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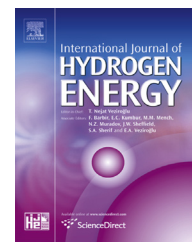

Identification of a gene cluster responsible for hydrogen evolution in *Vibrio tritonius* strain AM2 with transcriptional analyses

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ABSTRACT

Vibrio tritonius strain AM2 shows high-yield hydrogen production even under saline conditions (1.7 mol hydrogen/mol mannitol). However, the molecular mechanism of efficient hydrogen production has never been studied in the genus *Vibrio*. The aim of this study is to identify the genes responsible for hydrogen evolution in *V. tritonius* and the gene expression pattern. Complete genome analysis revealed an existence of a single 24-kb gene cluster containing 21 genes, which are essential for the formation of an energy-conserving formate hydrogen lyase (FHL) complex, to be more specific the vibrio FHL was structurally rather similar to the *hyf* (hydrogenase four) gene cluster found in *Escherichia coli*. Moreover, genes responsible to the formate dehydrogenase (FDH-H), *fhlA*-type transcriptional activator and hydrogenase maturation proteins (*hyp*) were also located downstream of the vibrio *hyf* gene cluster to form a "super-gene-set" of the FHL complex gene cluster. The vibrio gene for the large subunit of the FHL complex *hyfG* possessed typical motifs coordinating the [NiFe] center at the active site, which indicates the *V. tritonius* hydrogenase was able to be classified as a [NiFe]-hydrogenase. Furthermore, transcriptional analysis revealed that the expression level of the *hyfG* gene slightly increased upon pH decrease, which correlates to the pH-dependent hydrogen production of *V. tritonius*. Therefore, we can conclude that the FHL complex of *V. tritonius* is key enzyme in the hydrogen production under acidic conditions. Moreover *hyfABCDEFGHIJ-hycI-hydN-fdhF* and *hyp* genes could be

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co-transcribed respectively during the efficient hydrogen production state. Details of the gene cluster are discussed here.

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Introduction

Hydrogen (H_2) produced via biological processes, such as fermentation and photosynthesis, has been recognized as a renewable and carbon-neutral energy source for the future. Biological production of H_2 is commonly achieved by hydrogenase, which catalyzes both functions in evolution and oxidation of H_2, representing the following a simple chemical reaction: $2H^+ + 2e^- \rightleftarrows H_2$ [1–3]. Currently, hydrogenases can be classified into [NiFe]-hydrogenases, [FeFe]-hydrogenases, and [Fe]-hydrogenases based on the types of functional core which contain distinctive catalytic metal centers [1,4]. The [NiFe]-hydrogenases are widely distributed in bacteria belonging to the domains of *Bacteria* and *Archaea*, and have been the most extensively studied. The [NiFe]-hydrogenases which contribute to H_2 metabolism in bacteria are further classified into four groups based on the full sequence alignments of the large subunit and the small subunit [1,5]. Among the four groups of the [NiFe]-hydrogenases, those in group 4, which are defined as H_2-evolving, energy-conserving, and membrane-associated hydrogenases, play an important role in the disposal of excess reducing equivalents [6], acidic resistance [7], and energy-conservation [8–10].

Escherichia coli possesses two types of formate hydrogen lyase (FHL), each of which consists of a [NiFe]-hydrogenase and a formate dehydrogenase-H (Fdh-H), as the major H_2-evolving structural protein complexes; one is FHL-1 consisting of Hyc complex of hydrogenase-3 (encoded by *hyc* operon) and the other is FHL-2 consisting of Hyf complex of hydrogenase-4 (encoded by *hyf* operon) [8,11,12]. *E. coli* FHL-1 play an important role in circumventing the critical acidification of the cytoplasm due to the accumulation of formate (pKa = 3.75), when the pH of the medium drops below 6.8 [13]. The major structural differences between the Hyc- and the Hyf-type FHL complex are found in the integral membrane proteins; the Hyf-type complex is composed of three additional integral membrane components, which might be involved in proton translocation coupling with H_2 evolution [8]. Currently, however, we have less information on active Hyf-derived hydrogenase and the presence of Ni in HyfG, a large subunit of hydrogenase [14], nevertheless the structural and functional similarities of FHL to complex I have been discussed [3,15].

Vibrio tritonius strain AM2 isolated from the gut of the sea hare (*Aplysia kurodai*) is a facultative anaerobe which can produce H_2 via formate oxidation [16,17]. *V. tritonius* represents high-yield H_2 production (1.7 mol H_2/mol mannitol) even under saline conditions [17]. Vibrios are phylogenetically related to *Enterobacteriaceae* [18] and the genes related to FHL have been found in the complete genome sequence of *Vibrio furnissii* NCTC 11218 [19]. Therefore, it is expected that the *V. tritonius* genome also contains the genes responsible for the formation of FHL.

H_2 production in genus *Vibrio* is an atypical property; however, the details on biochemistry, genetics, genomics, and molecular biology involving transcriptional regulation of the H_2 production mechanism have not been elucidated yet. Fortunately, as mentioned above, the H_2 productivity of *V. tritonius* is high enough to conduct deeper studies in elucidating the H_2-producing mechanism in marine vibrios [17]. In this study, we focused on the genetic properties and the transcriptional patterns of the genes responsible for the H_2 evolution in *V. tritonius*. The results will also contribute not only to improvements in the H_2 productivity of *V. tritonius* but also help towards a better understanding of the H_2 metabolism and the evolutionary framework of vibrios.

Materials and methods

Bacterial strain and culture condition

In preculture and batch culture experiments, *V. tritonius* strain AM2 was grown in a synthetic medium containing 200 mM NaCl, 50 mM $MgSO_4 \cdot 7H_2O$, 10 mM KCl, 10 mM $CaCl_2 \cdot 2H_2O$, 20 mM glucose, 19 mM NH_4Cl, 0.33 mM K_2HPO_4, 50 mM MES and 0.2% (v/v) trace metal solution; 5 mM $NiCl_2$, 1.6 mM ferric citrate, 1 mM $Na_2MoO_4 \cdot 2H_2O$ and 1 mM Na_2SeO_3. The preculture was performed aerobically to achieve 0.6 ± 0.05 OD_{620} of cell density with shaking at 130 rpm at pH 7 and 37 °C. The batch culture was conducted by adding 1 mL of the preculture solution to 100 mL medium. The medium was stirred with a magnetic stirrer (RO 10 power IKAMAG, IKA, Staufensee, Germany) during cultivation. pH levels of the medium were maintained at pH 5.5, 6, 6.5 or 7 using a pH controller (DT-1023P, ABLE, Tokyo, Japan) with an electrode (FermProbe pH electrodes, Broadley-James Corp., Irvine, USA) by adding 5 N NaOH. The temperature of the medium was maintained at 37 °C.

Assays

The volume of the H_2 production was measured by analyzing the H_2 composition of the head space of the bottles using gas chromatography (GC2014, Shimadzu, Kyoto, Japan) with a thermal conductivity detector and a Shincarbon ST (Shinwa Chemical Industries Ltd., Kyoto, Japan). Dry cell weight (DCW) was calculated by measuring OD_{620}; 1 OD_{620} was estimated as 0.41 g DCW/L. The data of the H_2 production was analyzed using one-way analysis of variance (ANOVA) with Tukey–Kramer test for multiple comparisons. A probability less than 5% ($P < 0.05$) was considered statistically significant.

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