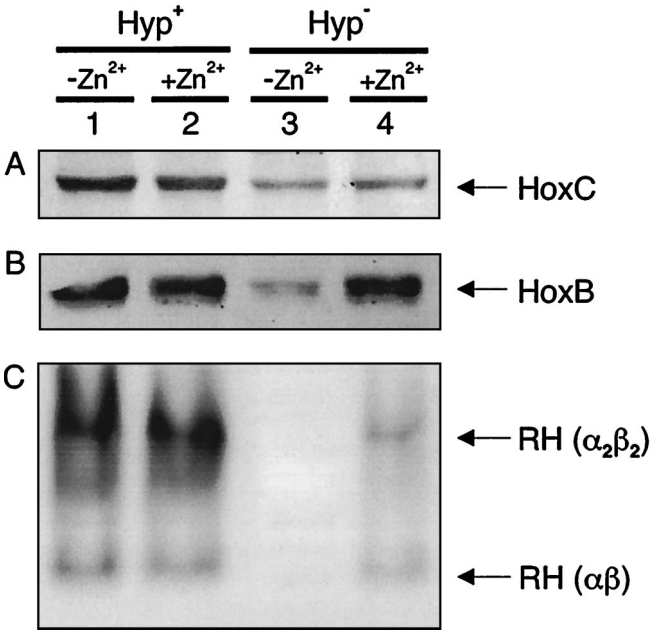


FIG. 5. RH protein stability and RH-mediated $H_2$-oxidizing activity in the *hyp*-negative strain HF581. *R. eutropha* strains were grown in FGN medium supplemented with or without 1 μM $ZnCl_2$ as indicated above the figures. (A) Immunoblot against the RH large subunit HoxC, with 20 μg of soluble protein in each lane. (B) Immunoblot against the RH small subunit HoxB, with 20 μg of soluble protein in each lane. (C) In-gel activity assay. A total of 500 μg of soluble proteins were separated by native PAGE. Dark-colored bands indicate $H_2$-oxidizing activity of the RH by the PMS-mediated reduction of NBT. The fact that the RH forms an $\alpha_2\beta_2$ oligomer was previously described (2). Lanes 1 and 2, HF573; lanes 3 and 4, HF581.

4, lane 5). This level was not enhanced by introducing the alternative copy *hypF2*. Moreover, plasmids harboring one of the other *hyp* genes had no complementation capacity at all (data not shown).

## DISCUSSION

The $H_2$-sensing hydrogenase of *R. eutropha* belongs to a new subclass of [NiFe] hydrogenases which exhibits some unique structural and biochemical features (2, 18). Although its active site has the spectral properties of a normal [NiFe] site, the active site can exist in only two redox states and does not react with $O_2$ or CO. In addition, RH has a very low $H_2$-oxidizing activity with artificial electron acceptors. The lack of a C-terminal extension in the large subunit indicates the absence of a proteolytic step in RH maturation and raised the question whether metal-center assembly requires auxiliary proteins as demonstrated for [NiFe] hydrogenases involved in energy metabolism (7, 25). In addition to the $H_2$-sensing hydrogenases (18), the CO-induced hydrogenase in *Rhodospirillum rubrum* (14) and the Ech hydrogenase from *Methanosarcina barkeri* (20), which are physiologically quite diverse, are devoid of a C-terminal extension in the large subunit. This observation suggests that the final proteolysis is not an obligate step in [NiFe] center assembly.

To examine whether Hyp proteins are involved in metal center assembly of the RH, a collection of *hyp* mutants of *R. eutropha* was analyzed for its regulatory capacity, for its ability to oxidize $H_2$ with redox dyes and for some structural features. It has been reported that the product of *hypD* is necessary for the synthesis of active HupUV protein in *R. capsulatus* (43) and that HypF participates in the regulation of hydrogenase synthesis through maturation of HupUV (8). In the present study we show that complete deletion of the known *hyp* genes in *R. eutropha* HF581 dramatically affects both the $H_2$-sensing and $H_2$-oxidizing activity of the RH. Only 10% of β-galactosidase activity, expressed from a Φ(*hoxK′-lacZ*) gene fusion, was recovered and trace amounts of hydrogenase activity were identified in a native gel after stabilization of the RH by the addition of $ZnCl_2$. This result clearly argues for a requirement of the Hyp proteins in the maturation of the $H_2$-sensing hydrogenase. The fact that mutants devoid of RH have completely lost the regulatory and enzymatic activities confirms the notion that the residual activity in the *hyp*-negative strains derived from some NiFe-containing RH molecules. A strict correlation between both the RH regulatory and enzymatic activity and the availability of nickel in the medium has been reported previously (18).

Our results showed that of the seven *hyp* gene products in *R. eutropha* HypX had barely any effect on the regulatory and enzymatic activity of the RH under all conditions tested. Even the CO- and $CN^-$-related infrared absorptions were not affected in extracts of the *hypX*-deficient strain. Therefore, the function of HypX which, under certain conditions, may participate in the delivery of the $C_1$ compounds (34) appears to be restricted to the maturation of the SH and MBH (6). From the phenotypic behavior of the corresponding mutants, it is inferred that the functional significance of the remaining *hyp* gene products follow a graded pattern. HypA, HypB, and HypC mutants showed a decrease of maximal 10% in MBH promoter activity which correlated with a slightly altered enzymatic activity. These mutations, however, had a severe effect on the level of enzymatic RH activity if the *hoxBC* genes were expressed from a multiple-copy plasmid. Obviously, the cells need these Hyp proteins when hydrogenase synthesis proceeds at a high level. Mutants with deletions of *hypD*, *hypE* and, in particular, *hypF* had low if any regulatory and enzymatic activity. Provided HypF of *R. eutropha* has a similar function as postulated for *E. coli* (29), incorporation of the nonprotein ligands CO and $CN^-$ is also a crucial reaction for metal-center assembly into the RH that can hardly be accomplished without the function of HypF. If HypF incorporates the $Fe(CO)(CN)_2$ moiety, then this reaction is essential for the $H_2$-sensing function of the RH. We previously showed that both the regulatory and enzymatic activity of the RH are dependent on Ni (18). Taken together, these two observations indicate that signal transduction requires an $H_2$ sensor with an intact [NiFe] active site. The question whether a simple binding of $H_2$ or further electron transfer processes are required for $H_2$-signaling remains open.

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