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Thermophilic hydrogen-producing bacteria inhabiting deep-sea hydrothermal environments represented by *Caloranaerobacter*

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Abstract

Hydrogen is an important energy source for deep-sea hydrothermal vent ecosystems. However, little is known about microbes and their role in hydrogen turnover in the environment. In this study, the diversity and physiological characteristics of fermentative hydrogen-producing microbes from deep-sea hydrothermal vent fields were described for the first time. Seven enrichments were obtained from hydrothermal vent sulfides collected from the Southwest Indian Ocean, East Pacific and South Atlantic. 16S rRNA gene analysis revealed that members of the *Caloranaerobacter* genus were the dominant component in these enrichments. Subsequently, three thermophilic hydrogen producers, strains H363, H53214 and DY22619, were isolated. They were phylogenetically related to species of the genus *Caloranaerobacter*. The H_2 yields of strains H363, H53214, DY22619 and MV107, which was the type species of genus *Caloranaerobacter*, were 0.11, 1.21, 3.13 and 2.85 mol H_2/mol glucose, respectively. Determination of the main soluble metabolites revealed that strains H363, H53214 and MV107 performed heterolactic fermentations, while strain DY22619 performed butyric acid fermentation, indicating distinct fermentation patterns among members of the genus. Finally, a diversity of forms of [FeFe]-hydrogenase with different modular structures was revealed based on draft genomic data of *Caloranaerobacter* strains. This highlights the complexity of hydrogen metabolism in *Caloranaerobacter*, reflecting adaptations to environmental conditions in hydrothermal vent systems. Collectively, results suggested that *Caloranaerobacter* species might be ubiquitous and play a role in biological hydrogen generation in deep-sea hydrothermal vent fields.

Keywords: *Caloranaerobacter*; Thermophilic microbes; Biological hydrogen production; [FeFe]-hydrogenase; Deep-sea hydrothermal vent environment

1. Introduction

Molecular hydrogen is an important metabolic intermediate in a wide range of microorganisms, formed by the degradation of organic matter and consumed coupling the oxidation of H_2 to the reduction of oxidized compounds [1]. Hydrogen is also the metabolic basis of many syntrophic associations that commonly occur in microbial communities, such as between sulfate-reducing bacteria and methanogenic archaea [2]. Hydrogen metabolism therefore plays a key role in the performance and maintenance of many microbial populations. Recently, hydrogen has been shown to be the primary fuel supporting a number of microbial assemblages [3–5], including those inhabiting deep-sea hydrothermal vent environments [6,7].

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Deep-sea hydrothermal vents are found globally, and are usually located in sea-floor spreading zones at mid-ocean ridges. Since they were first discovered in 1977, hydrothermal vents have been revealed as a vast and previously unknown domain of ecosystem on the Earth, hosting rich and diverse microbial populations. Currently, two major types of hydrothermal vent have been identified: ultramafic-hosted and basalt-hosted hydrothermal systems [8]. Fluids originating from basalt-hosted vents are hot (up to 405 °C) and acidic (pH 2–3), with high concentrations of H_2S and CO_2, but low concentrations of H_2 and CH_4 [9]. In contrast, ultramafic-hosted hydrothermal vents emit low (40–91 °C) temperature and highly alkaline (pH 9–11) fluids with high H_2, CH_4 and some low-molecular-mass hydrocarbons, but low H_2S concentrations and almost no dissolved CO_2 [8]. To our knowledge, the highest hydrogen concentration ever measured in hydrothermal systems was 26 mmol kg^{-1} in the Ashadze vent fields on the Mid-Atlantic Ridge [10]. The results indicated that hydrogen was widespread and particularly abundant in submarine hydrothermal vents, and thus it was postulated that H_2 could play an important role in vent ecosystems.

H_2 can be formed by both abiogenic and biogenic processes. In general, geological H_2 is produced through the reduction of water during serpentinization reactions in alkaline and ultramafic-rich environments [11,12]. H_2 generation also occurs through the hydrolysis of reduced water, catalyzed by basalt in acidic and basalt-hosted environments [13,14]. Biological hydrogen production is catalyzed by hydrogenases, with excess electrons generated during microbial metabolism transferred to protons to form H_2 [15]. Hydrogenases can be divided into three main groups based on their metallocenter composition: [NiFe] hydrogenases, [FeFe] hydrogenases and [Fe] hydrogenases. Two classes of hydrogenase are present in bacteria, [NiFe] and [FeFe] [15]. [FeFe] hydrogenases often have a higher turnover rate and are more active in environments with higher H_2 partial pressure than [NiFe] hydrogenase [16]. In general, [FeFe] hydrogenases are involved in the regeneration of reduced electron carriers, which is coupled to proton reduction. It was found that [FeFe] hydrogenases exist in multiple forms with different modular structures and are especially abundant in members of the genus *Clostridium* [17].

Recently, many studies have been dedicated to the characterization of the diversity of fermentative hydrogen-producing bacteria in diverse environments, including a saline microbial mat system [2], an H_2-generating anaerobic bioreactor [18], the surface waters of marine and freshwater environments [19] and geothermal springs in Yellowstone National Park [5], using functional genes, such as the *hydA* gene, as proxies. These studies demonstrated the widespread distribution and diversity of fermentative hydrogen producers in nature. However, little is known about deep-sea hydrothermal vent ecosystems. In contrast to other environments, there is a paucity of data on hydrogen turnover in submarine hydrothermal vents. Therefore, the aim of the present study was to: (1) investigate the diversity of fermentative hydrogen-producing thermophiles in hydrothermal vent environments using a culture-dependent method; (2) characterize and identify thermophilic hydrogen-producing bacteria; (3) investigate H_2 production properties of these novel themophiles; and (4) analyze the hydrogenase genes in members of the genus *Caloranaerobacter*.

2. Materials and methods

2.1. Enrichment and isolation

During the DY115-20, 21 and 22 oceanographic cruises, deep-sea hydrothermal vent sulfide samples were collected using a grabber from different depths on the Southwest Indian Ridge, East Pacific and South Atlantic. Aboard the research vessel *Dayang Yihao*, samples were immediately transferred into sterile plastic bags (Whirl Pak sample bags, Nasco, USA) and stored at 4 °C until enrichment in the laboratory. About 1 g of sample was inoculated in anaerobic tubes (18 × 150 mm) containing 10 ml MJYTG medium. The MJYTG medium contained 1 g yeast, 1 g tryptone, 2.5 g glucose, 1 ml vitamin solution [20], 0.5 g cysteine-HCl.H_2O and 0.5 mg resazurin per liter MJ synthetic seawater. The MJ synthetic seawater consisted of (L^{-1}): NaCl, 30 g; K_2HPO4, 0.14 g; $CaCl_2.2H_2O$, 0.14 g; NH_4Cl, 0.25g; $MgSO_4.7H_2O$, 3.4 g; $MgCl_2.6H_2O$, 4.18 g; KCl, 0.33 g; $NiCl_2.6H_2O$, 0.5 mg; $Na_2SeO_3.5H_2O$, 0.5 mg; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.01 g; trace mineral solution, 10 ml [20]. The medium was prepared under a N_2 gas phase and the pH was adjusted to 7. After 72 h of incubation at 60 °C, the enrichment results were checked by H_2 production and microscopy observations. A total of 7 samples were successfully enriched. After a three-time transfer, the enriched cultures were further purified by spreading onto MJYTG agar medium and incubated in an anaerobic chamber. Pure isolates were obtained after three successive transfers onto fresh MJYTG agar media. These isolates were grown routinely on YTG medium (composition per liter): 1 g yeast, 1 g tryptone, 2.5 g glucose, 30 g sea salt, 6.05 g PIPES buffer, 1 mg resazurin, and the pH was adjusted to 7 [21] at 60 °C for characterization studies.

2.2. DNA extraction, construction of clone libraries and DNA sequencing

After three-time transfer, the bacterial diversity of enrichments was analyzed. The bacterial 16S rRNA gene fragments were PCR-amplified from total DNA extracted from enriched cultures using the primers Eubac21F and Eubac1492R [22]. Total DNA extraction, PCR amplification and clone library construction were performed as previously described [23]. The reactions were performed in a T3 thermocycler (Biometra, Germany) using the following protocol: 1 min at 95 °C; 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min, followed by a final extension step at 72 °C for 10 min. A product of nearly 1500 bp was cloned into the pMD18-T vector supplied with the TA cloning kit (TaKaRa, Dalian, China) following the manufacturer's instructions. Ten clones per enrichment culture were picked up first, and then 10 clones more for enrichments samples HP3, HP8, HP6, and 20 clones