Universal degenerate oligonucleotide-primed-polymerase chain reaction for detection and amplification of NiFe-hydrogenase genes

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Abstract

Hydrogenase plays an important role in biological hydrogen production. Many researchers have paid attention to this enzyme in attempts to enhance the efficiency of biological hydrogen production. In contrast to the well-characterized Fe-hydrogenase, NiFe-hydrogenase, a heterodimeric metalloenzyme composed of two subunits, has not been examined in detail for biotechnological application. It has been reported that NiFe-hydrogenases have relatively higher tolerance to oxygen than Fe-hydrogenases and particularly, some enzymes are highly oxygen-tolerant. Because inhibition by oxygen is one of the serious problems in biological hydrogen production, the development of techniques for the effective detection of oxygen-tolerant NiFe-hydrogenase genes is important. In this study, we designed a universal degenerate primer pair based on highly conserved motifs at the N-terminus of the small subunit and C-terminus of the large subunit. We successfully demonstrated that the universal degenerate oligonucleotide-primed-polymerase chain reaction (DOP-PCR) amplified 2.9–3.0 kb NiFe-hydrogenase genes from various microorganisms. This technique was applied to detect and reveal unknown sequences from the large subunit of oxygen-tolerant NiFe-hydrogenase in Hydrogenovibrio marinus. Thus, this universal DOP-PCR procedure can be successfully employed to identify or amplify novel/known oxygen-tolerant NiFe-hydrogenase genes for engineering biological hydrogen production.

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1. Introduction

Hydrogen provides clean, eco-friendly energy [1]. Hydrogen does not involve the emission of carbon dioxide leading to the greenhouse effect and has a higher energy density per unit mass compared to other sources (e.g. 2.4 times that of methane). In addition, it is easily converted to electricity through fuel cells. Several methods of hydrogen production have been developed, and are classified into nuclear, solar, electrical, chemical, and biological according to the energy source. Among these, the biological approach is superior to photochemical and thermochemical methods in terms of being environmentally friendly [2,3]. Biological hydrogen production uses renewable biomaterials and does not produce pollutants such as CO₂ and NOₓ. In addition, it requires minimal energy consumption and simple, small-scale facilities. However, the biological method is only performed at the laboratory level at present, due to low production efficiency [2].

Biological hydrogen production is basically mediated by specific enzymes. While hydrogen-producing enzymes catalyze a simple chemical reaction (2H⁺ + 2e⁻ → H₂), they contain complex metallo-clusters as active sites and are synthesized by complicated procedures with the aid of auxiliary proteins. Two enzymes catalyzing this reaction are nitrogenase and hydrogenase. Nitrogenase consumes ATP for reducing protons and requires additional machinery for its function. Moreover, the turnover rate is extremely slow (6.4 s⁻¹) [4]. Hydrogenases are categorized into two major groups according to the metal content at the active center, specifically, Fe-hydrogenase and NiFe-hydrogenase [5]. Metal atoms are involved in formation of the active site that directly catalyzes proton reduction and hydrogen oxidation. NiFe-hydrogenase is a heterodimeric protein composed of small and large subunits. The small subunit has one [3Fe–4S] and two [4Fe–4S] clusters involved in electron transfer to the active center. The large subunit contains a unique nickel–iron active center buried deeply in the protein complex coordinated to two CN and one CO [6]. NiFe-hydrogenase generally functions as an uptake hydrogenase that provides reducing power via hydrogen oxidation. However, this enzyme

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also mediates reverse reaction with a turnover rate of 98 s\(^{-1}\) in \textit{Desulfovibrio fructosovorans} even though its activity is variable in different species [4,7,8]. \textit{Fe}-hydrogenase contains a unique complex iron–sulfur center in which one of the Fe atoms is coordinated with CO and CN [9,10]. This enzyme is more involved in hydrogen evolution and 10–100 times biologically active than NiFe-hydrogenase [11,12]. Therefore, many investigators have focused on mainly Fe-hydrogenase in attempts to enhance the efficiency of biological hydrogen production [13,14]. However, despite lower biological activity, NiFe-hydrogenases have specific advantages, including higher affinity for the substrate, hydrogen and in particular, lower sensitivity to inhibition by oxygen and carbon monoxide [7,15]. It has been reported that some NiFe-hydrogenases are highly oxygen-tolerant [8,15]. Oxygen tolerance of hydrogenase is an important target feature for engineering biological hydrogen production [15].

To date, limited biotechnological analyses of NiFe-hydrogenases, such as recombinant protein expression and protein engineering, have been performed compared to the well-characterized Fe-hydrogenase enzymes. Therefore, effective detection techniques for oxygen-tolerant NiFe-hydrogenase gene are necessary to obtain potential sources for improved hydrogen production. The degenerate oligonucleotide-primed-polymerase chain reaction (DOP-PCR) technique has been developed to allow an unselected amplification of any source of DNA, which has conserved amino acid motifs [16]. In the present work, based on several conserved motifs in its core enzyme subunits of NiFe-hydrogenase, we designed a universal degenerate primer pair and performed, for the first time as far as we know, DOP-PCR to detect and amplify NiFe-hydrogenase genes from various microorganisms with the ultimate aim of identifying an efficient oxygen-tolerant gene source for engineering biological hydrogen production.

### 2. Materials and methods

#### 2.1. Strains

\textit{Escherichia coli} Top10 (Invitrogen, USA) was used as the host strain for genetic manipulation. \textit{E. coli} K12 MG1655 (CGSC 6300), \textit{Ralstonia eutropha} (KCCM 41690), \textit{Citrobacter freundii} (KCTC 2509), \textit{Azotobacter vinelandii} (KCCM 40419), and \textit{Hydrogenovibrio marinus} (KCTC 2426), and \textit{Hydrogenovibrio marinus} MH-110 (DSM 11271) were selected as target microorganisms to detect and amplify genes encoding group 1 type NiFe-hydrogenase. \textit{Synechocystis} sp. PCC6803 and \textit{Erwinia uredovora} (KCCM 40419) were employed as negative controls. Cells were grown using the conditions recommended by each cell stock institution.

#### 2.2. PCR and sequence analysis

The Wizard genomic DNA purification kit (Promega, USA) was used to isolate chromosomes from microorganisms. The reaction mixture for amplification contained 5 \(\mu\)l of 10 \(\times\) reaction buffer with 15 mM MgCl\(_2\) (Bioneer, Korea), 1 \(\mu\)l (100 ng/ml) of chromosomal DNA, 4 \(\mu\)l of 10 mM dNTP mix, each 0.35 \(\mu\)l of 100 \(\mu\)M forward and reverse degenerate primers (Table 1), 0.4 \(\mu\)l of 5 \(U\) Taq polymerase (Bioneer), and distilled water up to a volume of 50 \(\mu\)l. The following conditions were employed: initial denaturation at 94 \(^\circ\)C for 5 min, followed by 30 cycles of denaturation at 94 \(^\circ\)C for 1 min, annealing at 55 \(^\circ\)C for 40 s, and extension at 72 \(^\circ\)C for 3 min, and final extension at 72 \(^\circ\)C for 5 min. PCR products were examined on an agarose gel, cloned into pGem-T easy vector (Promega), and sequenced using a commercial service (Genotech, Korea). Alignment analysis of hydrogenase sequences was performed using the ClustalW algorithm of BioEdit software [17].

#### 3. Results and discussion

NiFe-hydrogenase is synthesized through a highly complicated process requiring a number of accessory proteins, and is mostly encoded by several genes located in the same cluster [17]. The large and small subunits forming heterodimeric proteins play key roles in enzymatic activity [18]. NiFe-hydrogenase is an ancient enzyme in the evolutionary process, which contains several highly conserved motifs. Among the conserved regions, the small subunit contains a signal sequence at the

![Fig. 1](image-url)
Fig. 2. Sequence alignment of the large subunit of NiFe-hydrogenase from five microorganisms (E. coli, R. eutropha, A. vinelandii, C. freundii, and Hydrogenovibrio marinus). Alignment analysis was performed using the ClustalW algorithm. Newly identified sequences in the large subunit of NiFe-hydrogenase from H. marinus are underlined. Symbols: '*', identical in all sequences; ':', conserved substitution; '.', semi-conserved substitution.
N-terminus that facilitates the secretion of fully assembled NiFe-hydrogenase into periplasmic space through the twin-arginine translocation pathway, while the large subunit has an endopeptidase recognition sequence at the C-terminus, which is removed following nickel incorporation [19–21]. However, the information on these conserved sequences has not been used for detection or isolation of NiFe-hydrogenase genes. Accordingly, we used these sequences as templates to design a universal degenerate primer pair for detecting and amplifying group I type NiFe-hydrogenase genes (designated by Vignais et al. [20]), following alignment analysis of the respective genes from various microorganisms on the basis of available literature and databases (Table 1, Fig. 1A).

DOP-PCR was performed to assess the specificity of degenerate primers using each chromosomal DNA from four microorganisms (E. coli, R. eutropha, C. freundii, and A. vinelandii) having group 1 type NiFe-hydrogenase gene, and two negative control strains (Synechocystis sp. PCC6803 and E. uredovora) that do not contain the same gene type. We successfully amplified 2.9–3.0 kb DNA, consistent with the sizes of known NiFe-hydrogenase genes from all tested microorganisms (Fig. 1B, lanes 1–4), with the exception of the two negative control strains, as expected (Fig. 1B, lanes 5 and 6). Sequencing data revealed that all amplified gene products are identical to the known NiFe-hydrogenase genes of H. marinus [8]. However, the full gene sequence of H. marinus NiFe-hydrogenase is not available in Genbank (Access # E55242). Elucidation of the full sequence of the core NiFe-hydrogenase of H. marinus should provide helpful information on the amino acid sequences and protein structure that might attribute to oxygen tolerance. We successfully amplified a ~2.9 kb PCR product corresponding to the small and large subunits of H. marinus NiFe-hydrogenase (data not shown). Sequencing data revealed consistency with the reported partial sequence. The C-terminal sequence of the large subunit of NiFe-hydrogenase from H. marinus was newly determined in this study (Fig. 2; underline indicates the novel sequence). Alignment analysis of five NiFe-hydrogenase sequences disclosed relatively high homology between the enzymes, except at the C-terminus of the small subunit (data not shown) and residues 450–470 of the large subunit (Fig. 2). In particular, variations in residues 450–470 of the large subunit were due to the insertion of extra amino acids in H. marinus and R. eutropha. In view of the oxygen-tolerant properties of NiFe-hydrogenases from these two strains [8,15], we might propose that the amino acid variations at positions 450–470 of the large subunit contribute to differences in oxygen tolerance and oxygen sensitivity. However, further investigation is necessary to confirm this surmise.

Our universal PCR primer pair can thus be applied to identify novel NiFe-hydrogenase genes and amplify known genes. The NiFe-hydrogenase genes acquired may be subjected to protein engineering techniques, such as gene shuffling, to improve the efficiency of biological hydrogen production through modulation of enzyme property or for use as an electrocatalyst for biofuel cells and biosensors. Furthermore, this DOP-PCR analysis technique can be effectively employed to monitor temporal and spatial community variations of hydrogen-producing microorganisms in biological hydrogen production.

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