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Increased biological hydrogen production by deletion of hydrogen-uptake system in photosynthetic bacteria

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Summary

Hydrogenases are the key enzymes for the biological hydrogen production, which can be classified as H_2-uptake hydrogenase and H_2-production hydrogenase. The genes encoding a membrane-bound [NiFe]-hydrogenase (MBH), which is mainly responsible for hydrogen uptake, from the photosynthetic bacterium *Allochromatium vinosum* was cloned and sequenced. It consist of two structural genes (*hydS*, *hydL*) and two intergenic genes (*isp1*, *isp2*), which are therefore organized as *hydS–isp1–isp2–hydL*. This is different from the arrangement of other typical hydrogenase gene clusters. A deletion mutant-strain Φ*hydSL*, lacking *isp1*, *isp2*, partial *hydS* and *hydL* genes, was constructed by marker-exchange mutagenesis. Under dark fermentative conditions, the hydrogen production yield by this mutant increased by 62%. The result suggests that the disruption of MBH could greatly improve the hydrogen production in the cells by decreasing the hydrogen uptake.

1. Introduction

Hydrogen is considered as the most clean energy carrier (Cammack et al. 2001). Biological hydrogen production using fermentative microorganisms provides an excellent possibility to produce hydrogen economically on large scales. This process depends upon the presence of hydrogen-producing enzymes (Rita and Andreas 2004). Hydrogenases catalyze the reversible reaction, $H_2 \leftrightarrow 2H^+ + 2e^-$, and play a vital role in the energy metabolism of many microorganisms. They have been classified into [NiFe]-, [FeFe]- and [Fe]-hydrogenases, based on their metals contained in the active center (Vignais et al. 2001). A number of hydrogenase genes from aerobic-, anaerobic-, photosynthetic- and cyanobacteria have been cloned and sequenced. Hydro-

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genase is a complex enzyme whose synthesis requires the structural genes as well as a number of accessory genes (Kovács et al. 2002). The structural genes of hydrogenases are usually transcribed together with accessory genes as a unit. Most hydrogenases consist of a large subunit (45–72 kDa) and a small subunit (20–35 kDa). The genes encoding the small subunits are usually located upstream of the genes encoding the large subunits.

Allochromatium vinosum is a purple sulfur photosynthetic bacterium which contains at least one membrane-bound hydrogenase (MBH) (Bleijlevens et al. 2004) and one soluble hydrogenase (SH) (Minnan et al. 2007). The MBH with high hydrogen-uptake activity consists of a large (64 kDa) and a small subunit (35 kDa), and has been studied in detail as described recently (Bleijlevens et al. 2004). It contains two [4Fe–4S] clusters and one [3Fe–4S] cluster. In this study, we report the cloning and genetic deletion of the MBH gene. Analysis of the MBH-null bacteria shows that disruption of MBH significantly increases hydrogen production, likely through a decrease in hydrogen uptake by the cells.

2. Materials and methods

2.1. Bacterial strains and cultivation

A. vinosum DSM 185 was routinely grown in Pfennig's minimal medium (Pfennig and Trüper 1992) supplemented with 4 mmol/L sodium thiosulfate. The cells were cultivated anaerobically in a closed glass bottle under continuous illumination with a light intensity of 500 W/m^2 at 35 °C for 1 week.

2.2. Preparation of genomic DNA

A. vinosum cells were harvested by centrifugation (10,000*g*, 10 min) and washed twice with NaCl solution (1 mol/L). The cell paste was suspended in cold (4 °C) TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 8.0) and kept in an ice-bath for 1 h. Subsequently, the cells were incubated in lysis buffer (0.1 mol/L Tris–HCl, 0.1 mol/L EDTA, 1% lysozyme, pH 8.0) at 37 °C for 20 min. The mixture was then supplemented with RNase (100 μg/ml), SDS (1%) and proteinase K (50 μg/ml), and kept at 60 °C for 1 h. Finally, the lysate samples were extracted twice with phenol–chloroform–isoamyl alcohol (25:24:1, v/v), and with an equal volume of a mixture of chloroform–isoamyl alcohol (24:1, v/v). The genomic DNA was precipitated with ethanol and washed with 70% cold ethanol, followed by careful dissolution in TE buffer.

2.3. Analysis of N-terminal sequences of MBH

Purified MBH from *A. vinosum* was prepared as previously described (Bleijlevens et al. 2004). It was denatured and subjected to an SDS-PAGE. The protein bands were observed after Coomassie-brilliant blue staining, and the molecular weights determined. The proteins were then electroblotted onto a PVFM membrane with an electroblot instrument (Semi-dry transfer, Bio-Rad). N-terminal amino-acid analysis of the purified protein bands was performed by automated Edman degradation on an Applied Biosystem 470A gas-phase protein sequencer.

2.4. Cloning of *hydSL* genes

In the usual arrangement of typical hydrogenase structural genes, the gene encoding the small subunit is located upstream of the one encoding the large subunit. Primer hyd-SF (5′-CCSTCGGTCNTCTGGCTG-3′) corresponding to the N-terminal sequence (PSVIWL) of the small subunit, and primer hyd-SR (5′-GGTGAYSGGATCGACGAC-3′) corresponding to the N-terminal sequences (VVDPIT) of the large subunit were designed according to the codon usage of *A. vinosum*. The DNA fragment coding for the small subunit was amplified by standard PCR procedures (Sambrook and Russell 2001). The PCR fragments were then isolated from agarose gels and cloned into cloning vector pMD18-T. DNA sequencing was completed by the TaKaRa Biotechnology Company (Dalian, China).

To obtain the complete sequence of the MBH-gene cluster, the genomic DNA was partial digested with restriction endonuclease *Bam*HI. It was then ligated with an adaptor (5′-GATCAGCGGATAA-CAATTTCACAAG-3′) containing *Bam*HI cohesive ends. To get the upstream sequence of the small subunit and the downstream sequence of the large subunit, PCR primers were designed according to the N-terminal sequences of the small or large subunits and the sequence of the adaptor, respectively. The ligation product was purified and used as the template of PCR. DNA sequencing of the double strands was performed by the TaKaRa Biotechnology Company (Dalian, China).