J. Microbiol. Biotechnol. (2005), **15**(5), 1115–1119

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Improvement of Photoheterotrophic Hydrogen Production of *Rhodobacter sphaeroides* by Removal of B800-850 Light-Harvesting Complex

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Received: November 18, 2004
Accepted: January 17, 2005

Abstract The photoheterotrophic H_2 production of *Rhodobacter sphaeroides* was significantly increased through disruption of the genes coding for uptake hydrogenase and poly-β-hydroxybutyrate (PHB) synthase (Lee *et al.*, *Appl. Microbiol. Biotechnol.* **60:** 147–153, 2002). In this work, we further removed the B800-850 light-harvesting (LH) complex from the strain and found an increase in H_2 production at the light-saturating cell growth (≥10 Watts [W]/m^2). Neither the mutant nor the wild-type produced more H_2 at the brighter light. Accordingly, light does not appear to be limited for the H_2 production by the presence of B800-850. However, increase in the level of the spectral complexes resulted in decrease of H_2 production. Thus, although the B875 is essential for light harvesting, the consumption of cellular energy for the synthesis of B800-850 and the surplus LH complexes may reduce the energy flow into the H_2 production of *R. sphaeroides*.

Key words: Photosynthetic spectral complexes, H_2 production, *Rhodobacter sphaeroides*

The energy required for the nitrogenase-mediated H_2 evolution of *Rhodospirillaceae* [6, 25] is provided through photophosphorylation [7, 18]. Two light-harvesting (LH) pigment-protein complexes are involved in trapping light, which is transmitted as excited energy from the B800-850 to the B875 complex and to a photochemical reaction center (RC), the site of primary photochemistry [19]. There have been reports of the enhancement of H_2 production by alterations in the quantity of spectral complexes of *R. sphaeroides* [20, 23]. In the case of using solar energy, the strong light (ca. ≥1.0 kW/m^2) needs to be dispersed to raise the H_2 evolution by *R. sphaeroides* [24]. However, light absorption by the cells at the facing side of the culture vessel could result in reduction of light penetration [13, 20], and therefore, lower the H_2 production.

Previously, *Rhodobacter sphaeroides* KCTC 12085, a natural isolate, showed higher production of H_2 than the laboratory strain, *R. sphaeroides* 2.4.1 [15]. Accumulation of H_2 by the isolate was further elevated, following the suppression of H_2 uptake by mutation in *hupSL* coding for H_2-uptake hydrogenase and the disruption of poly-β-hydroxybutyrate (PHB) biosynthesis by mutation in *phbC* coding for PHB synthase [10, 15]. In this work, we examined the effect of the spectral complexes on the photoheterotrophic H_2 production, and found that the synthesis of B800-850 and the surplus LH complexes resulted in decrease of the H_2 production at the illumination saturating the growth of *R. sphaeroides*.

R. sphaeroides KCTC 12085 [15] and *R. sphaeroides* 2.4.1 were used as wild-type strains and were grown aerobically or photoheterotrophically at 28°C in Sistrom's succinate minimal medium [22], as described previously [1, 15]. *R. sphaeroides* HP1, derived from KCTC 12085, is a mutant having disruptions in *hupSL* and *phbC*. Light intensity for photoheterotrophic growth was measured with a photometer (Li-Cor, Inc., U.S.A.) [3]. Cell growth was monitored with a Klett-Summerson colorimeter (Manostat, U.S.A.) equipped with a KS-66 filter. Appropriate antibiotics were used as described previously [15].

Absorption spectra to detect photosynthetic complexes were examined with a UV 2041-PC spectrophotometer (Shimadzu, Japan). Protein content was determined by the modified Lowry method [17], and the quantity of the B800-850 and B875 complexes were calculated as described previously [19].

The modified Sistrom's medium supplemented with DL-malate (30 mM) and L-glutamate (7 mM) was used for the measurement of H_2 production as described previously [15]. The amount of hydrogen gas accumulated in the serum vial was analyzed with a GC-17A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a thermal conductivity

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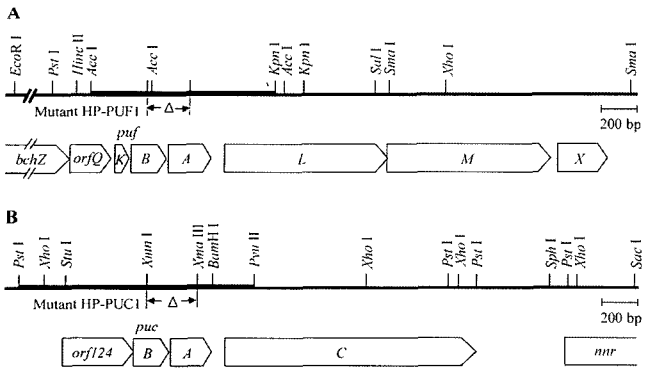

Fig. 1. Restriction maps of the *pufBA* (A) and the *pucBA* (B) of *R. sphaeroides* 2.4.1.

(A) Restriction map of the *R. sphaeroides* 2.4.1 DNA containing *pufBA* (B875-β and B875-α polypeptides, respectively) is shown, and *pufBA* is in-frame deleted in mutant HP-PUF1 from its parent strain, *R. sphaeroides* HP1. Genes *bchZ* (BchZ subunit of chlorophyllide *a* reductase), *orfQ* (bacteriochlorophyll-carrier protein [5]), *pufLM* (RC-L and RC-M polypeptides, respectively), and *pufX* (intrinsic membrane protein) are shown. (B) Restriction map of *R. sphaeroides* 2.4.1 DNA containing *pucBA* (B800-850-β and B800-850-α polypeptides, respectively) is shown, and *pucBA* is in-frame deleted in mutant HP-PUC1. *pucC* (assembly protein for the B800-850 complex [14]) and *nnr* (LuxR-type transcriptional regulator) are shown.

detector [12] and a column containing 60/80 mesh Molecular Sieve 5A (Supelco, Bellefonte, PA, U.S.A.). Argon was used as a carrier gas. The analyses of H_2 accumulation in this work were performed at least three separate times, and the data presented here were reproduced within standard deviations of 10–15%.

The nucleotide sequence of *R. sphaeroides* 2.4.1 was obtained from the genome site at http://mmg.uth.tmc.edu/sphaeroides/, and each DNA fragment encoding *puhA*, *pufBA*, and *pucBAC* was derived from *R. sphaeroides* 2.4.1. A 900-bp fragment extending from 40-bp upstream from *puhA* (reaction center H polypeptide) [4] to its 80-bp downstream was cloned into pRK415 [9] to generate pRKH100, in which *puhA* was oriented in the same direction as the *tet* promoter of the plasmid. The DNA containing *pufBA* (B875-β and B875-α polypeptides, respectively) [11] (Fig. 1A) was provided with pRKF100, a recombinant pRK415 containing the 1,997-bp *Eco*RI-*Kpn*I fragment extending from the second nucleotide of the 216th residue of *bchZ* to the first nucleotide of the 117th residue of *pufL*. A 3,386-bp *Xho*I-*Sac*I DNA (Fig. 1B) harboring *pucBAC* (B800-850-β, B800-850-α polypeptides, and an assembly protein for the B800-850 complex, respectively) [14] as well as its 465-bp upstream and 980-bp downstream DNA was cloned into pRK415 to yield pRKC100. Plasmids were mobilized from *E. coli* S17-1 into *R. sphaeroides* through conjugation as described previously [2].

Genes of *pufBA* and *pucBA* of *R. sphaeroides* HP1 were disrupted through homologous recombination using appropriate DNA of *R. sphaeroides* 2.4.1. The *pufBA*-deleted cell, HP-PUF1, lacking of B875 complex, was constructed as follows. The 220-bp DNA from the first nucleotide of the 15th residue of *pufB* to the first nucleotide of the 33rd residue of *pufA* was in-frame deleted from the 0.9-kb *Acc*I-*Kpn*I DNA (Fig. 1A, thick line) of *R. sphaeroides* 2.4.1. A 404-bp DNA fragment immediately upstream from the deletion region was PCR amplified using F1 (5'-GTC AAC ACG CCG GTC CAT-3') and R1 (5'-CTG CGC CTG **CTG CAG** TCC-3', mutated sequence underlined, unless otherwise noted) having the 14th and 15th codons of *pufB* changed into *Pst*I site (shown in bold). The 424-bp DNA downstream from the deletion region was also amplified using F2 (5'-CCA CC**A TGC ATC** CTG CTG A-3') having the 33rd and 34th codons of *pufA* changed into *Nsi*I site (shown in bold), and 3' primer R2 (5'-CAA GGG CCG GCG GGT AGA C-3'). The 329-bp *Acc*I-*Pst*I upstream fragment and 375-bp *Nsi*I-*Kpn*I downstream fragment were cloned into the *Acc*I and *Kpn*I sites of pBS, and the insert DNA was then subcloned into a suicide plasmid, pLO1 [8, 16]. In-frame deletion of *pufBA* was confirmed by the sequence analysis. The resulting plasmid pLO1-Δ*pufBA*, which harbored *aph* (Km^r) and *sacB*, was mobilized from *E. coli* S17-1 into *R. sphaeroides* HP1 through conjugation as described previously [2]. The Km^r exconjugant, which was generated from a single crossover, was isolated and subjected to segregation to double-crossover recombination, showing Km^s phenotype on the sucrose (15%)-containing Sistrom's minimal medium. The resulting mutant HP-PUF1, whose chromosomal deletion of *pufBA* was confirmed by genomic Southern hybridization analysis [21], did not show any B875 complex under the photoheterotrophic conditions, and the level of B800-850 complex was not changed, compared with its parental strain HP1 (data not shown). In order to construct the *pucBA*-deleted mutant HP-PUC1, the 267-bp *Xmn*I-*Xma*III DNA spanning from *pucB* to *pucA* was removed from the 1.34-kb *Pst*I-*Pvu*II fragment (Fig. 1B, thick line) of *R. sphaeroides* 2.4.1. The resulting fragment was cloned into pLO1 to generate pLO1-Δ*pucBA* and then mobilized into *R. sphaeroides* HP1. Double-crossover recombination events were selected as described above for HP-PUF1. The

Table 1. Relative level of LH complexes of *R. sphaeroides* HP1 carrying pRKH100 (*puhA*), pRKF100 (*pufBA*), pRKC100 (*pucBAC*), and pRK415 (vector plasmid control) under the photoheterotrophic conditions at 10 W/m^2.

Strains carrying plasmids	Level of LH complexes (nmol/mg of cell protein)	
	B875	B800-850
HP1 (pRK415)	10.3	12.3
HP1 (pRKH100)	10.7	18.1
HP1 (pRKF100)	13.5	19.3
HP1 (pRKC100)	8.6	21.0

Table 2. Maximum H_2 accumulation of *R. sphaeroides* HP1 carrying pRKH100 (*puhA*), pRKF100 (*pufBA*), pRKC100 (*pucBAC*), and pRK415 (vector plasmid control) under the photoheterotrophic conditions.

Light intensity (W/m^2)	Strains carrying plasmids	Maximum H_2 accumulation (μl/ml culture)
10	HP1 (pRK415)	3,553
	HP1 (pRKH100)	2,956
	HP1 (pRKF100)	2,176
	HP1 (pRKC100)	2,003
100	HP1 (pRK415)	3,037
	HP1 (pRKH100)	2,842
	HP1 (pRKF100)	2,125
	HP1 (pRKC100)	2,402

resulting mutant HP-PUC1, whose chromosomal deletion of *pucBA* was confirmed by genomic Southern hybridization analysis, did not reveal any B800-850 complex under the photoheterotrophic conditions, and the level of B875 complex was not changed in comparison with HP1 (data not shown).

The cellular levels of B875 and B800-850 complexes were increased, when the genes *puhA*, *pufBA*, and *pucBAC* were maintained in *trans* in *R. sphaeroides* HP1 (Table 1). The B875 complex was increased by approximately 30% only when pRKF100 was provided. However, all the recombinant cells showed enhancement in the level of B800-850 complex by approximately 50 to 70%.

The cells containing the recombinant plasmids were examined for the photoheterotrophic production of H_2 at light intensities of 10 and 100 W/m^2. All the cells grew with doubling time between 5 and 6 h (data not shown). Thus, the light level of as much as 10 W/m^2 was saturating cell growth. The recombinant plasmid-bearing cells produced less H_2 than the control cells having pRK415 at the light intensities examined (Table 2). The same decrease was also observed with the H_2 accumulation rates (data not shown), which were in parallel with the maximum levels of H_2 production [15]. The H_2 production was not enhanced at the brighter light of 100 W/m^2 (Table 2). These results suggest that the reduced level of H_2 production in the presence of additional spectral complexes cannot be ascribed to light-limiting due to the shadow by the higher level of spectral complexes, since it should have been diminished at 100 W/m^2. The energy waste for the synthesis of excess spectral complexes appears rather to be the primary reason for the decrease in H_2 production.

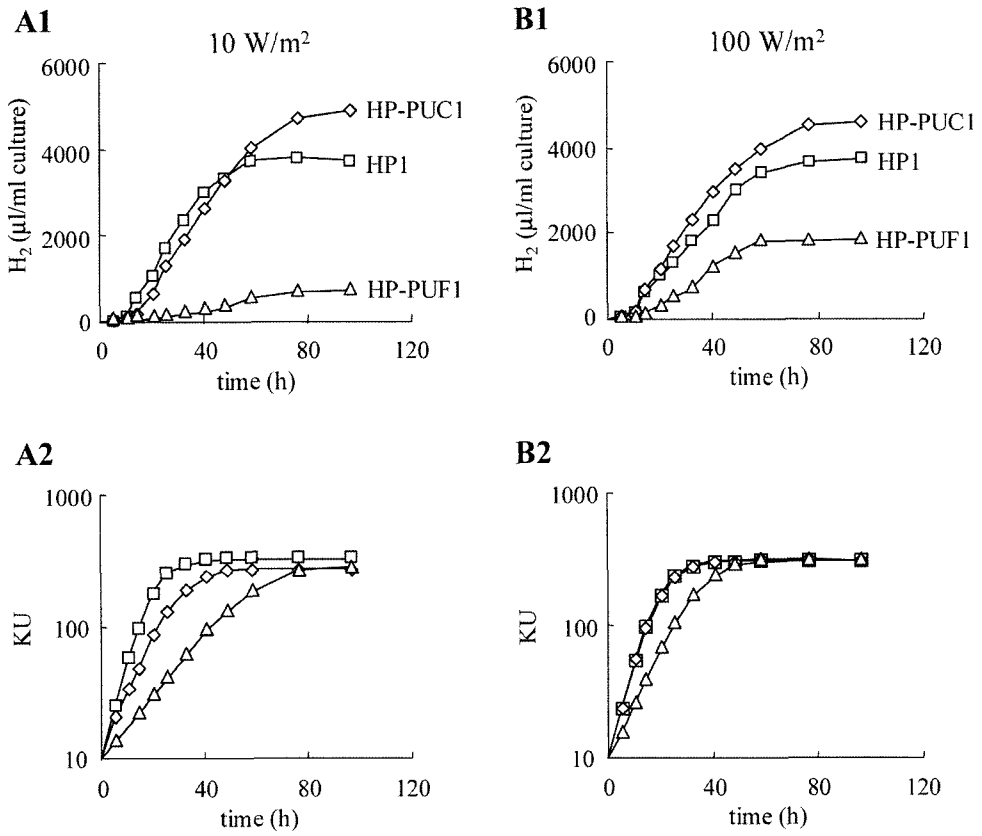

Fig. 2. H_2 accumulation and cell growth of *R. sphaeroides* HP1, HP-PUF1, and HP-PUC1.
H_2 accumulation (A1, B1) and cell growth (A2, B2) of *R. sphaeroides* HP1 (square), HP-PUC1 (diamond), and HP-PUF1 (triangle). Cells were grown under photoheterotrophic conditions at 10 (A) and 100 W/m^2 (B).

HP-PUF1 grew with a doubling time of approximately 13 h at 10 W/m^2, and its maximum cell density (KU) went up to about an 80% level of that of HP1, which doubled in every 5 h during the exponential growth (Fig. 2, A2). HP-PUF1, with 7-h doubling time, grew up to the same KU as that of wild-type at 100 W/m^2 (Fig. 2, B2). The results suggest that the B875 complex is essential for the efficient light harvesting and transfer of excitation energy to RC. The growth of HP-PUF1 was fully complemented with pRKF100 at both light intensities (data not shown). The H_2 production of HP-PUF1 at 10 W/m^2 amounted to only 20% of the HP1 level (Fig. 2, A1). HP-PUF1, however, produced H_2 up to the 50% level of HP1 at 100 W/m^2 (Fig. 2, B1), which was consistent with its growth restoration at the brighter light. Thus, the B875 complex is obligatory for the efficient harvesting of light energy.

HP-PUC1 grew with a doubling time of 6 h during the photoheterotrophic growth at 10 W/m^2, and the maximum KU was approximately 80% of HP1 (Fig. 2, A2). However, HP-PUC1 grew like HP1 at 100 W/m^2 (Fig. 2, B2), which implied that the B800-850 complex is required for light harvesting at the dimmer illumination ($\leq$10 W/m^2). The dependency on the complex can be completely abolished if exposed to the brighter light. The maximum level in H_2 production of HP-PUC1 during the photoheterotrophic growth at 10 W/m^2 was increased up to nearly 1.0 ml per ml of cell culture in addition to that of HP1. However, H_2 production of HP-PUC1 was not further increased at the brighter light (Fig. 2, A1, B1). The results suggest that the cellular energy for H_2 production of HP-PUC1 was not limited at 10 W/m^2, although the growth of HP-PUC1 was fully restored to the HP1 level at the brighter light (Fig. 2, A2, B2). The energy that may be saved by the lack of the B800-850 complex of HP-PUC1 appears rather to flow into the metabolism, leading to the enhanced production of H_2 under the conditions examined. Therefore, the B875 and RC complexes that comprise the fixed photosynthetic unit (PUF) are sufficient for the photoheterotrophic H_2 production of *R. sphaeroides* at the light-saturating cell growth.

Acknowledgment

This Research was supported by the Hydrogen Energy R&D Center, one of the 21st Century Frontier R&D Programs, funded by the Ministry of Science and Technology of Korea.

References

1. Baek, C. H. and K. S. Kim. 2003. *lacZ*- and *aph*-based reporter vectors for *in vivo* expression technology. *J. Microbiol. Biotechnol.* **13:** 872–880.
2. Davis, J., T. J. Donohue, and S. Kaplan. 1988. Construction, characterization, and complementation of a Puf-mutant of *Rhodobacter sphaeroides*. *J. Bacteriol.* **170:** 320–329.
3. Donohue, T. J., A. G. McEwan, and S. Kaplan. 1986. Cloning, DNA sequence, and expression of the *Rhodobacter sphaeroides* cytochrome c2 gene. *J. Bacteriol.* **168:** 962–972.
4. Donohue, T. J., J. H. Hoger, and S. Kaplan. 1986. Cloning and expression of the *Rhodobacter sphaeroides* reaction center H gene. *J. Bacteriol.* **168:** 953–961.
5. Gong, L., J. K. Lee, and S. Kaplan. 1994. The *Q* gene of *Rhodobacter sphaeroides*: Its role in *puf* operon expression and spectral complex assembly. *J. Bacteriol.* **176:** 2946–2961.
6. Hillmer, P. and H. Gest. 1977. H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: Production and utilization of H_2 by resting cells. *J. Bacteriol.* **129:** 732–739.
7. Jackson, J. B. 1988. Bacterial photosynthesis, pp. 317–376. *In* C. Anthony (ed.), *Bacterial Energy Transduction*. Academic Press, London.
8. Jeffke, T., N. H. Gropp, C. Kaiser, C. Grzesik, B. Kusian, and B. Bowein. 1999. Mutational analysis of the *cbb* operon (CO_2 assimilation) promoter of *Ralstonia eutropha*. *J. Bacteriol.* **181:** 4374–4380.
9. Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70:** 191–197.
10. Kho, D. H., J. H. Jang, H. S. Kim, K. S. Kim, and J. K. Lee. 2003. Quorum sensing of *Rhodobacter sphaeroides* negatively regulates cellular poly-β-hydroxybutyrate content under aerobic growth conditions. *J. Microbiol. Biotechnol.* **13:** 477–481.
11. Kiley, P. J., T. J. Donohue, W. A. Havelka, and S. Kaplan. 1987. DNA sequence and *in vitro* expression of the B875 light-harvesting polypeptides of *Rhodobacter sphaeroides*. *J. Bacteriol.* **169:** 742–750.
12. Kim, J. R., Y. K. Oh, Y. J. Yoon, E. Y. Lee, and S. Park. 2003. Oxygen sensitivity of carbon monoxide-dependent hydrogen production activity in *Citrobacter* sp. *J. Microbiol. Biotechnol.* **13:** 717–724.
13. Kim, N. J., J. K. Lee, and C. G. Lee. 2004. Pigment reduction to improve photosynthetic productivity of *Rhodobacter sphaeroides*. *J. Microbiol. Biotechnol.* **14:** 442–449.
14. Lee, J. K., P. J. Kiley, and S. Kaplan. 1989. Posttranscriptional control of *puc* operon expression of B800-850 light-harvesting complex formation in *Rhodobacter sphaeroides*. *J. Bacteriol.* **171:** 3391–3405.
15. Lee, I. H., J. Y. Park, D. H. Kho, K. S. Kim, and J. K. Lee. 2002. Reductive effect of H_2 uptake and poly-β-hydroxybutyrate formation on nitrogenase-mediated H_2 accumulation of *Rhodobacter sphaeroides* according to light intensity. *Appl. Microbiol. Biotechnol.* **60:** 147–153.
16. Lenz, O., E. Schwartz, J. Dernedde, M. Eitinger, and B. Friedrich. 1994. The *Alcaligenes eutrophus* H16 *hoxX* gene participates in hydrogenase regulation. *J. Bacteriol.* **176:** 4385–4393.
17. Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to

simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** 206–210.

18. McEwan, A. G. 1994. Photosynthetic electron transport and anaerobic metabolism in purple non-sulfur bacteria. *Antonie van Leeuwenhoek* **66:** 151–164.
19. Meinhardt, S. W., P. J. Kiley, S. Kaplan, A. R. Crofts, and S. Harayama. 1985. Characterization of light-harvesting mutants of *Rhodopseudomonas sphaeroides*. I. Measurement of the efficiency of energy transfer from light-harvesting complexes to the reaction center. *Arch. Biochem. Biophys.* **236:** 130–139.
20. Miyake, J., M. Miyake, and Y. Asada. 1999. Biotechnological hydrogen production: Research for efficient light energy conversion. *J. Biotechnol.* **70:** 89–101.
21. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., U.S.A.
22. Sistrom, W. R. 1962. The kinetics of the synthesis of photopigments in *Rhodopseudomonas sphaeroides*. *J. Gen. Microbiol.* **28:** 607–616.
23. Vasilyeva, L., M. Miyake, E. Khatipov, T. Wakayama, M. Sekine, M. Hara, E. Nakada, Y. Asada, and J. Miyake. 1999. Enhanced hydrogen production by a mutant of *Rhodobacter sphaeroides* having an altered light-harvesting system. *J. Biosci. Bioeng.* **87:** 619–624.
24. Wakayama, T. and J. Miyake. 2002. Light shade bands for the improvement of solar hydrogen production efficiency by *Rhodobacter sphaeroides* RV. *Int. J. Hydrogen Energy* **27:** 1495–1500.
25. Wall, J. D., P. F. Weaver, and H. Gest. 1975. Genetic transfer of nitrogenase-hydrogenase activity in *Rhodopseudomonas capsulata*. *Nature* **258:** 630–631.