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HupO, a Novel Regulator Involved in Thiosulfate-Responsive Control of HupSL [NiFe]-Hydrogenase Synthesis in *Thiocapsa roseopersicina*

Ildikó K. Nagy,[a] Kornél L. Kovács,[b] Gábor Rákhely,[b] Gergely Maróti[a]

Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary[a]; Department of Biotechnology, University of Szeged, Szeged, Hungary[b]

[NiFe]-hydrogenases are regulated by various factors to fulfill their physiological functions in bacterial cells. The photosynthetic purple sulfur bacterium *Thiocapsa roseopersicina* harbors four functional [NiFe]-hydrogenases: HynSL, HupSL, Hox1, and Hox2. Most of these hydrogenases are functionally linked to sulfur metabolism, and thiosulfate has a central role in this organism. The membrane-associated Hup hydrogenases have been shown to play a role in energy conservation through hydrogen recycling. The expression of Hup-type hydrogenases is regulated by H_2 in *Rhodobacter capsulatus* and *Cupriavidus necator*; however, it has been shown that the corresponding hydrogen-sensing system is nonfunctional in *T. roseopersicina* and that thiosulfate is a regulating factor of *hup* expression. Here, we describe the discovery and analysis of mutants of a putative regulator (HupO) of the Hup hydrogenase in *T. roseopersicina*. HupO appears to mediate the transcriptional repression of Hup enzyme synthesis under low-thiosulfate conditions. We also demonstrate that the presence of the Hox1 hydrogenase strongly influences Hup enzyme synthesis in that *hup* expression was decreased significantly in the *hox1* mutant. This reduction in Hup synthesis could be reversed by mutation of *hupO*, which resulted in strongly elevated *hup* expression, as well as Hup protein levels, and concomitant *in vivo* hydrogen uptake activity in the *hox1* mutant. However, this regulatory control was observed only at low thiosulfate concentrations. Additionally, weak hydrogen-dependent *hup* expression was shown in the *hupO* mutant strain lacking the Hox1 hydrogenase. HupO-mediated Hup regulation therefore appears to link thiosulfate metabolism and the hydrogenase network in *T. roseopersicina*.

Hydrogenases are ancient metalloenzymes that catalyze the reversible oxidation of molecular hydrogen. They can be found in many bacteria and archaea, as well as in eukaryotic microalgae. Three major groups of hydrogenases are distinguished according to their metal content: the [FeFe]-hydrogenases, [NiFe]-hydrogenases, and the iron-sulfur-cluster-free hydrogenase enzymes (1–3). The [NiFe]-hydrogenases contain a large (60- to 65-kDa) catalytic subunit and a small electron transfer subunit (25 to 35 kDa). Additional proteins are required for the posttranslational maturation of the enzyme. These accessory proteins participate in a complex multistep assembly process of the core catalytic center with well-defined specific functions (4–6). The [NiFe]-hydrogenases can be further classified according to their localization, function, and possible associated subunits. Four major groups have been distinguished: the membrane-associated uptake hydrogenases, the hydrogen-sensing hydrogenases, the bidirectional NADP/NAD-reducing mostly cytoplasmic hydrogenases, and the energy-converting membrane-associated hydrogen-evolving hydrogenases (3). A number of bacteria possess more than one [NiFe]-hydrogenase enzyme (e.g., *Escherichia coli*, *Cupriavidus necator* [formerly *Ralstonia eutropha*], and *Thiocapsa roseopersicina*). In these organisms, each hydrogenase enzyme is assumed to have specific physiological functions.

T. roseopersicina BBS is a Gram-negative photosynthetic purple sulfur bacterium in the *Chromatiaceae* family (7). It utilizes reduced sulfur compounds (predominantly $S_2O_3^{2-}$) as an electron source during anaerobic photochemolithoautotrophic growth. *T. roseopersicina* was shown to possess four functional [NiFe]-hydrogenases (HynSL, HupSL, Hox1, and Hox2), with differences in their localizations, structures, and metabolic contexts (8–11). The HynSL and HupSL enzymes are membrane associated; HynSL was shown to be tightly connected to the sulfur metabolism of *T. roseopersicina*, while HupSL is considered to play a role in energy conservation under nitrogen-fixing and possibly under thiosulfate-depleted conditions (12–14). The Hox1 and Hox2 enzymes are localized in the cytoplasm. The cyanobacterium-type bidirectional Hox1 is composed of five functional subunits: HoxEFUYH, in which YH represent the hydrogenase subunits, FU refer to the diaphorase subunits, and the function of the E subunit is still unclear (10). HoxE is essential for the *in vivo* activity of the Hox1 enzyme, as it has a crucial role in electron transfer. Hox2 has four subunits (Hox2FUYH), and this enzyme is functional under photoheterotrophic conditions in the presence of glucose (10, 11, 15).

The expression of [NiFe]-hydrogenases can be regulated by various environmental factors, like oxygen or nitrate levels in *E. coli* (16) or nickel concentration in *Nostoc* species (17). The Hup-type hydrogenases are regulated by H_2 in *C. necator* and *R. capsulatus* (18, 19). In these organisms, H_2 triggers the expression through a hydrogen-sensing regulatory hydrogenase (HoxBC-HupUV) and a two-component signal transduction system (HoxJA-HupTR) (18, 19). Only limited data are available on the

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Address correspondence to Gábor Rákhely, rakhely.gabor@brc.mta.hu, or Gergely Maróti, maroti.gergely@brc.mta.hu.

TABLE 1 Bacterial strains and constructs used in this study

Strain or plasmid	Relevant genotype/phenotype or description[a]	Reference or source
Strains		
T. roseopersicina		
BBS	Wild type	7
GB11	*hynSL*Δ::Sm^r	10
HOD1	GB11 Δ*hupO*	This work
HOD1comp	HOD1/pDSK*hupO*comp	This work
GB1131	*hynSL*Δ::Sm^r *hox1H*Δ::Er^r	13
HOD13	GB1131 Δ*hupO*	This work
HOD13comp	HOD13/pDSK*hupO*comp	This work
E. coli		
S17-1(λpir)	294 (*recA pro res mod*) Tp^r Sm^r (pRP4-2-Tc::Mu-Km::Tn*7*) λ*pir*	26
Plasmids		
pK18*mobsacB*	Km^r *sacB* RP4 *oriT* ColE1 *ori*	25
pDSK6CrtKm	pDSK509 replicon with *T. roseopersicina crtD* promoter region, Km^r	27; T. Balogh, unpublished data
pK*hupO*up	Upstream region of *hupO* in pK18mobsacB	This work
pK*hupO*D	Upstream and downstream regions of *hupO* in pK18mobsacB; construct for in-frame deletion of *hupO*	This work
pDSK*hupO*comp	*hupO* gene in pDSK6CrtKm, construct for complementation	This work

[a] Sm^r, streptomycin resistance; Er^r, erythromycin resistance; Tp^r, trimethoprim resistance; Km^r, kanamycin resistance.

transcriptional regulation of the multiple hydrogenases in *T. roseopersicina*. The expression of the HynSL enzyme is induced under anaerobic conditions by a fumarate and nitrate reductase regulatory (FNR) homologue, FnrT, and is apparently unaffected by H_2 (20). The two-component signal transduction system, composed of the HupR regulator and the HupT kinase originally discovered in *R. capsulatus*, was functional in *T. roseopersicina*, and the coding sequences (*hupUV*) of a putative hydrogen-sensing enzyme were also identified (21). However, the *hupUV* genes were found to be silent under various tested conditions (21). The transcript level of HupSL hydrogenase was relatively low and hydrogen independent in the *T. roseopersicina* GB11 (Δ*hynSL*) strain. This unusual feature was attributed to the lack of a functional hydrogen-sensing hydrogenase (21). Further studies revealed that thiosulfate was an important factor in the regulation of the *hupSL* operon (13). Increased *hupSL* expression by the GB1131 (Δ*hynSL* Δ*hox1*) strain was observed in response to decreasing thiosulfate levels (13). Therefore, increased *in vivo* hydrogen uptake by HupSL was expected under low-thiosulfate conditions in this strain.

Our aim was to perform a detailed investigation of the regulation of HupSL activity and the identification of elements influencing the Hup-mediated energy conservation processes, i.e., utilization of hydrogen as an energy source. We analyzed a previously described but functionally not characterized open reading frame (ORF) in the *hupSL* operon and provided clues for its role in the control of *hupSL* expression under specific conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *T. roseopersicina* strains were grown photoautotrophically in Pfennig's mineral medium (22). Cells were grown anaerobically in liquid cultures under illumination using incandescent light bulbs of 60 W (50 μE) at 28°C. Pfennig's medium was used with various sodium thiosulfate concentrations (PC0.5, PC1, PC2, and PC4 represent Pfennig's medium supplemented with 0.5 g liter^{-1}, 1 g liter^{-1}, 2 g liter^{-1}, and 4 g liter^{-1} [3.162 mM, 6.325 mM, 12.65 mM, and 25.3 mM] sodium thiosulfate, respectively). Plates (plate count agar [PCA]) were solidified with 7 g liter^{-1} Phytagel and supplemented with kanamycin or 3% sucrose when selecting for transconjugants (23). The plates were incubated in anaerobic jars (Anaerocult; Merck) for 2 weeks. *E. coli* strains were maintained in Luria-Bertani (LB) liquid medium and on Luria-Bertani agar plates at 37°C (24). Antibiotics were used at the following concentrations: 5 μg ml^{-1} streptomycin, 5 μg ml^{-1} gentamicin, 50 μg ml^{-1} erythromycin, and 25 μg ml^{-1} kanamycin.

In-frame deletion of the *hupO* gene. The primers used in the study are listed in Table 2. The vector construct used for in-frame deletion was derived from the pK18*mobsacB* vector (25). The upstream region of the *hupO* gene was amplified with the hupOupFw and hupOupRev primers. The PCR product was ligated into the polished EcoRI-XbaI site of pK18*mobsacB*, yielding pKhupOup. The downstream region was amplified from the genome using the hupOdownFw and hupOdownRev primers. The fragment was cloned into the MluI-HindIII-digested pKhupOup vector, yielding pKhupOD. pKhupOD was transformed into *E. coli* strain S17-1 and then conjugated into the *T. roseopersicina* GB11 (Δ*hynSL*) and GB1131 (Δ*hynSL* Δ*hox1H*) strains, as described previously (26). Single recombinants were selected on kanamycin-containing PCA plates. Double recombinants were selected on 3% sucrose-containing PCA plates. The sucrose-resistant and kanamycin-sensitive colonies were selected, and their genotypes were confirmed by PCR and subjected to capillary sequencing. The *hupO* gene was deleted from the GB11 strain, resulting in

TABLE 2 Primers used in this study

Primer name	5′→3′ sequence
hupOupFw	GCATAAGAATTCATCAAGCCCCGCTGCTGC
hupOupRev	TTATGGTCTAGAACGCGTCCCGAAAGCGAGCATCTC
hupOdownFw	AAGTGGACGCGTGAGACTCCGGCATGAGC
hupOdownRev	TATGCCAAGCTTGCACCGCGGCGACCCTGT
hupOcompFw	ATGACCACACCGATAGACCT
hupOcompRev	CATTCGTTGGATTTCGTTCT
hupOqFw	CGATCCGATCCAAAAACATC
hupOqRev	GCATCGGGTTAACGTCAAAG
hupLqFw	CCTCGAAGAATCTGCTCCTG
hupLqRev	GAATACTTGGCCTGCTCGTC

strain HOD1, and from the GB1131 strain, resulting in strain HOD13. Homologous complementation was performed using a pDSK509-based vector, pDSK6crtKm (reference 27 and T. Balogh, unpublished data). The *hupO* gene was amplified from genomic DNA using the hupOcompFw and hupOcompRev primers, and the product was ligated into pDSK6CrtKm, resulting in pDSKhupOcomp. pDSKhupOcomp was conjugated into HOD1, resulting in the HOD1comp strain, and into HOD13, resulting in the HOD13comp strain.

RNA isolation, RT, and quantitative real-time PCR (qPCR). For RNA isolation, *T. roseopersicina* strains were grown in 50 ml of liquid medium in sealed Hypo-Vial bottles, 12-ml cultures were pelleted at 3,750 × *g* for 15 min, the pellets were resuspended in 400 μl of SET buffer (20% sucrose, 50 mM EDTA [pH 8.0], and 50 mM Tris-HCl [pH 8.0]), and 350 μl of SDS buffer was added [20% SDS, 1% $(NH_4)_2SO_4$ (pH 8.0)]. Five hundred microliters of saturated NaCl was also added, and the solution was gently mixed. The samples were centrifuged at 17,000 × *g* for 10 min at room temperature, and the clear supernatants were transferred into clean tubes. A 0.7 volume of 2-propanol was added to the solutions, and the mixtures were centrifuged at 17,000 × *g* for 20 min. The pellets were washed twice with 1 ml of 70% ethanol. The dried pellets were resuspended in 35 μl of RNase-free Milli-Q water. DNase I treatment was performed for each sample at 37°C for 30 min. Reverse transcription (RT) was performed using random hexamers for the cDNA synthesis (SuperScript VILO cDNA synthesis kit; Invitrogen, Life Technologies, USA). RT-coupled PCRs were carried out using SYBR green real-time PCR master mix (Life Technologies) using specific primers (Table 2, hupLq and hupOq primer pairs) for the reactions.

Preparation of *T. roseopersicina* crude extract. The crude extracts were prepared from 50 ml of 7-day-old *T. roseopersicina* cultures grown in Pfennig's medium containing thiosulfate at various concentrations. The cells were harvested by centrifugation at 3,750 × *g* for 15 min, resuspended in 2 ml of 20 mM potassium phosphate (K-P) buffer (pH 7.0), and then disrupted by ultrasonication (VialTweeter, UIS250v, at 90% amplitude for 4 min; Hielsche). The broken cells were centrifuged at 3,750 × *g* for 10 min. The debris (sulfur globules and remaining whole cells) was discarded, and the supernatant was considered a bacterial crude extract.

***In vivo* hydrogen uptake measurement.** *T. roseopersicina* (50-ml) strains were grown in Pfennig's medium containing thiosulfate at various concentrations (PC1 and PC4) under a nitrogen atmosphere in sealed 100-ml Hypo-Vial bottles. Anaerobiosis was established by flushing the gas phase with N_2 for 5 min. One milliliter of pure H_2 (89.1 μmol H_2) was injected into the bottles at the start of the experiments. The cultures were grown under continuous illumination, and the H_2 content of the gas phase was monitored by gas chromatography (7890A gas chromatograph; Agilent Technologies) on each day of cultivation. Hydrogen uptake was calculated as the difference in hydrogen content between the start and the actual measurement point. Three biological replicates were used for each *in vivo* hydrogen uptake measurement.

***In vitro* hydrogen uptake activity measurement.** The *in vitro* uptake activities were measured using 100 μl of crude extracts. One milliliter of 20 mM K-P buffer containing 0.8 mM oxidized benzyl viologen was added to the crude extracts in cuvettes of 3 ml in volume. The cuvettes were sealed with Suba-Seal rubber stoppers. The gas phase was flushed with H_2 for 5 min, and the rate of hydrogen uptake was measured using a spectrophotometer, as described previously (13).

Western hybridization. The crude extracts of the *T. roseopersicina* strains grown in Pfennig's medium supplemented with various concentrations of sodium thiosulfate were analyzed. Proteins (50 μg of total protein in each sample) were separated in a 4 to 12% gradient Bis-Tris gel by SDS-PAGE and were blotted onto a nitrocellulose membrane (Bio-Rad). Nonspecific binding of proteins was blocked (blocking solution of 5% nonfat milk powder in TBST buffer [150 mM NaCl, 0.05% Tween 20, 10 mM Tris-HCl {pH 7.5}]). Anti-HupL antibody (kindly provided by Qing Xu, J. Craig Venter Institute [JCVI], USA) was used as the primary antibody at a 1:10,000 dilution in blocking solution. The secondary antibody (goat-anti-rabbit horseradish peroxidase [HRP] H+L) was used at a 1:5,000 dilution in blocking solution. For detection of the proteins, 1 ml each of the enhancer and peroxide solutions (Millipore) were used, and a chemiluminescence signal was detected by a Li-COR C-DiGit blot scanner. The Image Studio Lite software was used to evaluate the results. The nitrocellulose membrane was stained with Ponceau solution (0.1% [wt/vol] Ponceau S in 5% [vol/vol] acetic acid) to control the amounts of the loaded total proteins (thereby serving as the internal loading control).

RESULTS

Thiosulfate-dependent *in vivo* uptake activity of the HupSL hydrogenase. The membrane-associated HupSL hydrogenase is considered the main energy-conserving hydrogenase in *T. roseopersicina*; its proposed function is to recycle molecular hydrogen as an energy source under specific conditions, primarily under nitrogen-fixing conditions. The Δ*hynSL* Δ*hox1H* mutant (strain GB1131) is suitable for the selective investigation of the *in vivo* hydrogen uptake exerted by the HupSL hydrogenase (the Hox2 hydrogenase activity is detectable exclusively in the presence of glucose [2 g/liter]). The thiosulfate concentration was previously shown to affect the expression level of the *hupSL* genes (13). Here, we investigated HupSL *in vivo* uptake activity using various thiosulfate concentrations in the culture medium. Hydrogen gas (89.1 μmol H_2) was introduced in the headspace immediately after inoculation. HupSL activity strongly correlated with the thiosulfate content of the medium, whereas decreasing thiosulfate concentrations (from 4 g $liter^{-1}$ to 1 g $liter^{-1}$) resulted in a significant increase in the *in vivo* hydrogen uptake (Fig. 1). The HupSL hydrogenase of the GB1131 strain utilized about 20% of the added hydrogen in 7 days when grown in medium containing 1 g $liter^{-1}$ thiosulfate. In contrast, HupSL showed barely detectable hydrogen consumption when the medium was supplemented with 4 g $liter^{-1}$ thiosulfate (Fig. 1).

Identification of a novel ORF (*hupO*) in the *hupSL* operon. *In silico* analysis of the *hupSL* operon revealed that the previously published operon (*hupSLCDHIR*) contains an open reading frame between the *hupI* and *hupR* genes (8). The *hupI* gene encodes a rubredoxin-type protein, which was proposed to take part in the maturation of the hydrogenase small subunit, while HupR is a response regulator protein shown to be essential for *hupSL* transcription (21, 28). In the last submission of this locus (GenBank accession no. L22980), an *orf1* gene of 432 nucleotides (nt) in length (encoding the 143-amino-acid ORF1) was annotated in this region. The resequencing of the region confirmed the presence of an extra nucleotide in the sequence, resulting a frameshift and a stop codon after the 174th nt. The reannotation of this region disclosed a shorter *orf* coding for 57 amino acids. The role of this 174-nt *orf*, now denominated *hupO*, has been completely unknown so far. Except for the last few amino acids, the sequence of translated HupO was the same as the N terminus of ORF1. According to sequence comparisons, five homologous hits were found in the NCBI databases with at least 75% identity at the nucleotide level. Interestingly, all similar sequences were identified in the publicly available genomic regions of *T. roseopersicina*; no homologous sequences were found in any other organism by a BLAST search of the available databases with the entire *hupO* sequence. Nonannotated *hupO*-homologous regions were identified in various *T. roseopersicina* operons (Fig. 2, top); *hupO* shares the highest similarity with a putative gene with the inverse orientation located in the vicinity of the Hox2-soluble hydrogenase of *T. roseopersicina*. The nucleotide identity between *hupO* and this

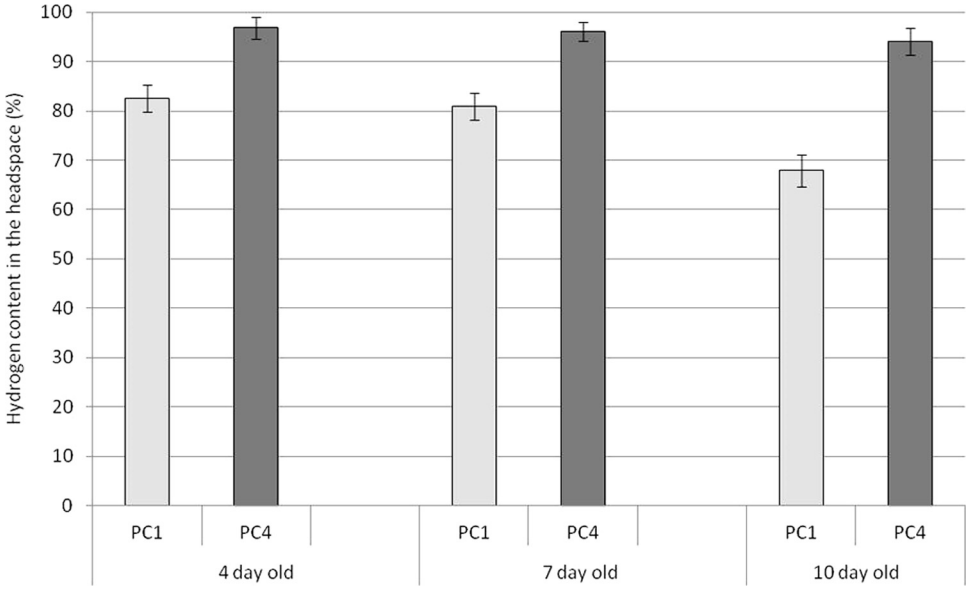

FIG 1 *In vivo* hydrogen uptake of HupSL in GB1131. The headspace hydrogen contents of GB1131 samples were measured on the 4th, 7th, and 10th days of growth; hydrogen uptake was calculated on the basis of the consumed hydrogen (a lower percentage represents higher hydrogen uptake). The initial hydrogen content represents 100%. The samples varied in the initial thiosulfate content of the medium (PC1 medium contains 1 g liter^{-1}, while PC4 contains 4 g liter^{-1} thiosulfate). Four biological replicates were used for the experiments.

putative ORF (here *hox2O*) was 85%. Homologous putative ORFs (HupO-like proteins) were discovered in the photosynthetic gene cluster (between the *ppsR2* and *bchP* genes), at the beginning of the carotenoid biosynthesis operon (preceding the *crtC* gene) (29), in the operon coding for the elements of the light-harvesting complex (between the *astE* and a putative glutamate-cysteine ligase-coding gene), and in a genomic region encoding proteins of the polyhydroxyalkanoate (PHA) biosynthesis pathway (between *phaZ* and a NAD-dependent epimerase-coding gene) in *T. roseopersicina* (30). The multiple alignments of the predicted proteins revealed a clear similarity between the translated HupO, Hox2O, and other HupO-like proteins (the identity values shared between HupO and the similar translated proteins were 75% [Hox2O], 76% [HupO-like 1], 46% [HupO-like 2], 60% [HupO-like 3], and 42% [HupO-like 4]). A highly conserved FNILNRADSNGR short consensus sequence was found in the middle of HupO, Hox2O, and HupO-like proteins (Fig. 2, bottom). A comprehensive search in the databases revealed that diverse proteins showed remarkable similarities to this conserved domain at short regions. A number of regulator proteins can be found among these hits, i.e., a short fragment of the MarR family transcriptional regulator of *Pseudomonas chlororaphis* or a similar fragment of the DNA-binding transcription factor ADR1 of *Saccharomyces cerevisiae* (31, 32). Additionally, similarities of this region were shown to the DNA-directed RNA polymerase sigma-70 factor of *Pseudoalteromonas undina* and to ABC transporter permeases of various bacteria among a large number of hits for hypothetical proteins of various organisms.

Deletion of *hupO* gene dramatically increased HupSL activity and expression. Mutant analysis was performed in order to investigate the role of the putative protein product of the *hupO* gene. In-frame deletion mutagenesis was used to inactivate the *hupO* gene in *T. roseopersicina* GB11 and GB1131. The generated mutant strains are referred to here as HOD1 and HOD13, respectively. Major alterations from strain GB1131 were observed in the HupSL *in vivo* hydrogen uptake activity of the HOD13 mutant strain. The *in vivo* hydrogen uptake was monitored daily starting on day 4 and finishing on day 10 of growth; GB11 and HOD1 were not measured for *in vivo* HupSL activity due to the presence of the active bidirectional Hox1 hydrogenase. The absence of *hupO* resulted in a significant increase in the HupSL activity of GB1131, which was observed exclusively under low-thiosulfate conditions (PC1 representing 1 g liter^{-1}) (Fig. 3). Strain GB1131 was able to utilize a maximum of 20% of the initial hydrogen content from the headspace in 7 days under low-thiosulfate conditions, while the HOD13 strain consumed 65% of the added hydrogen during the same period (Fig. 3, top). Moreover, in 10 days, the HOD13 strain utilized almost all hydrogen from the headspace, while GB1131 used only 35% of the total hydrogen. Interestingly, no significant differences were observed in the HupSL hydrogen uptake between GB1131 and HOD13 under high-thiosulfate conditions (PC4 representing 4 g liter^{-1}) (Fig. 3, bottom).

Homologous complementation of HOD13 (Δ*hupO*) was performed using the *T. roseopersicina crt* promoter for the expression of the *hupO* gene (HOD13comp). The introduction of the *hupO* gene in this expression vector fully restored the original low *in vivo* HupSL hydrogen uptake in strain HOD13comp (Fig. 3, top). Thus, the observed differences in the hydrogen uptake activities of GB1131 and HOD13 strains could be attributed only to the lack of the *hupO* gene.

Along with the *in vivo* Hup hydrogen uptake measurements, the *in vitro* activity of HupSL was investigated using crude extracts. Similar trends and differences were observed *in vitro*, i.e., the Δ*hupO* strain had significantly elevated *in vitro* hydrogen uptake activity compared to that of the GB1131 strain when crude extracts were prepared from cultures grown in medium containing thiosulfate at low concentrations (PC1 and PC2) (data not

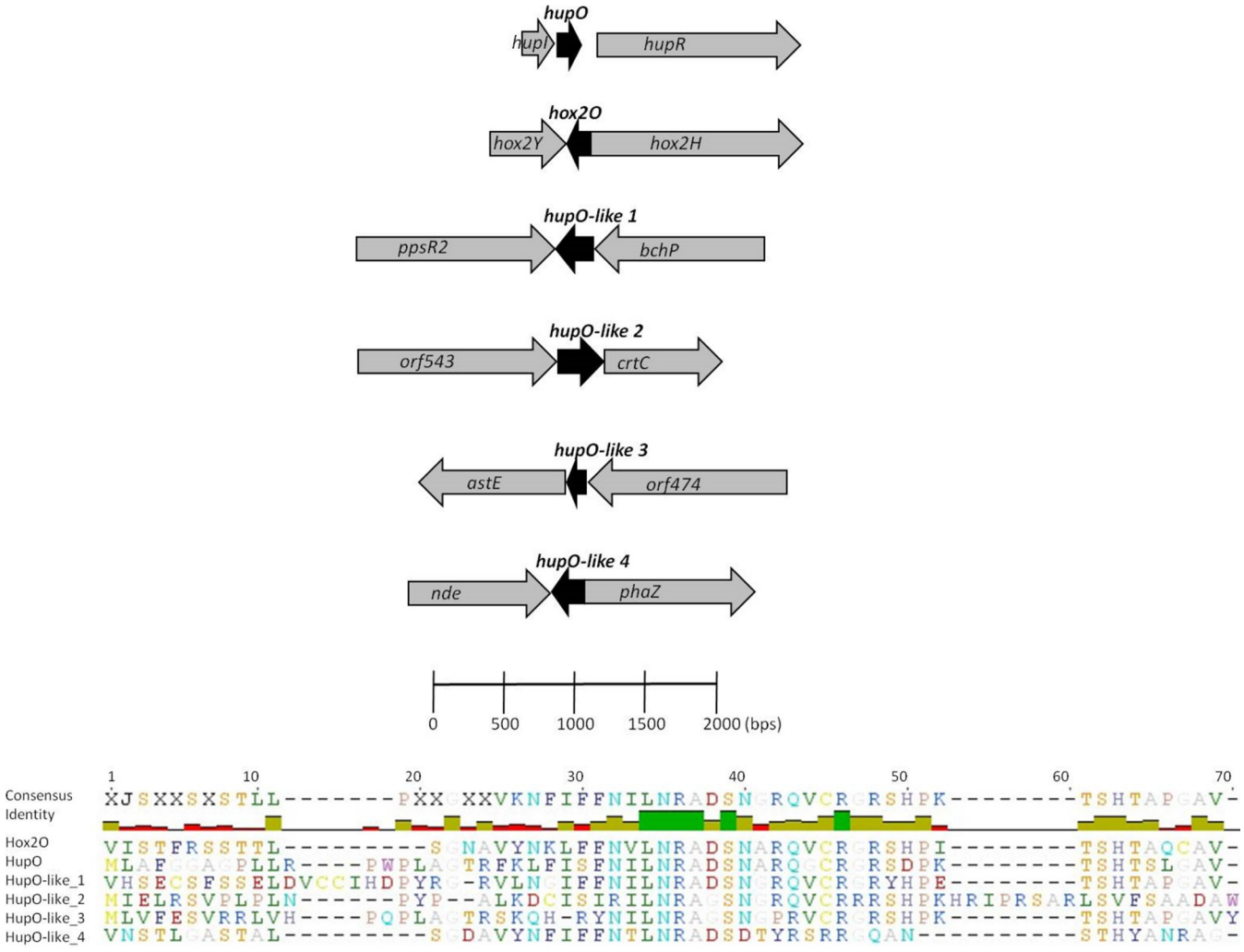

FIG 2 Location and sequence of the *hupO* gene. The *hupO* gene was found in the *hup* operon between *hupI* and *hupR*. (Top) Sequences showing high similarity to *hupO* were identified at multiple sites of the *T. roseopersicina* genome; no homologous sequences were found in any other organism. The *hupO*-like sequence localized in the *hox2* operon was named *hox2O*, while further similar sequences were named *hupO*-like sequences (*hupO*-like 1, 2, 3, and 4). (Bottom) Translated protein sequence alignment of HupO, Hox2O, and HupO-like proteins. A strongly conserved NILNRADSN domain was found in each HupO-like protein. For the genomic contexts, see the text.

shown). Interestingly, a clear difference was observed in the growth characteristics of the strains in PC1: GB1131 had a significantly lower initial growth rate than that of the Δ*hupO* mutant (a difference of 30% ± 7% was observed at 72 h); however, the numbers of cells of the strains were identical by the 7th day. The growth characteristics of the *hupO* mutant were highly similar to those of the wild-type *T. roseopersicina* BBS strain. Thus, more efficient hydrogen uptake coincided with a higher early growth rate in *T. roseopersicina* under low-thiosulfate conditions.

Beside the detailed HupSL activity and growth characterization, we have analyzed Hup expression at both the RNA and protein levels (Fig. 4). Western hybridization experiments were carried out using the appropriate strains (BBS, GB11, HOD1, GB1131, HOD13, and HOD13comp), and HupL was detected using polyclonal anti-HupL antibody. The results revealed a strongly decreased level of HupSL in GB1131 compared to that in BBS and GB11, indicating a prominent effect of the Hox1 hydrogenase on the regulation of HupSL. However, the wild-type level of HupL was restored in the Δ*hupO* mutant (HOD13). The results of the Western studies corroborated the activity analyses; i.e., a significantly elevated level of mature HupL protein was detected in the *hupO* mutant strain under low-thiosulfate conditions (PC0.5, PC1, and PC2) (10- to 15-fold increases in PC1 compared to the HupL level in GB1131), while no difference was observed in the low HupL levels of the GB1131 and HOD13 strains when 4 g liter^{-1} thiosulfate (PC4) was added to the culture medium (Fig. 4). All membranes were stained with Ponceau solution, which revealed the unvarying loading of the samples.

Similar patterns were observed for the transcript levels of the *hup* structural genes when reverse transcription-quantitative PCR (qRT-PCR) quantification of the *hupL* gene was performed under the described growth conditions (PC1 and PC4) using the same strains (BBS, GB11, HOD1, GB1131, HOD13, and HOD13comp) (Fig. 5). Samples were taken on the 4th and 7th days of growth. As expected, the *hupL* transcript level in the GB1131 strain was close to zero on day 4, irrespective of the thiosulfate content of the

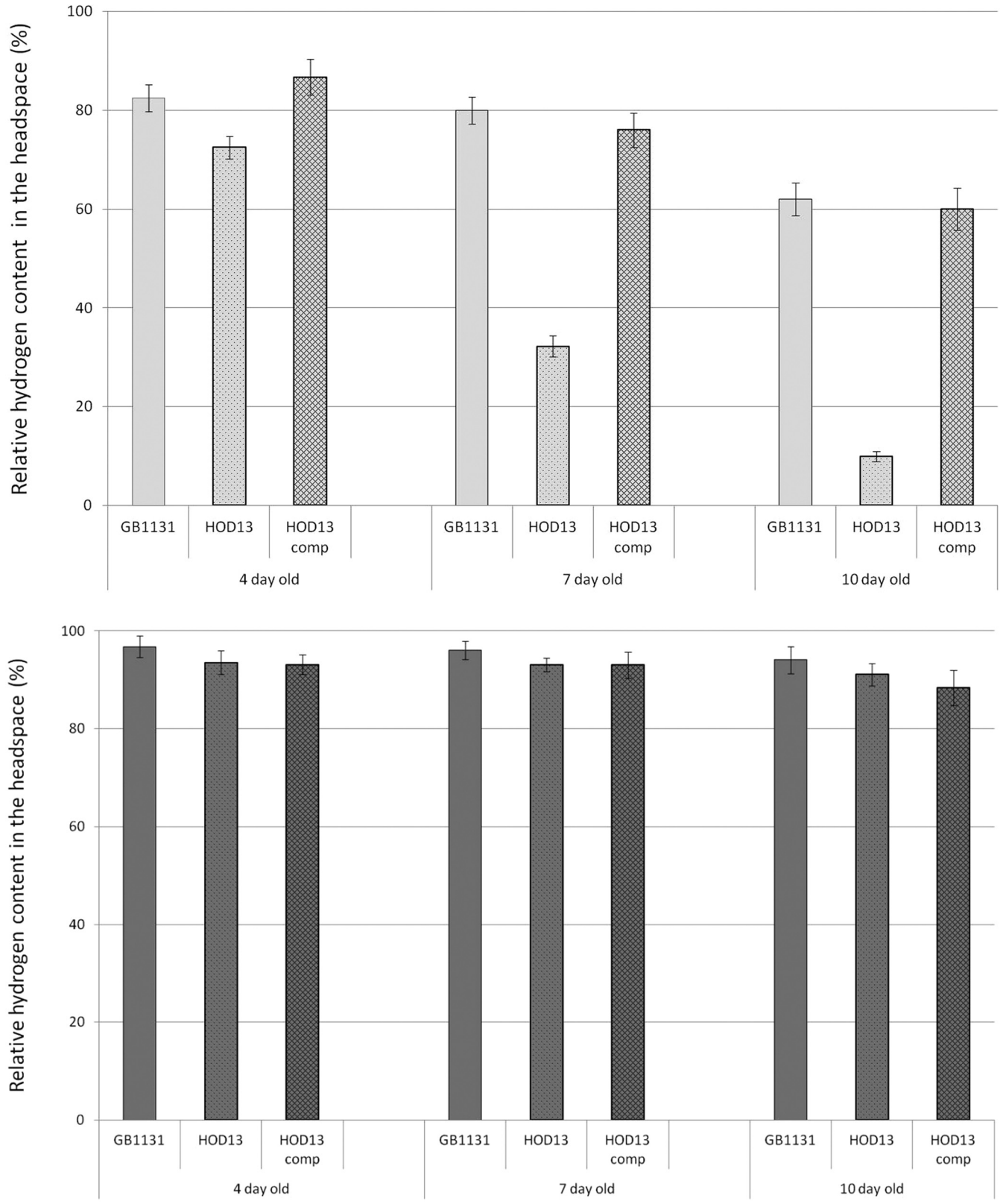

FIG 3 *In vivo* HupSL hydrogen uptake activity in the Δ*hupO* mutant strain. The headspace hydrogen contents of GB1131, HOD13 (Δ*hupO*), and HOD13comp samples were measured on the 4th, 7th, and 10th days of growth. Hydrogen uptake was calculated on the basis of the consumed hydrogen (a lower percentage represents higher hydrogen uptake). The initial hydrogen content represents 100%. Samples were grown in PC1 medium containing 1 g liter^{-1} thiosulfate (top) and in PC4 medium containing 4 g liter^{-1} thiosulfate (bottom). Four biological replicates were used for each experiment.

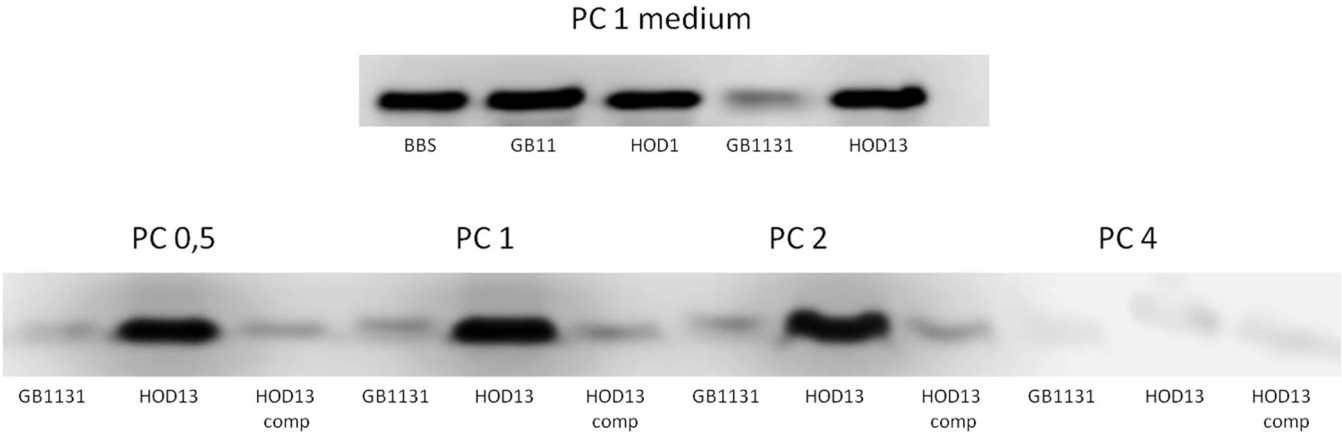

FIG 4 HupL expression analysis. The HupL protein level was analyzed in the appropriate strains using anti-HupL antibody for Western hybridization. The cultures were grown for 7 days in PC medium containing thiosulfate at different concentrations (0.5 g liter^{-1}, 1 g liter^{-1}, 2 g liter^{-1}, and 4 g liter^{-1}). (Top) The HupL protein level was studied in BBS, GB11, HOD1 (Δ*hupO*), GB1131, and HOD13 (Δ*hupO*) strains in PC1 medium. (Bottom) The HupL protein level of the GB1131, HOD13 (Δ*hupO*), and HOD13comp strains was tested in PC medium containing thiosulfate at different concentrations (0.5 g liter^{-1}, 1 g liter^{-1}, 2 g liter^{-1}, and 4 g liter^{-1}). Four biological replicates were done for the Western blotting.

medium. By day 7, a general increase was observed in the transcript levels in all strains, although this increase was much higher in the BBS, GB11, HOD1, and HOD13 strains than those in GB1131 and HOD13comp (e.g., the transcript level of the *hupL* gene was more than 2 orders of magnitude higher in the Δ*hupO* mutant than in GB1131 in PC1 medium) (Fig. 5). Thus, the *hupL* gene was strongly upregulated in the *hupO* mutant strain compared to its level in GB1131, and this phenomenon was more pronounced under low-thiosulfate conditions. Similarly, a relatively high *hupL* level was observed in the wild-type, GB11, and HOD1 strains, all of which contain the Hox1 soluble hydrogenase.

Investigation of the *hupO* transcript level. The *hupO* transcript was investigated under various growth conditions in the BBS, GB11, and GB1131 strains containing the complete *hupSL* operon. The samples harvested on day 4 of growth showed extremely low *hupO* expression according to qRT-PCR. An increased *hupO* transcript level was detected in samples collected on day 7 of growth; therefore, the data derived from these samplings are displayed in Fig. 6. The expression level of *hupO* was slightly influenced by the thiosulfate concentration in all strains; a 2-fold increase was observed in the PC4 medium compared to the *hupO* expression level in PC1. Neither the presence/absence of hydrogen in the headspace nor the presence/absence of Hox1 hydrogenase influenced the *hupO* gene expression levels under any applied thiosulfate concentrations.

Hydrogen-dependent HupSL expression in the Δ*hupO* mutant strain. In the previous investigations, the expression of the *T. roseopersicina* HupSL hydrogenase was independent of the presence or absence of molecular hydrogen (21). Our experiments corroborated this finding when HupSL synthesis was investigated in the GB1131 strain (and also in BBS, GB11, and HOD1), regardless of the applied thiosulfate concentration. However, the clear hydrogen dependence of HupSL synthesis was observed in the *hupO* mutant GB1131 strain (HOD13) in samples grown under

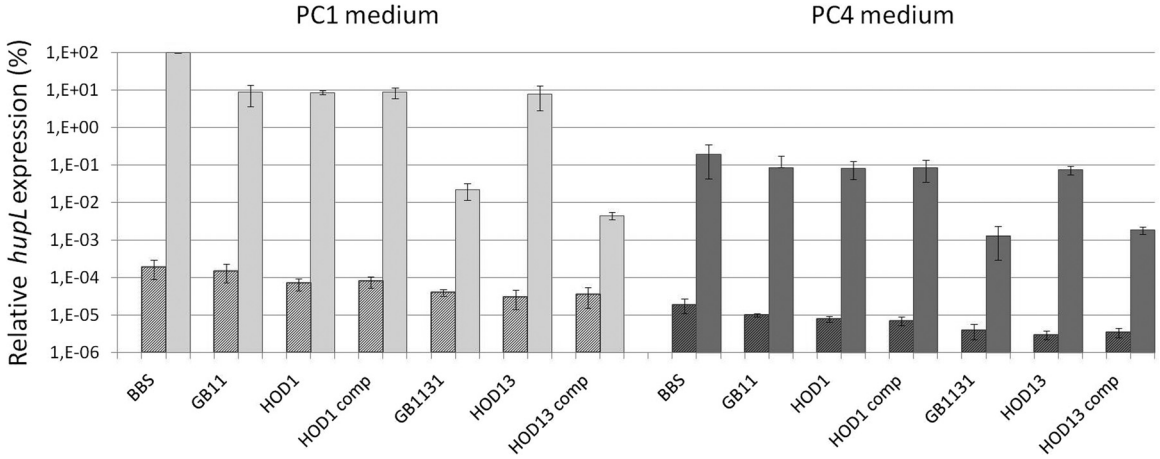

FIG 5 *hupL* transcript analysis by qRT-PCR. The relative transcript level of the *hupL* gene was determined in the BBS, GB11, HOD1 (Δ*hupO*), HOD1comp, GB1131, HOD13 (Δ*hupO*), and HOD13comp strains. The expression level of *hupL* in BBS in PC1 was considered 100%. The cultures were grown under various conditions (PC1 and PC4), and the cells were harvested on the 4th and 7th days of growth. Four biological replicates in triplicate were used. The stippled columns show the expression level of the 4-day-old culture, and the solid columns shows the 7-day-old culture.

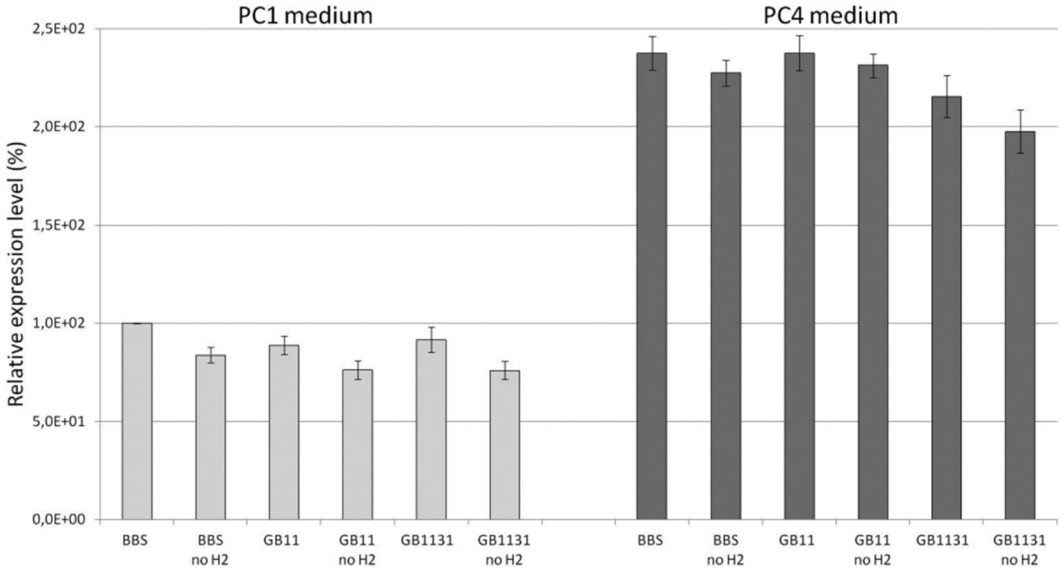

FIG 6 *hupO* is transcribed in a hydrogen-independent manner. The relative expression level of the *hupO* gene was investigated in the BBS, GB11, and GB1131 strains. Samples were grown under various conditions (PC1 and PC4 with and without hydrogen in the headspace), and the cells were harvested at the 7th day of growth. Four biological replicates were measured in triplicate.

low-thiosulfate conditions (PC1) (Fig. 7). Western hybridization experiments using the anti-HupL antibody were carried out on BBS, GB11, HOD1, GB1131, and HOD13 strains cultivated under various thiosulfate conditions for 7 days. Hydrogen (89.1 μmol H_2) was either added or omitted at the beginning of the experiment. The generally low level of HupL synthesis showed only a minor change in response to the addition of hydrogen in GB1131, while the level of HupL showed significant differences in HOD13 between cultures grown with and without hydrogen in PC1 medium. The mutant cultures (HOD13) grown under hydrogen expressed a multiplied amount of HupL protein compared to that with the same strain grown in the same medium (PC1) without hydrogen in the headspace (Fig. 7). However, hydrogen dependence of HupL protein synthesis was not observed in strains containing the Hox1 hydrogenase (BBS, GB11, and HOD1). It should be noted that although H_2 was added at the beginning of the experiment, the headspace still contained H_2 at the time of sampling on day 7 (Fig. 3, top). The strains grown in PC4 medium showed a significantly lower level of HupL synthesis, and this was only slightly influenced by the presence or absence of hydrogen (data not shown). The hydrogen dependence of the *hupSL* transcript level was investigated by qRT-PCR; the obtained data corroborated the results of the protein analysis (Fig. 8). The expression level of the *hupL* structural gene in GB1131 showed only minor differences in the presence or absence of hydrogen. Contrarily, the *hupL* gene expression level in the HOD13 (GB1131 Δ*hupO*) strain was strongly influenced by hydrogen under low-thiosulfate conditions (Fig. 8). The *hupO* (HOD13) mutant strain grown in the presence of hydrogen showed higher *hupL* transcript levels than those of the corresponding cultures without hydrogen. It is noteworthy that the effect of hydrogen is specific, as the addition of alternative electron donors (organic acids) had an effect similar to that of the elevated thiosulfate concentration.

DISCUSSION

Hup-type membrane-associated [NiFe]-hydrogenases are the major energy-conserving hydrogenases utilizing molecular hydrogen as an electron and energy source (3). It has been demonstrated in cyanobacteria that HupSL hydrogenase expression is induced under nitrogen-depleted conditions, and the important role of the Hup hydrogenase in recycling and utilization of molecular hydrogen generated by the nitrogenase enzyme as a by-product of the bacterial nitrogen fixation process has been established (33). *T. roseopersicina* also harbors a Hup-type [NiFe]-hydroge-

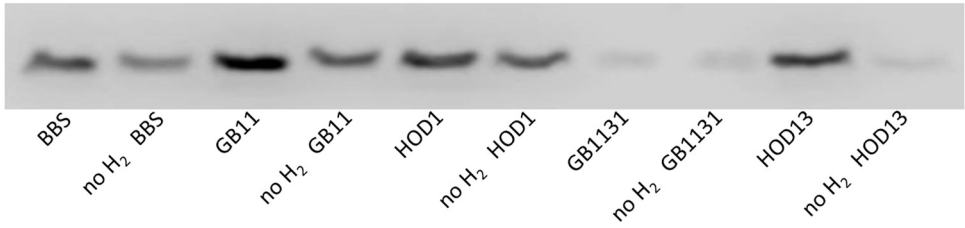

FIG 7 Hydrogen dependence of HupL synthesis. A Western hybridization approach was used to investigate the hydrogen dependence of Hup expression. The HupL protein levels were analyzed in the BBS, GB11, HOD1 (Δ*hupO*), GB1131, and HOD13 (Δ*hupO*) strains using anti-HupL antibody. The cultures were grown for 7 days in PC medium containing 1 g $liter^{-1}$ thiosulfate (with and without hydrogen in the headspace). Four biological replicates were done for the Western blotting.

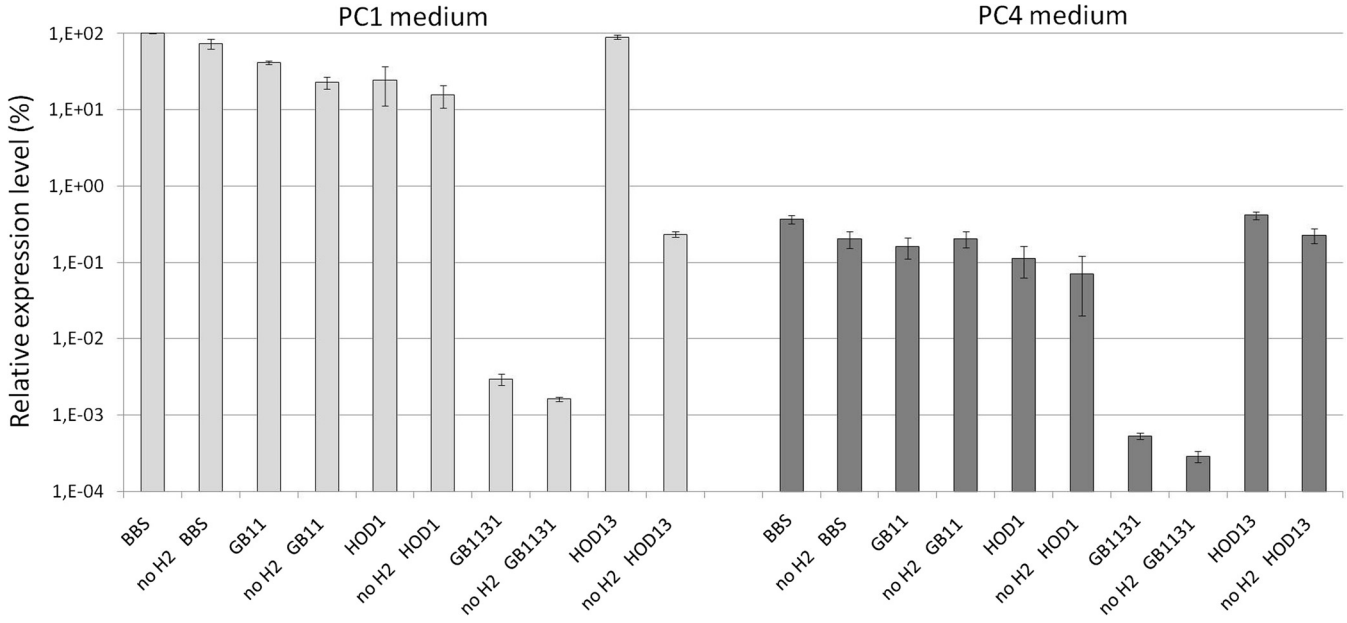

FIG 8 Hydrogen dependence of *hupL* transcription. The relative transcript levels of the *hupL* gene were determined in the BBS, GB11, HOD1 (Δ*hupO*), GB1131, and HOD13 (Δ*hupO*) strains. The expression level of *hupL* in BBS in PC1 was considered 100%. The cultures were grown in PC1 and PC4 medium with and without hydrogen in the headspace, and the cells were harvested on the 7th day of growth. Four biological replicates were used.

nase and a nitrogenase; thus, it can grow in nitrogen-depleted environments. Previous studies indicated that the HupSL enzyme was constitutively expressed in this organism at a relatively low level, and under the tested conditions in the investigated strains, the presence of hydrogen could not regulate *hupSL* expression (21). This was an unexpected result, especially because all elements of the hydrogen-sensing apparatus were shown to be present in *T. roseopersicina* at the genetic level. This issue seemed to be resolved by the observation that the *hupTUV* genes were inactive in this organism; thus, hydrogen sensing was not observed. Thiosulfate was also shown earlier to be an important influencing factor in *hupSL* expression (13). A significant increase was observed in the *hupSL* expression level when the concentration of thiosulfate, the major electron donor for *T. roseopersicina*, was decreased in the medium of the GB1131 strain, wherein HupSL was the only functional hydrogenase (13).

We have identified homologous short ORFs in several operons related to redox reactions in *T. roseopersicina*. One of them, designated *hupO*, was located in the *hup* operon preceding the *hupR* gene, coding for the regulator element of the HupR-HupT two-component signal transduction system. In-frame deletion mutagenesis was applied to assess the possible role(s) of *hupO*, which was shown to be expressed under various tested conditions. The *hupO* gene was deleted in the GB11 (*hynSL* mutant) and GB1131 (*hynSL* and *hox1H* mutant) strains (resulting in the HOD1 and HOD13 strains, respectively). Dramatically increased HupSL hydrogen uptake activity was observed in the HOD13 mutant strain compared to that in GB1131, but this increase was observable exclusively under low-thiosulfate conditions. The thiosulfate concentration dependence of the elevated *in vivo* HupSL hydrogen uptake was assessed in detail in the GB1131 and in the HOD13 (GB1131 Δ*hupO*) strain, and a clear correlation was observed between the thiosulfate content and HupSL activity. The Hup activity-promoting effect of the *hupO* mutation was eliminated when a high thiosulfate concentration was applied, leading to the conclusion that the supposed release (achieved by deletion) of the *hupO*-mediated HupSL repression was masked by high thiosulfate concentrations. Essentially the same conclusions were drawn from the *hupL* expression studies at both the RNA and protein levels. *hupL* expression was strongly increased in the GB1131 *hupO* mutant strain compared to that in GB1131, and this increase was more pronounced under low-thiosulfate conditions. The fact that the HupSL protein level in HOD13 is similar to what is observed in BBS (wild type) and GB11 (*hynSL* mutant) suggests a sophisticated interconnection of the various [NiFe]-hydrogenases in this bacterium and the dramatic effect of Hox1 hydrogenase on the regulation of the HupSL enzyme. The deletion of Hox1 results in strongly decreased HupSL synthesis, and this decrease can be reversed by the deletion of the *hupO* gene. Thus, HupO has a mediating role between Hox1 and HupSL, as the *hupO* gene supposedly encodes a repressor, which is active exclusively in the absence of Hox1. However, changes were not observable either at the RNA or protein level of HupO under any tested conditions, indicating possible posttranslational regulation. It is also known that the Hox1 hydrogenase plays an essential role in the redox homeostasis of *T. roseopersicina* by functioning as a redox valve (15). Hox1 has a central position among the [NiFe]-hydrogenases in this organism; HupO might represent a molecular switch between Hox1 and HupSL, and in the absence of the central component, the switch (HupO) might be arrested in repressing mode.

Also, clear hydrogen dependence of *hup* expression was observed exclusively in the GB1131 strain. The observed hydrogen-related elevated *hup* expression in the *hupO* mutant in the absence of Hox1 is a rational step for the bacterial cell, since HupSL has to replace Hox1 in energy conservation and in the maintenance of the cell's redox balance by hydrogen uptake and consequent electron supply under conditions of electron shortage (i.e., low thiosulfate levels).

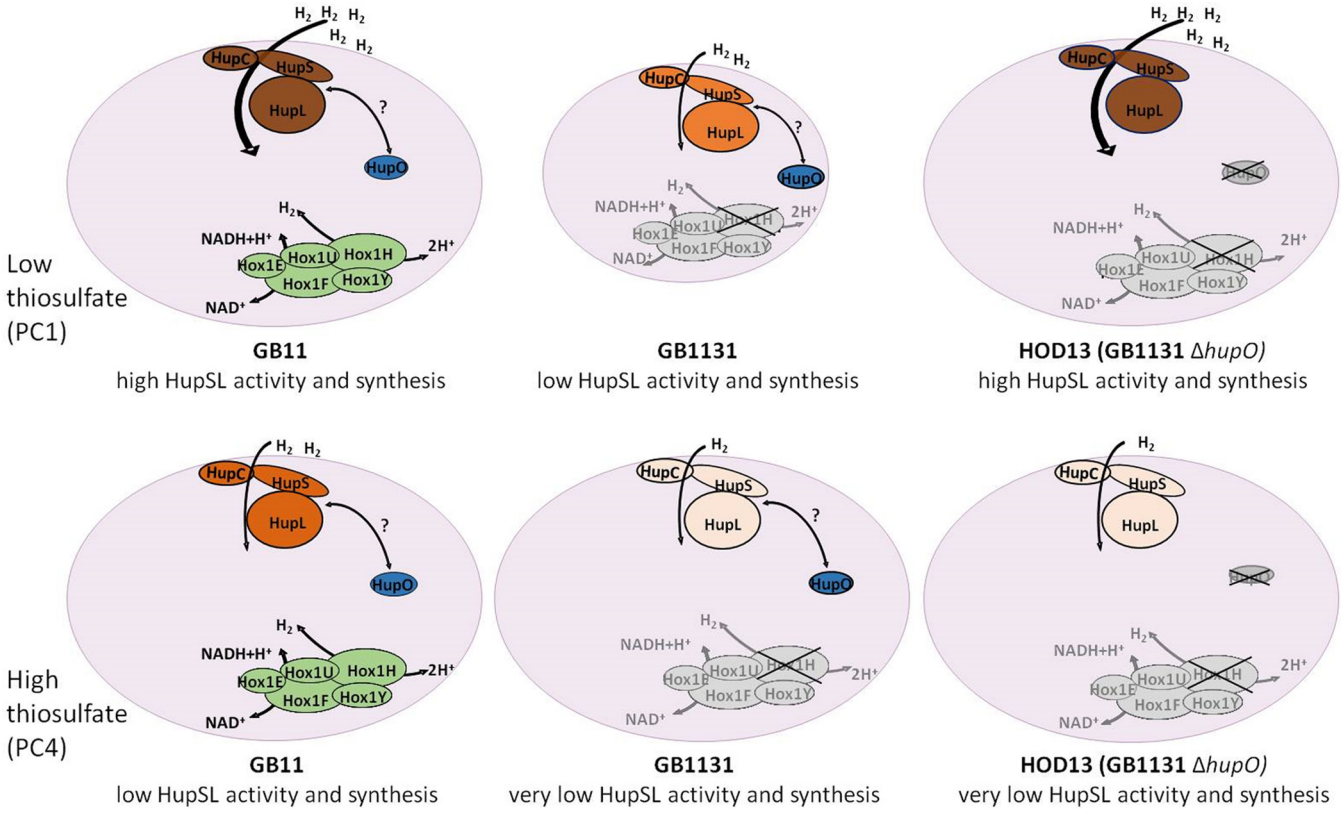

FIG 9 Summary of HupSL activity and synthesis in *T. roseopersicina*. (Top) Cells were grown under low-thiosulfate conditions (PC1). (Bottom) Cells were grown under high-thiosulfate conditions (PC4). The sizes of the cells correlate with the observed different growth characteristics of the strains. The shades of HupSL and HupO proteins reflect the synthesis levels (darker color represents higher protein expression). The proteins in gray represent mutations. The number of H_2 molecules represents the different HupSL hydrogen uptake activities.

The results suggest a triple mechanism of control of the HupSL hydrogenase in *T. roseopersicina*, as summarized in Fig. 9. In our model, thiosulfate is the primary regulator; when thiosulfate concentration in the environment is high, the HupSL hydrogenase is efficiently repressed in all strains, irrespective of the presence or absence of the *hupO* gene and of the presence of further hydrogenases in the cell. Under low-thiosulfate conditions, the expression of the HupSL enzyme is elevated in each strain except those lacking the Hox1 hydrogenase. Both the HupSL activity and HupL protein amount are much lower in the GB1131 strain than those in strains harboring Hox1 hydrogenase (BBS and GB11), which implies to an as-yet-uncharacterized connection between Hox1 and HupSL. However, the low Hup activity and expression in GB1131 are significantly increased by elimination of the *hupO* gene, which supposedly encodes a repressor acting as a second-level regulator. Moreover, hydrogen seems to serve as an additional modulator of Hup functions by influencing *hup* expression in the *hox1* mutant strain when the *hupO* gene, coding for a putative repressor, is deleted (HOD13).

A number of questions remain open for further research. What is the rationale behind holding the *hupSL* operon under permanent repression, mediated by the product of the *hupO* gene even under low-thiosulfate conditions, when HupSL might be an efficient tool for energy conservation through hydrogen uptake? Most probably, the explanation is hidden in the sophisticated interhydrogenase communication network of the *Thiocapsa* cell. The possibly specific roles of additional *hupO*-like sequences identified in a number of *T. roseopersicina* operons represent further questions to address. Interestingly, all of these operons code for enzymes, pathways participating in the maintenance of the redox homeostasis of the cells. Is it possible that these pathways are also in connection with Hox1 through these *hupO*-like elements, which were shown to be conserved and similar to various regulator proteins?

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