

Metabolically Engineered *Rhodobacter sphaeroides* RV strains for Improved Biohydrogen Photoproduction Combined with Disposal of Food Wastes

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Abstract: Three differently metabolically engineered strains, 2 single PHA⁻ and Hup⁻ mutants and one double PHA⁻/Hup⁻ mutant, of the purple nonsulfur photosynthetic bacterium *Rhodobacter sphaeroides* RV, were constructed to improve a light-driven biohydrogen production process combined with the disposal of solid food wastes. These phenotypes were designed to abolish, singly or in combination, the competition of H₂ photoproduction with polyhydroxyalkanoate (PHA) accumulation by inactivating PHA synthase activity, and with H₂ recycling by abolishing the uptake hydrogenase enzyme. The performance of these mutants was compared with that of the wild-type strain in laboratory tests carried out in continuously fed photobioreactors using as substrates both synthetic media containing lactic acid and media from the acidogenic fermentation of actual fruit and vegetable wastes, containing mainly lactic acid, smaller amounts of acetic acid, and traces of higher volatile acids. With the lactic acid-based synthetic medium, the single Hup⁻ and the double PHA⁻/Hup⁻ mutants, but not the single PHA⁻ mutant, exhibited increased rates of H₂ photoproduction, about one third higher than that of the wild-type strain. With the food-waste-derived growth medium, only the single Hup⁻ mutant showed higher rates of H₂ production, but all 3 mutants sustained a longer-term H₂ photoproduction phase than the wild-type strain, with the double mutant exhibiting overall the largest amount of H₂ evolved. This work demonstrates the feasibility of single and multiple gene engineering of microorganisms to redirect their metabolism for improving H₂ photoproduction using actual waste-derived substrates.

Key words: metabolic engineering, *Rhodobacter sphaeroides* RV, biohydrogen production, food waste disposal.

INTRODUCTION

Hydrogen (H₂) is rapidly emerging as a key component of the clean and sustainable energy systems of the future.

Hydrogen from biomass and biowastes is one potentially economical and renewable source of H₂, with both thermal and biological conversion processes providing plausible approaches. Photosynthetic bacteria can use sunlight to drive H₂ evolution reactions from organic substrates in so-called photofermentations, thereby recovering a greater fraction of the energy stored in biomass than is possible with dark fermentations (Benemann, 1977, 1998; Sasikala

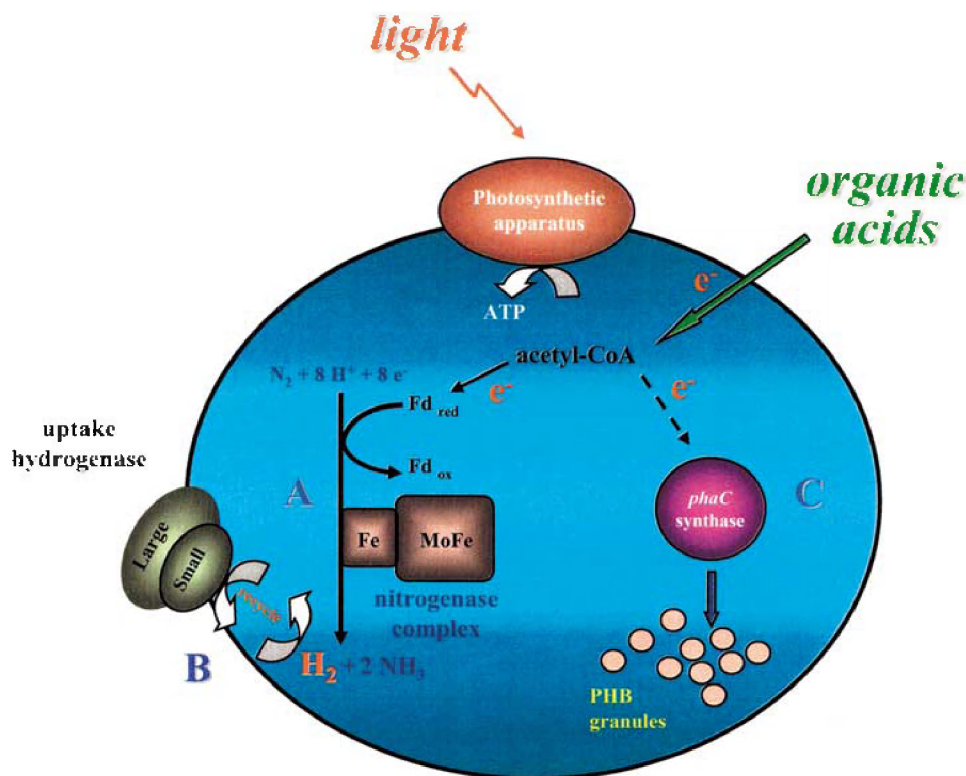


Figure 1. Schematic of metabolic pathways of H₂ evolution/uptake and competing reactions. **A:** Nitrogenase complex, catalyzing H₂ evolution. **B:** Membrane-bound uptake hydrogenase, responsible for H₂ uptake (recycling). **C:** PHB synthase, involved in biosynthesis of poly-3-hydroxybutyrate granules.

et al., 1993; Ikuta et al., 1998; Das and Veziroglu, 2001; Miyake et al., 2001). Our research target is to maximize H₂ photoproduction from food wastes (Fascetti and Todini, 1995; Fascetti et al., 1998), and the specific objective of the present work was to genetically improve the H₂ evolution performance of the purple nonsulfur photosynthetic bacterium *Rhodobacter sphaeroides* RV, a wild-type strain extensively studied in Japan (Mao et al., 1986; Miyake et al., 1999).

In photosynthetic bacteria the amount of H₂ that evolves anaerobically from organic substrates is determined by the interaction of several metabolic pathways, as schematically shown in Figure 1: H₂ evolution, mediated by the enzyme nitrogenase; H₂ uptake (recycling), catalyzed by a membrane-bound uptake hydrogenase that reduces the net amount of gas evolved (Vignais et al., 1985); and biosynthesis of alternative electron sinks for reductants, in particular, poly-3-hydroxybutyrate (PHB) in the form of cytoplasmic granules.

In batch cultures with synthetic growth media, uptake hydrogenase-negative mutants of *Rhodobacter capsulatus* and *Rhodospirillum rubrum* showed increased H₂ photoproduction, depending on the nitrogen and the carbon sources employed (Jahn et al., 1994; Kern et al., 1994; Zorin et al., 1996). Similarly, the biosynthesis of storage

energy reservoirs, specifically PHB (Brandl et al., 1991; Katipov et al., 1998), reduced nitrogenase-mediated H₂ evolution by photosynthetic bacteria, as demonstrated with batch cultures using synthetic media (Husted et al., 1993; Maeda et al., 1997; Vincenzini et al., 1997). With *R. sphaeroides* RV in continuous-flow cultures, using both synthetic and waste-derived growth media, H₂ photoproduction slowed as the bacteria started producing PHB in response to carbon excess and nitrogen and light limitations (Fascetti and Todini, 1995; Fascetti et al., 1998). In the present study, 3 mutants of *R. sphaeroides* RV were constructed, 2 single PHA⁻ and Hup⁻ mutants and one double PHA⁻/Hup⁻ mutant, to demonstrate the possibility of improving the nitrogenase-dependent H₂ photoproduction rate and duration, compared to the wild-type strain, with both synthetic and food-waste-derived fermentation substrates.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study, together with their relevant characteristics, are listed in Table 1.

Table 1. Bacterial Strains and Plasmids Used in this Study

Strains or plasmids	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
TG1	SupE <i>thi-1</i> Δ (<i>lac-proAB</i>) Δ (<i>mcrB-hsdSM</i>)5 (<i>r_k-m_k-</i>) [<i>F' traD36proAB lacI^q ZΔM15</i>]	Sambrook and Russell (2001)
S17-1	C600::RP4-2 (<i>Tc</i> ::Mu)(<i>Km</i> ::Tn7) <i>thi pro hsdR recA</i>	Simon et al. (1983)
<i>R. sphaeroides</i> strains		
RV 8703	Wild type	Mao et al. (1986)
SMV047	Spontaneous Rif ^r mutant of <i>R. sphaeroides</i> RV	This work
SMV071	<i>R. sphaeroides</i> RV PHA ⁻ mutant	This work
SMV089	<i>R. sphaeroides</i> RV Hup ⁻ mutant	This work
SMV087	<i>R. sphaeroides</i> RV PHA ⁻ /Hup ⁻ double mutant	This work
Plasmids		
pUC18	Ap ^r , lacZa	Yanisch-Perron et al. (1985)
pSUP2021	pSUP202::Tn5, Ap ^r , Cm ^r , Tc ^r	Simon et al. (1983)
p34E-Tp	R388 derivative, Tp ^r	De Shazer and Woods (1996)
pWRK102A	<i>mob</i> , Gm ^r , Cm ^r	Colonna-Romano et al. (1990)
pSM774	pUC18-derivative containing 1.7 kb of <i>R. sphaeroides</i> RV DNA (<i>phaC</i>) Ap ^r	This work
pSM872	pUC 18 derivative containing 2.1 kb of <i>R. sphaeroides</i> RV DNA (<i>hupSL</i>), Ap ^r	This work
pSM583	pWRK102A derivative containing <i>R. sphaeroides</i> RV <i>phaC</i> gene interrupted by the Km ^r cassette, Gm ^r Cm ^r Km ^r	This work
PSM825	pWRK 102A derivative containing <i>R. sphaeroides</i> RV <i>hupSL</i> genes interrupted by the Tp ^r cassette, Gm ^r Cm ^r , Tp ^r	This work

Escherichia coli strains were grown at 37°C in LB medium (Sambrook and Russel, 2001) supplemented, when required, with the following antibiotics: ampicillin 100 µg/ml, kanamycin 20 µg/ml, chloramphenicol 20 µg/ml, trimethoprim 1.5 mg/ml. *R. sphaeroides* RV strain 8703 was provided by the National Institute of Bioscience and Human Technology, Tsukuba, Japan. A spontaneous rifampicin-resistant mutant of *R. sphaeroides* RV (SMV047 strain) was used as a wild-type control in all comparative experiments. *R. sphaeroides* strains were routinely grown in liquid cultures and on plates at 30°C, either aerobically in the dark or anaerobically in the light, using synthetic media prepared by adding appropriate carbon and nitrogen sources to the basal medium (Miyake et al., 1984). The maintenance medium, aSy, was prepared by adding to the basal medium (NH₄)₂SO₄ 1.25 g/L, Na succinate 9.8 g/L (wt/vol), and yeast extract 1 g/L (Miyake et al., 1984). The gL medium, used for H₂ photoproduction in tubes and bottles, contained the basal medium and Na D-L lactate 98% 7.4 ml/L, Na-L-glutamate 1.88 g/L, and NaHCO₃ 1.5 g/L (Nagamine et al., 1996). The medium used to induce PHB

synthesis (acetate medium) contained the basal medium and NH₄Cl 0.2 g/L, Na acetate 0.75 g/L, and yeast extract 0.2 g/L. Growth media were supplemented, when required, with the following antibiotics: rifampicin 150 µg/ml, 1gentamicin 5 µg/ml, trimethoprim 25 µg/ml, kanamycin 20 µg/ml. Photoheterotrophic growth was carried out under anaerobic conditions using completely filled 40-ml screw-capped bottles illuminated with tungsten lamps (9.5 klux) in a water bath maintained at 30°

Hydrogen Photoproduction Tests

H₂ photoproduction tests of *R. sphaeroides* RV wild-type and mutant strains were performed under artificial light using 1-L cylindrical photobioreactors equipped with a magnetic stirrer and operated continuously. Initial comparative tests were carried out using a synthetic medium containing, in addition to the basal medium, lactic acid 5 g/L, yeast extract 0.25 g/L, and NH₄Cl 0.254 g/L. Further tests used a liquor derived from the acidogenic fermentation of discarded fruits and vegetables with

an average composition of 4.8% total solids (TS), of which 72% were volatile solids (VS), lactic acid 41 g/L, acetic acid 3.8 g/L, traces of propionic and butyric acids, total nitrogen 0.59 g/L, of which NH₄⁺ nitrogen 0.26 g/L, total carbon 15.8 g/L, TOC 14.9 g/L, COD 5.9 g/L, Ca²⁺ 5.9 g/L, Na⁺ 0.25 g/L, sulfates 0.25 g/L, and phosphates 23 mg/L (Fascetti et al., 1998). Owing to batch-to-batch variability in the composition of the food-waste-derived substrate, which was found to affect both the rate and duration of H₂ photoproduction, all comparative experiments were carried with the wild type in parallel to the mutant strain under evaluation. Reproducibility of the results was confirmed by several side-by-side comparative tests performed with different batches of the waste-derived medium. For the H₂ photoproduction tests, precultures of *R. sphaeroides* strains were prepared from glycerinate stocks in 10 ml of aSy medium, then incubated at 30°C in aerobic/dark conditions for 2 days, and then transferred to anaerobic/light conditions at 30°C in 250-ml bottles containing the medium used for the subsequent growth in the photobioreactor and incubated for 2 days. These anaerobic inocula were added to 1-L photobioreactors (930 ml liquid volume, V), flushed with sterile oxygen-free argon, and maintained in batch culture for 24 hours before continuous feeding of the substrate, which was kept under argon atmosphere. The flow rate (*F*) was fixed at 31 ml/h, giving a dilution rate ($D = F/V$) of 0.033/h and, thus, hydraulic residence time ($HRT = 1/D$) of 30 hours. Illumination was provided by two 100-W halogen lamps, and the light intensity was 6.0 klux measured by a LSI luxmeter on the external surface of the reactor. The pH was automatically maintained at 7 by a pH controller through 1 N NaOH or HCl addition. The volume of gas evolved was measured with a Triton wet gas meter.

Analytical Measurements

Bacterial growth was measured by optical density at 600 nm. Cell dry weight (g/L) was determined by centrifugation of 10 ml of cell suspension, washing the pellet once in distilled water, and drying it in a vacuum oven. One OD₆₀₀ unit corresponded to a dry weight of 3.3 g/L. Poly(3-hydroxybutyrate) was quantitatively assayed according to the gas chromatographic method previously described (Brandl et al., 1989). Protein content of *R. sphaeroides* soluble extracts was determined according to Bradford (1976), using a bovine serum albumin standard. Lactic acid was measured with a Varian 3400

GC equipped with a 250 × 2-mm glass column packed with Carbowax B-DA 4% Carbowax 20 M 80-120 mesh (Supelco). The composition of the effluent gas was assayed by injecting 200 µl of the gas phase into a Varian 3400 gas chromatograph equipped with a 200 × 3-mm steel column packed with Carbosieve II 80-100 mesh (Supelco). Uptake hydrogenase activity of intact *R. sphaeroides* RV cells was assayed by following the reduction of methylene blue (MB) under a H₂ atmosphere at 570 nm. Reactions were carried out at room temperature in H₂-filled sealed glass cuvettes, containing 50 µl of cell suspension and 120 µM MB in 20 mM Tris HCl buffer, pH 8. One unit of hydrogenase activity is the amount of enzyme that catalyzes the reduction of 1 µmol MB/min. Nitrogenase activity was determined by the acetylene assay with culture samples (6 ml) transferred to 20-ml glass vials sealed with rubber serum stoppers and preincubated at 30°C for 5 minutes under an argon atmosphere with stirring and illumination (150 W/cm²). The reaction was started by the injection of 2 ml of acetylene with a gas-tight syringe. The gas phase samples of 50 µl at intervals (0, 5, 15, and 20 minutes) were analyzed by a Varian 3400 GC with a HayeSep S column (2-m length, 1-mm i.d.). One unit of nitrogenase activity reduces 1 µmol acetylene/min.

Recombinant DNA Techniques

All restriction endonucleases, nucleic acid-modifying enzymes, and nylon membranes for Southern blot were products of Roche Molecular Biochemicals. Oligonucleotides for polymerase chain reaction (PCR) experiments, DNA sequencing analyses, and Southern blots were synthesized with an Oligo 1000 DNA synthesizer (Beckman). Plasmid isolation, DNA ligation, restriction mapping, and transformation were performed as described by Sambrook and Russell (2001). PCRs were performed with a 480 PerkinElmer Cetus thermal cycler. A PCR program optimized for high G+C content genomic DNA was employed (initial denaturation at 98°C for 2 minutes, then 25 cycles with denaturations at 98°C for 1 minute, annealing at 60°C for 1 minute, and elongation at 72°C for 2 minute). Reaction samples (100 µl) included 500 ng of template DNA, 200 µM dNTPs, 100 pmol of each primer, 10 mM Tris-HCl, pH 8.85, 25 mM KCl, 5 mM (NH₄)SO₄, 2 mM MgSO₄, and 2.5 U of *Pwo* DNA polymerase. The following oligonucleotides were used to amplify a 1.7-kb DNA fragment corresponding to the *R. sphaeroides* RV *phaC* gene sequence: *phaC* forward,

5'-CGTGACGCTCAGTTCGAGCGTCTAAC-3'; *phaC* reverse, 5'-CGGCGCGAGCTCGGGATGTTTCAATC-3'. The following oligonucleotides were used to amplify a 2.1-kb DNA fragment corresponding to *R. sphaeroides* RV *hupS-hupL* gene sequences: *hupS* forward, 5'-AATACAAGGGCAACTACATTG-3'; *hupL* reverse, 5'-GCCCTCGACCTGGTTCTTGATGTCCT-3'.

The resulting amplicons were used for the insertion of the interposon in mutagenesis experiments aimed to inactivate *phaC* and *hupL* genes, respectively, on the genome of *R. sphaeroides* RV. Southern blot analyses were performed on genomic DNA extracted from *R. sphaeroides* RV wild-type and mutant strains according to a standard protocol (Wilson, 1987). For the genetic characterization of *R. sphaeroides* RV mutants by Southern blot analysis, 10 µg of genomic DNA was digested with *EcoRI* (PHA⁻ mutants) or *BstXI* (Hup⁻ mutants). DNA digestion products were separated by agarose gel electrophoresis, and after the electrophoretic run the DNA transfer was carried out in a PosiBlot apparatus (Stratagene). Nylon membranes were hybridized according to the DIG System method (Roche Molecular Biochemicals) using 3'-labeled oligonucleotides specific for *phaC* and *hupL* genes as probes.

DNA sequencing was performed by an ABI PRISM 373A automatic DNA sequencer (Applied Biosystems). DNA and protein databanks were surveyed with the BLAST server of the National Center for Biotechnology Information, National Institutes of Health, Bethesda, Md. The OMIGA software (Oxford Molecular) was used to process DNA and protein sequence data.

Construction of *R. sphaeroides* RV Mutants by Interposon Mutants by Interposon Mutagenesis

The kanamycin resistance cassette (1.6 kb), comprising the kanamycin resistance gene *neo*, together with the upstream promoter sequence used for the inactivation of the *R. sphaeroides* RV *phaC* gene, was recovered as a *SalI-HindIII* fragment from the plasmid pSUP2021 (Simon et al., 1983). The cassette was inserted in the unique restriction site *StyI* within the *phaC* gene (1.7 kb), previously isolated by PCR and cloned into the vector pUC18 (pSM744). The location of the interposon at the *StyI* site in the *phaC* gene was verified by restriction analysis. The *phaC* gene interrupted by the kanamycin cassette was then recovered as an *Asp718-XbaI* fragment and cloned into the suicide mobilizable vector pWKR102A (Colonna-Romano et al., 1990). The resulting plasmid pSM583 was

isolated by selection of *E. coli* transformants on LB agar plates containing 20 µg/ml chloramphenicol and 20 µg/ml kanamycin. The trimethoprim resistance cassette (620 bp), consisting of the trimethoprim-resistant dihydrofolate reductase gene *dhfrIIb*, together with the strong promoter P₁ used for the inactivation of *R. sphaeroides* RV *hupL* gene, was recovered as a *PstI* fragment from the vector p34E-Tp (De Shazer and Woods, 1996). The cassette was inserted in one of the 2 *PstI* restriction sites within the *hupL* gene (1.45 kb), previously isolated by PCR together with 650 bp of the upstream *hupS* gene, and cloned into the vector pUC18 (pSM872). By restriction analysis it was verified that the location of the interposon was at the *PstI* site, corresponding to nucleotide 338 of the *hupL* gene, and that the insertion of the cassette resulted from partial *PstI* digestion. The *hupSL* genes, interrupted by the trimethoprim resistance cassette, were then isolated from the vector pSM872 by PCR and cloned into the suicide mobilizable vector pWKR102A. The resulting plasmid pSM825 was recovered by selection of *E. coli* transformants on LB agar plates containing 20 µg/ml chloramphenicol and 1.5 mg/ml trimethoprim.

To construct PHA⁻ and Hup⁻ mutant strains, plasmids pSM583 and pSM825 were separately mobilized from *E. coli* S17-1 into *R. sphaeroides* RV wild type by conjugation. The subsequent chromosomal *phaC* and *hupL* gene replacements were selected by kanamycin and trimethoprim resistance, respectively. The double mutant PHA⁻/Hup⁻ was obtained by mobilizing the plasmid pSM825 from *E. coli* S17-1 into *R. sphaeroides* RV PHA⁻ mutant (SMV071) by conjugation. The subsequent chromosomal *hupL* gene replacement was selected by kanamycin and trimethoprim resistance contemporarily. Mutant strains carrying interposons were first identified by tests for loss of the pWKR102A-encoded gentamicin resistance. Their further genetic characterization was carried out by Southern blot analysis to confirm double recombination events and correct location of interposons on the genome (not shown).

Nucleotide Sequence Accession Number

The *R. sphaeroides* RV *phaC* DNA sequence has been submitted to the EMBL Nucleotide Sequence Database under the accession number X97200. The *R. sphaeroides* RV uptake hydrogenase operon, including *hupS-hupL* genes, has been submitted to the same database under the accession number Y14197.

RESULTS

Construction and Characterization of *R. sphaeroides* RV PHA⁻ Mutant Strain

PHB biosynthesis is a multistep pathway involving the formation of β -hydroxybutyryl-coenzyme A from acetoacetyl-coenzyme A and its polymerization mediated by a PHA synthase. Inactivation of the synthase renders a microorganism unable to synthesize PHAs and eliminates the competition between PHA accumulation and H₂ photoproduction that can occur depending on the organic substrate used.

The *phaC* gene was isolated from the genome of *R. sphaeroides* RV by PCR, using forward and reverse primers designed on the basis of the already published sequence of the homologous gene from *R. sphaeroides* strain ATCC 17023 (Hustede and Steinbuchel, 1993). The amplicon obtained from strain RV included a 1827-bp open reading frame preceded by a putative ribosome-binding site and encoding a protein of 609 amino acids with a high degree of homology to sequences of other bacterial synthases, particularly of type I (classification of Steinbuchel et al., 1992). There was 98% identity between strain RV and ATCC 17023, but the former had 8 additional amino acid residues at the C terminus. The overall degree of homology with *R. capsulatus* synthase was lower (61% identity), and it lacks 12 amino acids at the C terminus when compared with *R. sphaeroides* RV. DNA sequence analysis of flanking regions suggests that in all 3 of these photosynthetic bacterial strains, the *phaC* gene is monocistronic, not being clustered with the other genes (*phaA* and *phaB*) involved in PHB biosynthesis.

The *phaC* gene was inactivated by inserting a Kanamycin resistance cassette in its reading frame and by subsequent chromosomal gene replacement (see "Materials and Methods"). The *R. sphaeroides* RV PHA⁻ mutant so obtained, named strain SMV071, was confirmed to carry the genomic interposon by Southern blot analysis (not shown). After 3 days of cultivation under anaerobic/light conditions, the PHA⁻ mutant SMV071 reached optical densities similar to those for the wild type in aSy, gL, and food-waste-derived medium, whereas it was unable to grow in the acetate medium that specifically induces PHB synthesis. Gas chromatographic analysis of the cellular PHB content confirmed that the PHA⁻ mutant was unable to produce PHB because no peak characteristic of β -hydroxybutyric acid methyl ester could be detected (data not shown).

Construction and Characterization of *Rhodobacter sphaeroides* RV Hup⁻ Mutant Strain

Membrane-bound H₂ uptake Ni-Fe hydrogenases are respiratory enzymes with a functional core consisting of a small and a large subunit having average masses of 30 and 60 kDa, respectively. Operons encoding Ni-Fe uptake hydrogenases are multicistronic. Besides structural genes, They include a considerable number of accessory genes involved in electron transfer, biosynthesis of metal clusters, maturation, and regulation of the enzyme, for a total of 21 open reading frames in the case of *Rhodobacter capsulatus* (Colbeau et al., 1993; Vignais et al., 2001), as shown in Figure 2.

In *R. sphaeroides*, DNA sequences of genes involved in uptake hydrogenase biosynthesis were only partially known for strain 2.4.1: namely, *hupTUV* accessory genes and *hupSL* structural genes (Gomelsky and Kaplan, 1995). More recently, the characterization of the *R. sphaeroides* strain 2.4.1 uptake hydrogenase operon has been extended (Figure 2).

To obtain a mutant strain unable to perform H₂ recycling, we disrupted the *hupL* gene that encodes the large (catalytic) subunit of the membrane-bound H₂ uptake hydrogenase. As a starting point we isolated by PCR and IPCR (inverse PCR) and then characterized about 10 kb of the *R. sphaeroides* RV uptake hydrogenase gene cluster (Figure 2). The physical organization of *R. sphaeroides* RV uptake hydrogenase operon was highly conserved compared with the homologous gene cluster from *R. capsulatus*, with an extra gene located immediately downstream from the structural *hupSL* genes. The product of this gene, named *hupE*, is a highly hydrophobic polypeptide of 191 amino acids with 34.5% identity to a putative membrane-spanning protein found in the uptake hydrogenase cluster of the bacterium *Rhizobium leguminosarum* and is required for hydrogenase activity (Hidalgo et al., 1992).

The DNA fragment (about 2100 bp) covering the 3' region of the *hupS* gene and the 5' region of the *hupL* gene was isolated from the genome of *R. sphaeroides* RV by PCR and IPCR, using forward and reverse primers designed on the basis of homologous sequences from *R. sphaeroides* strain 2.4.1. The *hupL* gene was inactivated by inserting a trimethoprim resistance cassette in its reading frame and by subsequent chromosomal gene replacement (see "Materials and Methods"). The *R. sphaeroides* RV Hup⁻ mutant so obtained, named strain SMV089, was confirmed to carry

Rhodobacter capsulatus
hoxH ORF2 hupTUV hypF hupSLCDFGHJK hypAB hupR hypCDE

Rhodobacter sphaeroides 2.4.1
hupTUV hypF hupSLEC ---HJK hypAB hupR hypCD

Rhodobacter sphaeroides RV
hupTUV hypF hupSLEC

Rhizobium leguminosarum
hupSLCDEFGHIJK hypABFCDEX ψhoxA

Features or function of the gene products

HupT	Histidine kinase
HupU	H ₂ sensor, small subunit
HupV	H ₂ sensor, large subunit
HupS	H ₂ ase small subunit
HupL HoxH	H ₂ ase large subunit
HupC	cytochrome <i>b</i>
HupD	maturation protease
HupF	
HupJ	rubredoxin-like
HupK	
HypA	Ni-insertion, maturation
HypB	GTPase, Ni donor and storage
HypF	CO and CN synthesis
HypC	chaperone, maturation
HypD	Fe/S protein, maturation
HypE	maturation
HypX	maturation
HupR ψHoxA	response regulator

Figure 2. Physical organization of homologous uptake hydrogenase gene clusters from *R. capsulatus* (Colbeau et al. 1993; Vignais et al., 2001), *R. sphaeroides* strain 2.4.1 (Gomelsky and Kaplan, 1995), *R. sphaeroides* strain RV (this work) and *Rhizobium leguminosarum* (Hidalgo et al., 1992). Also indicated are the features or the function of the proteins encoded by structural and accessory genes.

the genomic interposon by both Southern blot analysis and PCR amplifications using oligonucleotides based on *hupSL* gene sequences (data not shown).

After 3 days of cultivation under anaerobic/light conditions, the Hup[−] mutant SMV089 reached similar optical densities to the wildtype in aSy, gL, and food-waste-derived medium. Assay of the uptake hydrogenase activity demonstrated the lack of a functional enzyme in strain SMV089, as no methylene blue reduction was detected compared with the wild type (data not shown). This suggests that *R. sphaeroides* RV, like *R. capsulatus* but unlike *R. rubrum*, possesses a single H₂ recycling enzyme, because the inactivation of the uptake hydrogenase large subunit,

HupL, was sufficient to abolish the capacity of the bacterium to oxidize H₂.

Construction and Characterization of *Rhodobacter sphaeroides* RV PHA[−]/Hup[−] Double Mutant Strain

A double mutant, in which both PHA biosynthesis and uptake hydrogenase activity were contemporarily abolished, was constructed in order to combine the 2 negative phenotypes into a single strain and test if these could synergistically improve the overall nitrogenase-dependent H₂ photoproduction. This was the central objective of this research.

The *R. sphaeroides* RV double mutant PHA⁻/Hup⁻ was obtained by 2-step interposon mutagenesis in which the single PHA⁻ mutant SMV071, carrying the chromosomal *phaC* gene interrupted by the kanamycin resistance cassette, was used as a recipient strain for the replacement in the genomic DNA of the *hupL* gene, previously inactivated by the insertion of a trimethoprim resistance cassette. The *R. sphaeroides* RV double mutant so obtained, named strain SMV087, was confirmed to carry the genomic interposons by both Southern blot analysis and PCR amplifications using oligonucleotides based on *hupSL* gene sequences (data not shown).

The presence of the double mutation did not affect photoheterotrophic growth capacity under anaerobic/light conditions, compared with the wild-type strain, on aSy, gL, and food-waste-derived medium. Conversely, like the single PHA⁻ mutant, no growth was detected in acetate medium. The gas chromatographic analysis of cellular PHB content showed no peak characteristic of β -hydroxybutyric acid methyl ester, and the uptake hydrogenase activity assay showed no methylene blue reduction, indicating that both PHB accumulation and H₂ recycling were contemporarily abolished.

Comparative H₂ Photoproduction with Lactic acid-based Synthetic Medium

The performance of the 3 metabolically engineered strains was evaluated for H₂ photoproduction in comparison to that of the wild type in laboratory tests using 1-L continuous cultures fed with a lactic acid-based synthetic medium (see “Materials and Methods”) (Figure 3).

During the steady-state continuous culture period, from day 2 to days 10 to 12 (some 6 to 7 generations at the HRT of 30 hours), both the single Hup⁻ and the double PHA⁻/Hup⁻ mutants exhibited significantly higher rates (on average 30% and 36%, respectively) in H₂ photoproduction compared with the wild-type strain, whereas the H₂ evolution rate of the PHA⁻ mutant was not increased (Figure 3, A). The cause for the decline in H₂ photoproduction by all the cultures, starting on days 10 to 12, is unknown. Nitrogenase activity, measured in assays with small culture samples incubated under high light intensities (see “Materials and Methods”), was not a limiting factor in H₂ production, as it greatly exceeded whole culture H₂ evolution activity in all the experiments (data not shown). This suggests that the limiting factor for H₂ evolution was light limitation in the continuous cultures.

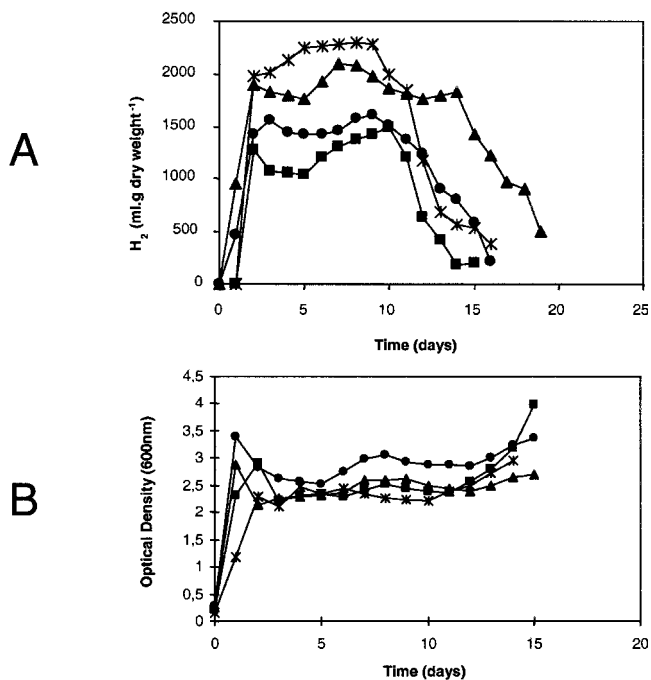


Figure 3. Comparative H₂ photoproduction tests performed in 1-L photobioreactors operated continuously at 30°C and 6 klux with a hydraulic residence time of 30 hours using the lactic acid-based synthetic medium. *R. sphaeroides* RV wild-type strain SMV047 (●), PHA⁻ mutant strain SMV071 (■), Hup⁻ mutant strain SMV089 (▲), PHA⁻/Hup⁻ double mutant strain SMV087 (*). **A:** Rate of H₂ photoproduction (ml g dry weight⁻¹). **B:** Cell growth (OD₆₀₀).

At day 10 the cultures were started on a fresh feed bottle, and this resulted in an increase in cell density (OD₆₀₀), which had been rather steady prior to that time (Figure 3, B). The increase in optical density, which would reduce light available to individual cells, corresponded to the decline in H₂ evolution by all the cultures, indicating a casual relationship. The increase in cell density led to an increased light limitation that was plausibly the reason for the decline in H₂ photoproduction. Although these increases in cell density were modest, similarly large changes in nitrogenase-dependent H₂ production were observed with cyanobacteria in response to small changes in cell density (Weare and Benemann, 1973). Continuous cultures monitored over a steady state of some 6 generations are generally considered to represent longer-term stability. Although not the case here, and regardless of the cause for this decline, the initial steady state period was sufficiently long to reproducibly establish the differences in H₂ evolution rates between the mutants and the wild-type strain. The lactic acid conversion efficiency into H₂, based on a stoichiometry of 6 mol H₂ per mole of lactic acid

consumed, was maximal for the double mutant (36% of the theoretical value). Somewhat lower but comparable values were obtained in the case of the wild-type strain and of the Hup⁻ mutant (31% and 30.6%, respectively), whereas the PHA⁻ mutant exhibited the least overall efficiency in lactic acid utilization (21%).

It can be concluded that with the lactic acid-based synthetic medium both the single Hup⁻ and the double mutants overall performed better than the wild-type strain as a result of an increased specific rate of H₂ evolution, whereas the single PHA⁻ mutant did not show an improvement in H₂ evolution performance.

Comparative H₂ Photoproduction Tests with Food-Waste-Derived Growth Medium

The performance of each of the mutants on food-waste-derived growth medium was compared with that of the wild-type strain in side-by-side H₂ photoproduction tests, to account for the effect of the batch-to-batch variability in this mixed substrate (see "Materials and Methods"). These experiments were performed in the same 1-L photobioreactors and under similar experimental conditions as for the synthetic medium (Figure 4).

As with the lactic acid-based synthetic medium, the continuous cultures exhibited an initial short period of increasing H₂ evolution, followed by a few days of maximal H₂ photoproduction, and then a more or less rapid decay in this activity. The data for the wild type in the 3 experiments demonstrate relatively large variations in terms of the longevity of the process, ranging from 5 to almost 14 days to the point of a 50% decline in initial rates of H₂ evolution. As with the synthetic medium, the decline in activities correlates to increases in cell densities (Figure 4, B and C), which could have been due to the variable nature of the substrate. However, the relative H₂ photoproduction rates between wild-type and mutant strains can be compared during the periods of maximal activities. This comparison shows that with the food-waste-derived growth medium, all 3 mutants sustained H₂ photoproduction longer than the wild-type strain, run in parallel with the same batch of growth substrate.

The single PHA⁻ mutant had a more active and stable uptake hydrogenase (Figure 4, A), suggesting that the difference in H₂ photoproduction was not due to the activity of this enzyme and that the reducing equivalents were channelled to nitrogenase for an extended period as a result of the lack of PHB formation. However, compared with the

Figure 4. Comparative H₂ photoproduction tests performed in 1-L photobioreactors operated continuously at 30°C and 6 klux with a hydraulic residence time of 30 hours using the food-waste-derived growth medium. In each side-by-side test, the wild-type is run in parallel to the mutant strain under evaluation employing the same batch of growth medium. In the 3 comparative tests, different batches of the food-waste-derived medium were used. **A:** Left panel, rate of H₂ photoproduction (ml g dry weight⁻¹) of *R. sphaeroides* RV wild-type strain SMV047 (●) and PHA⁻ mutant strain SMV071 (■). Right panel, uptake hydrogenase activity of *R. sphaeroides* RV wild-type strain SMV047 (●, day 10; ○, day 15) and PHA⁻ mutant strain SMV071 (■, day 10; □, day 20). The rate of decrease in A₅₇₀, the methylene blue reduction assay, is a direct measure of the activity of the uptake hydrogenase enzyme. **B:** Left to right panels, rate of H₂ photoproduction (ml g dry weight⁻¹), cell growth (OD₆₀₀), and cellular PHB content of *R. sphaeroides* RV wild-type strain SMV047 (●) and Hup⁻ mutant strain SMV089 (▲). **C:** Left to right panels, rate of H₂ photoproduction (ml g dry weight⁻¹), and cell growth (OD₆₀₀) of *R. sphaeroides* RV wild-type strain SMV047 (●) and PHA⁻/Hup⁻ double mutant strain SMV087 (*).

wild-type strain, this mutant did not exhibit any higher rates of H₂ evolution. The pH values remained constant at about 7 in the PHA⁻ mutant culture, whereas in that of the wild type the pH tended to increase, paralleled by PHB production. The single Hup⁻ mutant exhibited a significantly higher rate of H₂ photoproduction than the wild-type strain (Figure 4, B). In this experiment, for both the wild type and the Hup⁻ mutant, the decay in H₂ evolution tracks closely similar increases in cell densities and production of PHB (Figure 4, B). The double mutant exhibited the most sustained rate of H₂ photoproduction, after an initial decay of over half the activity (Figure 4, C). In this experiment initial cell density was considerably lower for the double mutant (average OD₆₀₀ around 1.7 versus 2.9 of the wild type), but reached comparable densities after 15 days of H₂ photoproduction (Figure 4, C).

These comparative experiments were repeated several times using different batches of food-waste-derived growth medium, as this was the major factor in the variability observed even with the wild-type strain. The overall data from these side-by-side tests are summarized in Table 2.

The improved performance in H₂ evolution by the mutant strains, in comparison with that of the wild-type strain, proved to be reproducible, regardless of the batch of waste-derived growth medium used. As in Figure 4, the single PHA⁻ mutant exhibits a significantly extended

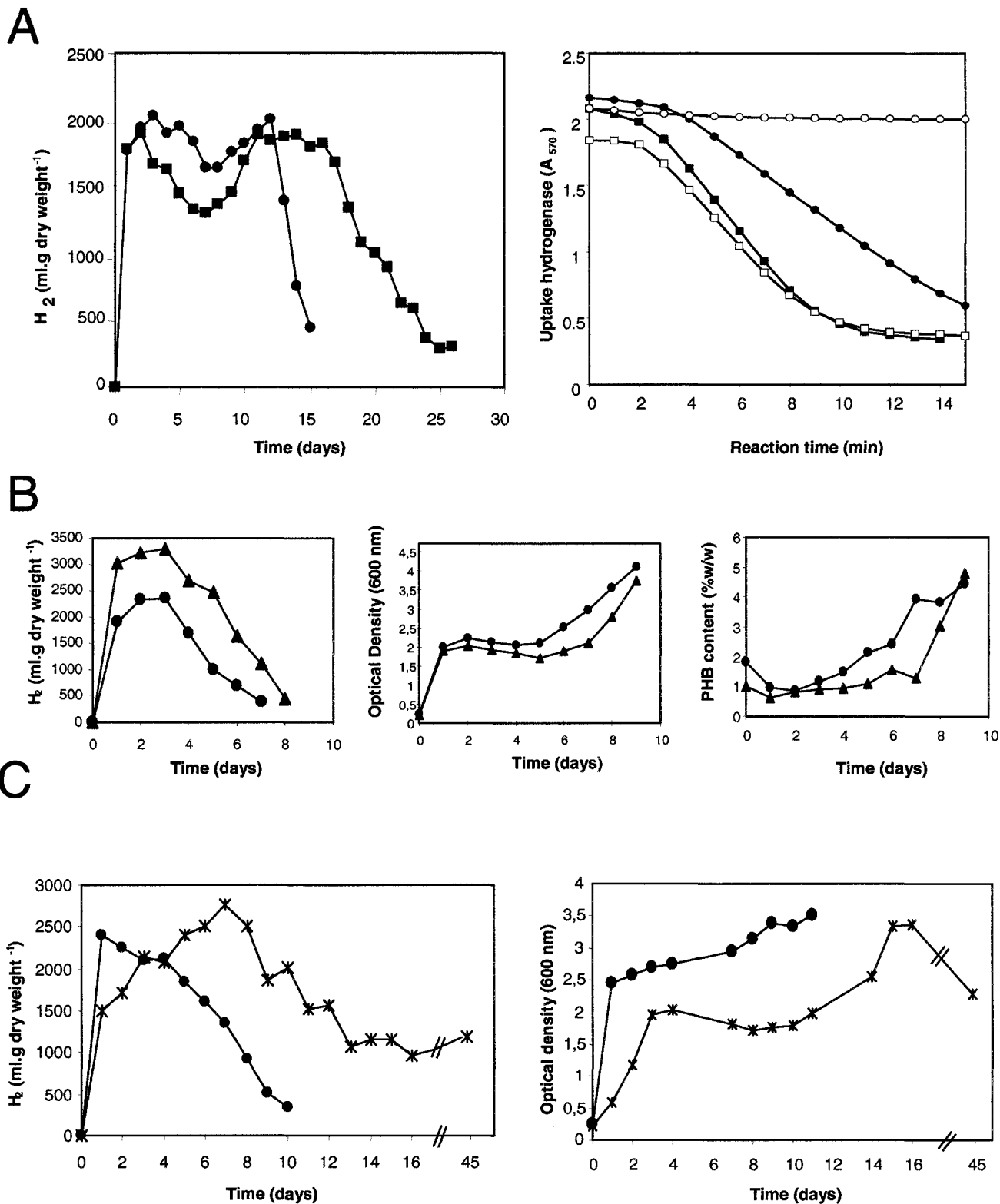


Figure 4.

duration of H₂ evolution but at a lower rate, both volumetric and specific. The single Hup⁻ mutant is able to produce H₂ with somewhat higher specific and volumetric rates, for a period similar to the wild-type strain.

Finally, the double mutant shows a several-fold longer duration of H₂ photoproduction with a daily rate, both volumetric and specific, in the same range of that of the wild-type strain. Thus the results in Figure 4 are repre-

Table 2. Comparative H₂ Photoproduction Tests Using Different Batches of Food-waste-Derived Growth Medium^a

	Wild-type SMV047	PHA ⁻ Mutant SMV071	Hup ⁻ Mutant SMV089	PHA ⁻ /Hup ⁻ mutant SMV087
Average daily rate				
1H ₂ ·L reactor ⁻¹ day ⁻¹	1.26	1.00	1.55	1.22
1H ₂ ·g dry weigh ⁻¹ day ⁻¹	1.90	1.55	2.75	1.85
Average duration (days)	9	18	8	30

^aComparative tests, in which the wild type was run in parallel to the mutant strain under evaluation using the same batch of food-waste-derived growth medium, were carried out as described in the legend to Figure 4. Average daily rates of H₂ evolution were computed for periods of maximal photo-production based on at least 3 independent side-by-side tests, repeated using different batches of the food-waste-derived growth medium. Standard deviations were less than ±10% from the mean. Duration was determined as the time for a 50% reduction in H₂ evolution.

sentative of the performance of these negative mutants on actual food-waste-derived media.

DISCUSSION

The objective of this research was to demonstrate the feasibility of metabolically engineering photosynthetic bacteria to improve biological H₂ production in a process for the management of solid food wastes. For this purpose the wild-type strain *R. sphaeroides* RV was genetically modified to enhance its nitrogenase-dependent H₂ photoproduction by removing, singly and in combination, its capacity to synthesize PHA storage compounds and to recycle H₂ produced by nitrogenase. The single PHA⁻ and Hup⁻ mutants were constructed by inactivating the synthase of the PHA biosynthetic pathway and the catalytic subunit of the uptake hydrogenase, while the double mutant PHA⁻/Hup⁻ was constructed to combine these negative phenotypes into a single strain. We then compared the performance of these mutants with that of the wild-type strain in continuous culture tests using both synthetic and actual food-waste-derived substrates.

With a lactic acid-based synthetic medium, both the single Hup⁻ and the double mutants showed increases in specific rates of H₂ photoproduction, of about 30% and 36%, respectively, compared with that of the wild-type strain. The mutation abolishing PHA accumulation singly did not result in an improved H₂ evolution, suggesting an absence of competition of PHA biosynthesis with H₂ photoproduction under these growth conditions. This is supported by the low PHB content, always below 1% on a dry weight basis, both in the wild-type strain and in the Hup⁻ mutant during these experiments, and it is in

agreement with data reported for *R. sphaeroides* and *R. rubrum* showing that acetate is the organic substrate most readily converted to PHB and, therefore, likely to result in a greater competitive effect (Brandl et al., 1991; Hustede et al., 1993; Katipov et al., 1998).

Comparative tests were then performed using a growth medium derived from the acidogenic fermentation of fruit and vegetable wastes, to more closely simulate an actual biohydrogen production process. With this medium containing mixed carbon sources, in particular acetate in addition to lactate, all 3 mutants showed somewhat enhanced overall performance relative to that of the wild type, mainly in duration of the H₂ production phase. Only the single Hup⁻ mutant exhibited an increased evolution rate, and its H₂ photoproduction was affected by PHB formation similarly to the wild type. Media variability resulted in significant differences in the performance of the wild-type control in the experiments reported in Figure 4 and additional tests summarized in Table 2. However, the general patterns of relative H₂ production of the mutants compared to wild type with this food-waste-derived medium were reproducible (Table 2).

The decay of nitrogenase activity in these continuous cultures, with both synthetic and waste-derived growth medium, was unexpected, and a cause was not immediately apparent from the experimental protocols. Assays for nitrogenase activity demonstrated that nitrogenase itself was not the limiting factor, as the volumetric rate of acetylene reduction in these assays was over an order of magnitude higher than the volumetric H₂ photoproduction rate by the continuous cultures. However, nitrogenase activity in photosynthetic bacteria can be rapidly regulated at the enzyme level by ammonia and is also highly dependent on reductant supply, which in turn is affected by the specific environmental conditions in the culture, particularly light

intensities. We surmise that the decline in H₂ photoproduction, in both the synthetic and the waste-derived medium, was triggered by relatively minor changes in the growth environment, including modest increases in metabolizable nitrogen, leading to the observed increases in cell densities and increased light limitation in the continuous cultures. Thus competition for electrons, the main objective of this study, from PHB synthesis and H₂ recycling, is only one aspect of the process, with competition from cell growth and metabolism also involved. Indeed, at most only about one third of the lactate consumed is accounted for in H₂ evolved. Thus the actual supply of electrons to nitrogenase by the photosynthetic reactions, presumed to occur through reverse electron flow, may be an even more important parameter in determining the overall H₂ photoproduction performance of the continuous cultures. It should be further noted that the 30-hour hydraulic residence time is relatively long, more characteristic of batch cultures, in which such instabilities are well known.

The reproducible differences in H₂ photoproduction between the wild type and the mutants in terms of rate during the initial phase of the experiments and duration, with both synthetic medium and food-waste-derived medium, can only be explained, however, by the mutations engineered into these organisms. For the 2 single mutants, the improved performance corresponded to total H₂ photoproduction about 50% higher than the wild-type strain using the waste-derived medium. Also, the single PHA⁻ mutant was not advantageous in the synthetic medium, where PHB biosynthesis is low or absent, but does perform better relative to the wild type with the waste-derived medium, where PHB biosynthesis takes place mainly due to the presence of acetate. Under these growth conditions the double mutant showed a remarkably sustained H₂ photoproduction, leading to a greatly increased H₂ evolution compared to the wild type and even the single mutants. Overall, the improvements of the mutants were more pronounced with the actual waste substrate than with the synthetic medium, and the combination of the 2 negative phenotypes conferred greater H₂ evolution capabilities than the single mutations.

The development of biological hydrogen production processes has attracted much research attention for several decades, and a number of different systems have been proposed, in particular, those based on nitrogenase-dependent H₂ evolution using cyanobacteria and photosynthetic bacteria. Here we demonstrate both the potential

and the challenges of applying molecular biology tools to such processes. We have advanced the state of the art from single to multiple gene engineering and from synthetic media to media derived from solid food waste. Overall, the results are promising in that significant improvements in H₂ production were observed, although the decay of H₂ evolution by the cultures, with both media and all strains, suggests that factors other than H₂ recycling and PHB formation also impinge on H₂ evolution. The future development of such biohydrogen production processes must also consider the practical issues of bioreactor costs and performance, as well as overall substrate conversion efficiencies.

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