Journal of Microbiology and Biotechnology
Vol. 7. No. 5, 360-362, 1997

NOTE

Isolation and Characterization of β-Hydroxybutyrate Dehydrogenase-deficient Mutant of *Rhodobacter sphaeroides* 2.4.1

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A transposon Tn*5* mutant of *Rhodobacter sphaeroides* 2.4.1 was isolated for its impaired ability of growth on minimal medium containing β-hydroxybutyric acid as a sole carbon source. The mutant, *R. sphaeroides* S7 showed approximately 6-fold decrease in β-hydroxybutyrate dehydrogenase activity compared with that of wild type. In *R. sphaeroides* S7 the Tn*5* was located in DNA region corresponding to a 4.2-kb *Eco*RI DNA fragment of *R. sphaeroides* 2.4.1 chromosome.

Poly-β-hydroxybutyric acid (PHB), a homopolymer of β-hydroxybutyric acid accumulates intracelluarly when optimal growth conditions are not met in both gram-positive and gram-negative bacteria (for a review, see ref. 1). The PHB functions as a carbon storage compound or as a sink for reducing equivalents, so it can be utilized again when cells are under carbon-limited growth conditions.

Although catabolic pathway of intracellular PHB has not been characterized clearly, PHB has been supposed to be degraded initially by PHB depolymerase into a monomer, β-hydroxybutyric acid which is subsequently oxidized into acetoacetate by β-hydroxybutyrate dehydrogenase with NAD^+ as a cofactor (1, 9). The acetoacetate is further converted into acetyl-CoA to enter energy-yielding metabolic process.

Recently we cloned, sequenced, and analyzed *phbC* expression of *Rhodobacter sphaeroides* 2.4.1, which codes for PHB polymerase (6). As an attempt to understand the PHB mobilization in *R. sphaeroides* 2.4.1, we tried to clone out gene coding for β-hydroxybutyrate dehydrogenase by employing transposon Tn*5* mutagenesis of the *R. sphaeroides* culture followed by selection of mutants defective in use of β-hydroxybutyric acid for its growth. In this work we isolated such a Tn*5*-generated mutant, and analyzed activity of β-hydroxybutyrate dehydrogenase which is responsible for the catabolic utilization of the β-hydroxybutyric acid. The chromosomal DNA structure where the Tn*5* is inserted was also examined.

The bacterial strains and plasmids used in this study are described in Table 1. *R. sphaeroides* was grown chemoheterotrophically at 28°C in Sistrom's minimal medium (11) containing acetate (30 mM). To check the ability to utilize β-hydroxybutyric acid as a sole carbon source, M9 minimal medium (8) subsituting β-hydroxybutyric acid (30 mM) for glucose was used after addition of vitamins and trace elements of the Sistrom's minimal medium. *Escherichia coli* strains were grown at 37°C in Luria medium (8). Ampicillin, chloramphenicol, and kanamycin (final concentrations, 50, 50, and 25 μg/ml, respectively) were added to the growth medium for *E. coli* carrying genomic DNA or plasmids encoding these drug resistance genes.

Plasmid pSUP2021 (10) containing transposon Tn*5* was mobilized into *R. sphaeroides* 2.4.1 by conjugation with a helper *E. coli* HB101 (pRK2013) as described previously (3), and Tn*5*-generated mutants were selected by Km^r (final concentration, 25 μg/ml).

Large- and small-scale plasmid DNA were prepared as described (8). DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes in accordance with the manufacturers' instructions. Southern hybridization was performed as described (8).

Determination of PHB was performed by spectrophotometric assay using the Law and Slepecky method (7). The relative amount of PHB between the culture samples was quantitated from the absorbance of crotonic acid at 235 nm divided by the absorbance of the culture at 600 nm.

For β-hydroxybutyrate dehydrogenase activity, *R. sphaeroides* cells were harvested by centrifugation followed by wash and resuspension in Tris-HCl buffer (50 mM, pH 8.0). The cells were broken at pressure of 10,000 psi with French® Press (SLM, IL, U.S.A.). Intact cells and cell debris were removed after centrifugation at 19,000 g

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Key words: *Rhodobacter sphaeroides*, β-hydroxybutyrate dehydrogenase, leaky mutant, Tn*5*

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristic (s)	Source or reference
E. coli		
DH5α*phe*	F⁻Φ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*($r_K^-m_K^+$)*supE44* λ^- *thi-1 gyrA relA1 phe*::Tn*10d*Cm	4
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl*-1	8
R. sphaeroides		
2.4.1	Wild type	W. Sistrom
S7	2.4.1::Tn*5* β-hydroxybutyrate dehydrogenase-deficient mutant	This study
Plasmids		
pSUP2021	pBR325-Mob Ap^r Cm^r Tc::Tn*5*	10
pRK2013	ColE1 Mob Km^r	5
pTn19	pUC19/*Hin*dIII, 3.4-kb *Hin*dIII fragment of Tn*5*	This study

for 10 min, and the supernatant was used for enzyme assay. The enzyme reaction was performed as previously described (2, 9). One unit of the enzyme actvity is defined as the amount of enzyme which catalyze the reduction of 1 μmole of NAD^+ per min.

By Tn*5* mutagenesis we have obtained about 500 mutants of *R. sphaeroides*. They were examined for their ability to utilize β-hydroxybutyric acid for growth by replica plating on M9 minimal medium containing β-hydroxybutyric acid as a sole carbon source. Only one mutant was observed to show very defective growth. The mutant, *R. sphaeroides* S7 grew with doubling time longer than 4 days while the wild type, *R. sphaeroides* 2.4.1 divides every 3 h. However, in Sistrom's minimal medium containing acetate *R. sphaeroides* S7 grew with ~4-h doubling time which is comparable with 3-h doubling of 2.4.1.

In order to understand the physiological basis for impaired growth of *R. sphaeroides* S7 in the M9 minimal medium containing β-hydroxybutyric acid, β-hydroxybutyrate dehydrogenas activity was measured and compared with that of 2.4.1. Mutant and wild-type cells were grown until mid-log phase in the Sistrom's minimal medium containing acetate, and used for the enzyme assay. As shown in Table 2, the specific activity of β-hydroxybutyrate dehydrogenase of *R. sphaeroides* S7 was lower by about 6 fold than 2.4.1. This result clearly indicates that the slow growth of *R. sphaeroides* S7 in the β-hydroxybutyric acid medium should be due to the reduced level of β-hydroxybutyrate dehydrogenase activity. The possibility of reduced uptake of the β-hydroxybutyric acid into the mutant cells is ruled out because cell-free extracts were used for the enzyme activity assay. The reason for the enzyme activity of *R. sphaeroides* S7 comprising as much as 16% of that of 2.4.1 could be due to incomplete disruption of the gene encoding β-hydroxybutyrate dehydrogenase by Tn*5*, or possibly presence of the gene(s) for isozyme(s) on its genomic DNA, or the both.

Table 2. β-Hydroxybutyrate dehydrogenase activities of *R. sphaeroides* 2.4.1 and S7.

Strains	Klett[a] units	Specific activity (units/mg protein)	Relative specific activity (%)
2.4.1	53	49.5	100
S7	66	8.1	16

[a] One Klett unit corresponds to about 10^7 cells/ml.

The level of PHB production of *R. sphaeroides* S7 was similar to that of the wild type (data not shown) indicating that the mutaion does not affect the regulation of anabolic pathway leading to PHB formation.

The choromosomal structure of the *R. sphaeroides* S7 was examined by Southern hybridization analysis. A 1.0-kb *Hin*dIII-*Sma*I DNA fragment of pTn19 was used as a probe. The probe DNA extends from internal region of IS50L to the middle of the km^r gene of Tn*5*. As shown in Fig. 1, the chromosomal DNA from wild type did not show any hybridization signal as expected (Fig. 1B, lane 1), while about 10-kb *Eco*RI DNA fragment was detected indicating the presence of the Tn*5* on the chromosome of the mutant (Fig. 1B, lane 2). Since the size of the Tn*5* is 5.8 kb, the Tn*5* in *R. sphaeroides* S7 is located in DNA region corresponding to a 4.2-kb *Eco*RI DNA fragment of *R. sphaeroides* 2.4.1 chromosome. We further isolated the DNA region flanking the Tn*5* from the mutant chromosome, and used it as a DNA probe for genomic Southern hybridization analysis with the 2.4.1 chromosomal DNA. In addition to the homologous DNA region, other hybridization signals heterologous to the probe were detected (data not shown). These could reflect the DNA regions coding for isozymes of β-hydroxybutyrate dehydrogenase.

This is the first report of *R. sphaeroides* mutant deficient in the β-hydroxybutyrate dehydrogenase activity, and further research is under way to characterize the

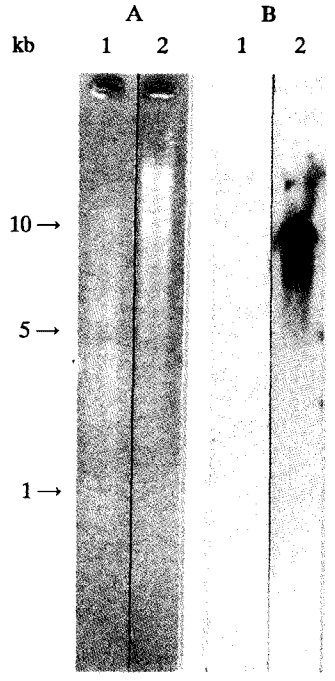

Fig. 1. Sorthern hybridization analysis of genomic DNA from *R. sphaeroides* 2.4.1 and S7 with Tn*5*-specific DNA probe.
A, Agarose gel of chromosomal DNA digested with *Eco*RI; B, Southern hybridization analysis of the gel shown in pannel A with 1.0-kb *Hin*dIII-*Sma*I DNA fragment of Tn*5*. Lane 1 has DNA from 2.4.1, while lane 2 harbors DNA from S7. The numbers shown on the left of the gel are the sizes of restriction endonuclease DNA fragments in kb.

DNA region of Tn*5* insertion.

Acknowledgements

This research was supported by the academic research fund of the Ministry of Education, Republic of Korea.

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(Received June 28, 1997)