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Title page

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

Highlights

- Genome sequence analysis of the *V. tritonius* reveals a 24-kb *hyf*-type gene cluster.
- The gene cluster consists of 21 genes for FHL complex, Fdh-H, FhlA, and Hyp proteins.
- The *hyfG* gene expression slightly increases along with the acidification of medium.

1. Introduction

Hydrogen (H₂) 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 H₂ is commonly achieved by hydrogenase, which catalyzes both functions in evolution and oxidation of H₂, representing the following a simple chemical reaction: $2\text{H}^+ + 2\text{e}^- \rightleftharpoons \text{H}_2$ [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 H₂ 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 H₂-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].

E. 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 H₂-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 ($pK_a=3.75$), 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 *V. 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.

2. Materials and Methods

2.1 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 NiCl₂, 1.6 mM ferric citrate, 1 mM Na₂Mo₄ · 2H₂O and 1 mM Na₂SeO₃. The preculture was performed aerobically to achieve 0.6±0.05 OD₆₂₀ 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.

2.2 Assays

The volume of the H₂ production was measured by analyzing the H₂ 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 OD₆₂₀; 1 OD₆₂₀ was estimated as 0.41 g DCW/L. The data of the H₂ 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.

2.3 Genome analysis

The whole genome sequence of *V. tritonius* strain AM2 was obtained using a 454 GS FLX Titanium sequencer (Roche Diagnostics, Branford, USA) and the sequence reads were assembled using Newbler software version 2.3. Gaps were filled manually by sequencing PCR products covering each gap region by Sanger sequencing. Illumina reads obtained by a MiSeq sequencer (Illumina, San Diego, USA) were used only for sequence error correction. Identification of protein-coding sequences (CDSs) was carried out using Microbial Genome Annotation Pipeline (MiGAP; <http://www.migap.org/>) [20-21] and GLIMMER [22]. Additional gene analyses were performed using In Silico Molecular Cloning software (In silico biology, Inc., Yokohama, Japan). Amino acid sequence alignments of hydrogenase subunits were conducted using CLUSTAL X2 program [23-24]. Characterization of the genes responsible for H₂ production was conducted using InterPro database [25]. Transcriptional terminators were predicted using ARNold [26]. Transmembrane helices were predicted by SOSUI [27] and protein localization by PSORTb 3.0 [28].

2.4. Total RNA extraction and real-time quantitative PCR

Total RNA was extracted from the cells grown in the synthetic medium described above to mid-logarithmic phase using a TRIzol LS Reagent (Life Technologies, Carlsbad, USA). The total RNA was treated with RNase-free DNase (Promega, Madison, USA) to remove any residual DNA, and was further purified using RNeasy Mini kit (Qiagen, Hilden, Germany). The first-strand cDNA was synthesized using SuperScriptTM III reverse transcriptase and random 6-mer (Life Technologies, Carlsbad, USA) at 46°C for 3 hours. The cDNA was used for real-time quantitative PCR (qPCR). The *hyfG* gene of *V. tritonius* was selected as a target gene of interest, and two genes, *proS* and *pyrH*, were selected as reference genes for the real-time qPCR. The two reference genes were selected on the basis of a rather stable expression level even under a wide range of pH conditions (pH 5-7) in the *V. tritonius* cells to increase the stability of the RT-qPCR experiments. Primers (*hyfG1F* and *hyfG1R*) for the real-time qPCR were designed using a Primer Express Software (Life Technologies, Carlsbad, USA). The quantitation was performed using SYBR Green PCR platform and StepOneTM real time PCR system (Life Technologies, Carlsbad, USA) on the basis of $\Delta\Delta C_t$ method. The PCR cycle conditions consisted of 95°C for 10 min., followed by 40 cycles of 95°C for 15 sec. and 60°C for 1 min. All expression levels were presented here are relative to those at pH 7.

2.5. Transcriptional unit analysis

The first-strand cDNA, which corresponds to a 24-kb region consisting of all of the genes in the formation of the FHL complex of *V. tritonius*, was synthesized on the basis of total RNA, which was purified from cells grown at pH 6, using SuperScriptTM III reverse transcriptase and gene specific primers (*fhlART* and *hypBRT*). The condition for the reverse transcription (RT) was 55°C for 90 min. for cDNA synthesis and 70°C for 15 min. for the termination. Using the two generated cDNAs, PCR reactions were performed with primer pairs listed in Table S1 that are able to amplify intergenic regions of the gene cluster of *V. tritonius* using a GoTaq Green Master Mix (Promega, Madison, USA). The PCR cycle was consisted of 95°C for 2 min. for an initial denaturation, and 30 cycles of 95°C for 1 min (denaturation), 55°C for 1 min. (annealing) and 72°C for 1 min. (extension). Positive and negative control reactions were set to use the genomic DNA of strain AM2 extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, USA) and the total RNA as a template, respectively. The resultant PCR products were analyzed by agarose gel electrophoresis with a 1.0% (w/v) gel in 0.5x TBE buffer.

3. Results

3.1. Identification of the *Vibrio tritonius* FHL-Hyp gene cluster on the large chromosome

The genome of *V. tritonius* strain AM2 consisted of two circular forms of DNA; the large chromosome (Chr. 1) of 3,434,724 bp in size and the smaller second one (Chr. 2) of 1,787,202 bp. The G+C% of Chr. 1 and Chr. 2 were 43.96 and 43.78, respectively. A total of 34 rRNA genes, which were encoded by *rrn* operons, were found on Chr. 1 but not on Chr. 2. The total CDSs of Chr. 1 and Chr. 2 were around 3,000 and 1,600, respectively. Possible origins of replication (*oriC*) were predicted by the OriC finder [29]. The *ori* region of Chr. 1 was confirmed by the co-localization of *gidA*, a *mioC*-like FMN-binding protein gene, *dnaA*, *dnaN*, *recF*, and *gyrB* [30-31]. The genes encoding plasmid partitioning proteins (ParAB) were also located at the *oriC* region of Chr. 1. The *ori* region of Chr. 2 was identified by the structural similarity to that of *V. cholera*; the *parAB* and *rctAB* genes were co-localizing as in *V. cholerae* [30-31]. A 5' inter-genetic region of *rctB* of *V. tritonius* possessed an AT rich sequence highly similar to those of the *V. cholera* and *V. parahaemolyticus* Chr. 2. Genome sequences of Chrs. 1 and 2 for *V. tritonius* strain AM2 were deposited in the DDBJ/EMBL/GenBank database under accession numbers AP014635 and AP014636, respectively.

Only two hydrogenase genes were found on the genome of *V. tritonius*; each encodes large and small subunits of hydrogenase. On further inspection of the region flanking the possible hydrogenase genes, a large gene cluster of 24 kb in length, which could be responsible for the H₂ production, was identified at the 481,700-505,700 bp region of Chr. 1 (Fig. 1A). We found that the gene cluster containing 21 genes required for the formation of FHL by BLAST and InterPro Scan searches (Fig. 1A and Table 1). Among the 21 genes, the first 10 genes showed a remarkable similarity to the *hyfABCDEFGHIJ* genes in the *hyf* operon of *E. coli* K-12, but the amino acid identity of each homolog ranged from 45-68%. Downstream of the *hyf*-like operon, we found the genes homologous to the *hycI*, *hydN*, *fdhF*, *fhlA*, and *fdhD* genes and the genes in the *hyp* operon of *E. coli* (Fig. 1A). *V. furnissii* also contain the homologs of these genes, which showed higher sequence similarities to the counterparts of *V. tritonius* than those of *E. coli*, but three genes corresponding to the *hyfDEF* genes were absent in *V. furnissii* (Table 2).

Hyp proteins have the role of forming the metal center of hydrogenase. Genes responsible to Hyp proteins were found downstream of the *hyf*, *fdhF* and *fhlA* genes (Fig. 1). The vibrio *hyp* genes consisted of six showing similarity to *hypFCDE* (cyanide ligands biosynthesis and iron transfer to the cyanide) and *hypAB* (Ni insertion). The order of the vibrio *hyp* genes was different from that of *E. coli*. Based on these sequence similarities, we refer to the gene cluster of *V. tritonius* as the FHL-Hyp gene cluster.

3.2. Properties of the predicted gene products of hyf-like gene cluster in *Vibrio tritonius*

The HyfA protein of *V. tritonius* (Vt-HyfA; 21.6 kDa) was predicted to contain four conserved motifs, each containing four cysteine residues constituting four [4Fe4S] clusters (Fig. S1). The 16Fe ferredoxins generally function as electron-accepting/donating components in oxido-reduction pathways [8]. The motifs are also found in the HycE protein of *E. coli* (Ec-HycE), which plays a role in electron transport between the FDH-H and Hyc complex [32].

Vt-HyfB (72.6 kDa), Vt-HyfD (51.2 kDa) and Vt-HyfF (56.6 kDa) were predicted to be transmembrane proteins belonging to the protein family *Oxidored_q1* (PF00361), in which NADH-ubiquinone/plastoquinone (complex I) are classified. The molecular function of the proteins belonging to this family was predicted to be the proton translocation across cytoplasmic membrane coupled to ATP synthesis. Vt-HyfBDF showed the high amino acid sequence homology (>56% in average) to Ec-HyfBDF.

Vt-HyfC (34.4 kDa) and Vt-HyfE (23.7 kDa) were predicted to be membrane proteins belonging to the protein families *NADHdh* (PF00146) including NADH dehydrogenase, and *Oxidored_q2* (PF00420) including NADH-ubiquinone/plastoquinone oxidoreductase chain 4L, respectively. Therefore both of the two proteins appear to have an oxidoreductase activity.

Vt-HyfG (66.0 kDa) corresponds to the large subunit of the [NiFe]-hydrogenase. The amino acid sequence alignment of the large subunits of the [NiFe]-hydrogenases belonging to group 4 revealed that two CxxC motifs coordinating the [NiFe] center at the active site were conserved at the N- and C-terminal regions of Vt-HyfG (Fig. 2A). The CxxC motif at the C-terminal region is a part of the DPCxxCxxR motif (Fig. 2A) with a C-terminal extension, which is usually cleaved off by protease [33-34]. As a notable feature of the gene, the start codon of *Vt-hyfG* gene was ATG, whereas the *Ec-hyfG* gene uses GTG [8].

Vt-HyfH (21.6 kDa) contained two CxxCxxCxxxCP motifs which were also found in Ec-HyfF, Ec-HyfH, and Vf-HyfH (Fig. S2). Therefore, Vt-HyfH possesses the two [4Fe4S] clusters and the proposed function of this is to play a role in electron transport. In addition, the CxxCx_nCPxC (n=29-36) motif was also found in Vt-HyfH and CooX of *C. hydrogenoformans* and *R. rubrum*, suggesting the presence of a third iron site.

Vt-HyfI (29.6 kDa) had a CxxCx_nGxCxxxGx_mGCPP (n=61-106, m=24-61) motif typically found in the small subunits of the [NiFe] hydrogenase (Fig. 2B). In addition, a CHGC sequence, which might be related to the active site, was observed at the C-terminal region of HyfI of *V. tritonius* and *V. furnissii* but not at those of Ec-HyfE and Ec-HyfG, and CooH of *C. hydrogenoformans* and *R. rubrum* (Fig. 2B).

Vt-HyfJ (16.5 kDa) shared a 48% amino acid sequence identity with Ec-HyfH, which might

be responsible for the maturation of the large subunit of hydrogenase-3 [35]. *Ec*-HyfJ is also known as the homologue of *Ec*-HycH, sharing a 47% identity, and the functions of HycH and HyfJ remain unknown but might be involved in the maturation of hydrogenase [8].

Vt-HycI (16.8 kDa) showed 53% homology to *Ec*-HycI (Table 1), which is proposed to play an important role of the maturation of HycE corresponding to the large subunit of hydrogenase-3 [34]. The *Vt-hycI* gene is located downstream of *Vt-hyfJ* (Fig. 1A).

3.3 Effects of pH of the medium on the H₂ production and *hyfG* expression in *Vibrio tritonius*

The pH dependency of H₂ production and expression level of *Vt-hyfG* gene, which encodes the large subunit of the [NiFe]-hydrogenase, was examined within the pH range of 5.5-7 in the batch culture (Fig. 3). In the batch culture after 12 hours, the organism produced higher amounts of H₂ per DSW in the medium maintained at a lower pH (Fig. 3A). In the 12 hour-batch culture at pH 7, apparent H₂ production was not detected. The *Vt-hyfG* gene expression also showed similar pH dependency; the expression level at pH 5.5, 6.0, and 6.5 were 1.7, 1.5 and 1.2 folds relative to that at pH 7, respectively (Fig. 3B).

3.4. Transcriptional unit analysis of the FHL-Hyp gene cluster during glucose fermentation

The FHL-Hyp gene cluster of *V. tritonius* consisted of 21 genes, all of which are oriented in the same transcriptional direction (Fig. 1A). In order to determine transcriptional units of the FHL-Hyp gene cluster, a RT-qPCR experiments were performed with a set of primer pairs capable of amplifying various regions of the FHL-Hyp gene cluster and the first-strand cDNA synthesized with two kinds of primers (*fhlART* and *hypBRT* in Fig. 1A). The cDNA synthesized by *hypBRT* contained the regions which were amplified by the primer pairs named as follows; *fhlAF* and *fdhDR*, *hypEF* and *hypDR*, and *hypEF* and *hypBR*, whereas any regions containing the *hyfABCDEFGHII*, *hycI*, *hydN* and *fdhF* genes were not amplified. On the other hand, cDNA synthesized by *fhlRT* contained regions which were amplified by the following primer pairs; *hyfAF* and *hyfDR*, *hyfDF* and *hyfHR*, and *hyfGF* and *fdhFR*, but not *hyf5F* and *hyfCR*. These electrophoretic profiles suggested that at least *hyfABCDEFGHII-hycI-hydN-fdhF* and *hypFCDEAB* were co-transcribed as a single transcript, respectively (Fig. 1B). Two Rho-independent terminator-like sequences were identified just downstream of the 3' end of *Vt-fdhF* and *Vt-hypB* by ARNold finding terminators [26], which corresponded with the transcription results.

4. Discussions

Physiology and biochemistry of the Hyf-type FHL complex compared to the Hyc-type FHLs are still a mystery because of ambiguity of the expression in *E. coli* cells, nevertheless the structural similarity to complex I is proposed [3,15]. The complete genome sequence of *V. tritonius* strain AM2 identified the FHL-Hyp genes cluster responsible for fermentative H₂ production on Chr. 1. More interestingly, the vibrio FHL is classified to Hyf-type FHL on the basis of gene-structural comparison between those of *E. coli* *hyc*, *hyf*, and *V. furnissii* *hyc*. The FHL-Hyp gene cluster consists of 24 kb in length in three parts. The first part consists of ten genes (*Vt-hyfABCDEFGHIIJ*) constituting a Hyf-type FHL (*hyf*). The second part is made up of the four genes encoding a formate dehydrogenase and a transcriptional activator, FhlA which controls the expression of the formate regulon of *E. coli*. The third is made up of six *hyp* genes involved in hydrogenase maturation. These genetic features are most likely to be common among the other strain (MA35) of *V. tritonius* (unpublished data). The vibrio FHL-Hype gene cluster consists of the full set of genes responsible for hydrogenase maturation, FHL complex formation and gene activation machinery, which had been never found in any bacteria until now. Furthermore, we revealed that the *Vt-hyfG* gene is up-regulated in slightly acidic conditions by real-time qPCR (Figs. 1 and 3). Therefore, the Hyf complex may also play a key role in H₂ production of *V. tritonius* in maintaining the pH homeostasis of the bacterium, which was proposed for Hyc complex of *E. coli* [7]. As both the function and the transcriptional regulation of the *Ec-hyf* operon (*Ec-hyfABCDEFGHIIJ*) have not been clarified yet due to the lack of significant enzymatic activities and the gene expressions in the wild-type strain of *E. coli* [8,14,36], the discovery of the active *hyf* genes in *V. tritonius* could greatly contribute to a better understanding of the physiology, biochemistry and mechanisms in the regulation and the function of the *hyf* genes.

Since Andrews *et al.* [8] first discovered the *hyf* operon in the *E. coli* genome in 1997, many hydrogenases have been assigned to group 4, the members of which shares sequence similarities with each of the proteins of the Hyf complex in *E. coli* [5,8,15]. Several proteins, however, could not be clearly identified as [NiFe]-hydrogenases due to the lack of the two conserved CxxC motifs located at the N- and C-terminal regions of the large subunits, which are residues acting as ligands for the [NiFe] center at the active site. For example, the four gene products of a *hyf*-like operon in *Mycobacterium tuberculosis* (Orf2, 3, 4 and 5) share similarities with HyfC (34%), HyfE (24%), HyfBDF (24, 21 and 36%, respectively) and HyfG (31%) of *E. coli*, respectively, but these proteins are not thought to correspond to the [NiFe]-hydrogenase components due to the lack of the CxxC motifs in Orf5 [8]. Likewise, Coppi [37] indicated that

the Ech homologue cluster of *Geobacter sulfurreducens* does not encode the [NiFe]-hydrogenase, and thus designated it to Ehr (Ech-hydrogenase-related). The comparison of the active center of Vt-HyfG protein indicated the *V. tritonius* hydrogenase could be identified as [NiFe] hydrogenase. Vt-HyfBDF are predicted to be the transmembrane proteins related to NADH-ubiquinone oxidoreductase (complex I), and Vt-HyfCD may have an oxidoreductase activity related to proton translocation [38] according to the results of InterPro Scan search [25]. Therefore, *V. tritonius* FHL consisting of the Hyf complex with the FDH-H could be thought to form a FHL-2 homologous to *E. coli*, which was proposed as an energy-conserving FHL coupled to proton translocation [8].

The type strain of *V. furnissii* LMG 7910^T shows a faint H₂ production, and the organization of the FHL-Hyp gene cluster obtained from the complete genome sequence differed markedly from that of *V. tritonius*; only 7 genes (*hyfABCGHIJ*) were identified. *V. furnissii* FHL gene cluster also possessed *fdhF*, *fhlA* and *hyp*, but one extra *hupE*-like gene was found (Fig. 1). The *V. furnissii* FHL is rather similar to the *E. coli* Hyc complex. The *E. coli* Hyf-type gene cluster does not contain *fdhF* and *hyp*, which are replaced by *focB*, a second formate channel protein. Hydrogenase complex showing both activities in H₂ evolution and energy-conservation have been well described in several [NiFe]-hydrogenases belonging to group 4, such as Mbh of *Pyrococcus furiosus* [9,39] and Ech of *Methanosarcina barkeri* [40-41]. Coo of *R. rubrum* and *C. hydrogeniformans* is also energy-conserving [42-43]. A study using the membrane vesicles of *P. furiosus* revealed that Mbh plays a role in proton reduction (H₂ evolution) together with proton translocation [44]. However, there were not found to be any gene structural similarities between those group 4 [NiFe]-hydrogenase and the *V. tritonius* Hyf-type FHL complex gene. Moreover, we could confirm structural similarity between the *V. tritonius* FHL complex consisting of 9 proteins and the complex I consisting of 14 proteins [15]. As vibrios are fast-growing microbes and there are few reports on maintenance of complex I by vibrios, *V. tritonius* could be a promising research material that may provide a lot of information on the physicochemical features of the Hyf complex, the bioenergetics in the group of marine microbes, and the evolutionary linkage to complex I. Recently, an pH dependent H₂ production and a dose-dependent inhibition by glucose of Hyf (Hyd-4) type FHL are also suggested in *E. coli* using *hyf* and/or *fhlA* deletion mutants [45,46]. Elucidation of universal roles in Hyf type FHLs in prokaryotes could also help us to understand the evolutionary link to the complex I.

For the biosynthesis of an active [NiFe]-hydrogenase, several proteins are required for complex maturation processes [35,47]. In the maturation process proposed in *E. coli*, a precursor of the large subunit (pre-HycE), which contains a C-terminal extension, is formed by the 6 Hyp

proteins (HypABCDEF). The *hyp* genes of *V. tritonius* (*Vt-hypFCDEAB*) are co-localized *hyf* genes, *fdhF*, and *fhlA* on the same strand (Fig. 1A and Table 1). The C-terminal extension that contains a DPCxxCxxR motif is usually removed by an endopeptidase, such as HycI and HupD in *E. coli* and *Rhodobacter capsulatus*, respectively, at the final stage of the maturation process [34,35,47,48]. *Vt-HyfG* also possesses the DPCxxCxxR motif (Fig. 2). The presence of the *Vt-hycI* gene and the DPCxxCxxR motif in *Vt-HyfG* suggests that it is plausible to consider that *Vt-HycI* may also be involved in the maturation process of *Vt-HyfG*.

Compared to the formate regulon in *E. coli*, the FHL, Fdh, FhlA, and Hyp gene clusters are co-localized on the vibrio genome (Fig. 1A). RT-qPCR experiments suggested that *hyf* and *hyp* gene clusters are likely to form operon during the mid-logarithmic phase of *V. tritonius* (Fig. 1A). A Rho-independent transcription terminator was predicted to be at the region 43-bp upstream of the 5' end of *Vt-fhlA*, the *E. coli*-homologue which encodes a transcriptional activator of the formate regulon (Fig. 1). Sequence similarity search against the *E. coli* genome sequence failed to identify the UAS (universal activating sequence) for the formate regulon [48] (data not shown). The absence of a gene homologous to the *E. coli hycA* gene, which encodes a negative regulator of the formate regulon, has also been pointed out in *V. tritonius* [14]. Analyses of 24 kb-long transcript were rather difficult both by cDNA mapping and northern analysis. Currently, we are conducting genome-wide transcriptome analyses for this vibrio, which will help us to elucidate the mechanisms for the transcriptional control of the FHL-Hyp gene cluster. The *hyfG* expression measured using by the RT-qPCR with the slight pH dependency (Fig. 3B) were also supported by RNA-Seq analyses (data not shown).

In enterobacteria, formate contributes to their energy metabolism by the workings of various enzymes rather than being simply excreted. The formate metabolism in marine vibrio may differ from those of enterobacteria. In addition to the *fdhF* gene encoding FDH-H in the *Vt*-FHL-Hyp gene cluster on Chr. 1, two genes may encode the proteins with Fdh activities in the *V. tritonius* genome sequences. A gene product (nucleotide position 1782569-1784875 on AP014636) contained a molybdopterin dinucleotide binding domain as in the catalytic subunits of FDH-N and FDH-O of *E. coli*. However, the Fdh protein was predicted to be located in the cytoplasm (the localization scores is 9.97) by PSORTb 3.0, whereas the catalytic subunits of the FDH-N and the FDH-O of *E. coli* are located on the periplasm (the localization scores are 10). On the other hand, a *fdh*-like gene (nucleotide position 2586173-2588029 on AP014635) appears to be a homologue of the NAD(P)-dependent formate dehydrogenase because it contains a domain of pyridine nucleotide-disulphide oxidoreductase responsible for NADH binding [49]. The protein was also predicted to be located on the periplasmic side (the localization scores is

9.97). The levels of H₂ production and the expression of the *hyfG* gene in *V. tritonius* were higher in slightly acidic conditions than in neutral conditions (Fig. 3), whereas the cell growth of *V. tritonius* showed the opposite [17]. These results suggest that the FHL of *V. tritonius* might contribute to the disposal of the cytoplasmic formate in order to avoid not only the critical acidification of the cytoplasm but also to conserve energy in unfavorable environments, such as under acidic conditions where cell growth is significantly repressed.

5. Conclusions

A 24-kb gene cluster required for the formation of FHL consisting of Hyf complex was identified on Chr. 1 of *V. tritonius* AM2. The results of sequence similarity analysis suggested that the Hyf complex of *V. tritonius* constitute an energy-conserving [NiFe]-hydrogenase. *hyfABCDEFGHIJ-hycI-hydN-fdhH* and *hypFCDEAB* were likely to be co-transcribed as a single transcript, respectively. The *hyfG* gene was up-regulated under slightly acidic conditions, which is likely to correspond to the H₂ production pattern. Therefore, the Hyf complex may be the key enzymes not only in avoiding the critical acidification of the cytoplasm due to the accumulation of formate under acidic conditions but also in the energy metabolism of *V. tritonius*. It is necessary to perform more detailed analysis of gene expression control, and the biochemical features of H₂ production in vibrios, because the gene organization of the FHL-Hyp gene cluster of *V. tritonius* differs significantly from the formate regulon of *E. coli*.

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References

[1] Vignais PM, Billoud B, Meyer J. Classification and phylogeny of hydrogenase. FEMS Microbiol Rev 2001;25:455-501.

444 [2] Vignais PM, Colbeau A. Molecular biology of microbial hydrogenases. Mol Biol
445 2004;6:159-188.

446 [3] McDowall, JS, Murphy, BJ, Haumann, M, Palmer, T, Armstrong, FA, Sargenta, F. Bacterial
447 formate hydrogenlyase complex. Proc Natl Acad Sci USA 2015; in press.
448 doi/10.1073/pnas.1407927111.

449 [4] Nicolet Y, Lemon BJ, Fontecilla-Camps JC, Peters JW. A novel FeS cluster in Fe-only
450 hydrogenases. Trends Biochem Sci 2000;25:138-143.

451 [5] Vignais PM, Billoud B. Occurrence, classification, and biological function of hydrogenases:
452 an overview. Chem Rev 2007;107:4206-4272.

453 [6] Nakashimada Y, Rachman MA, Kakizono T, Nishio N. Hydrogen production of *Enterobacter*
454 *aerogenes* altered by extracellular and interacellular redox states. Int J Hydrogen Energy
455 2002;27:1399-1405.

456 [7] Noguchi K, Riggins DP, Eldahan KC, Kitko RD, Slonczewski JL. Hydrogenase-3 contributes
457 to anaerobic acid resistance of *Escherichia coli*. PLoS One 2010;5:e10132.

458 [8] Andrews SC, Berks BC, McClay J, Ambler A, Quail MA, Golby P, Guest JR. A 12-cistron
459 *Escherichia coli* operon (*hyf*) encoding a putative proton-translocating formate hydrogenlyase
460 system. Microbiology 1997;143:3633-3647.

461 [9] Sapra R, Verhagen MFJM, Adams MWW. Purification and characterization of a
462 membrane-bound hydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. J
463 Bacteriol 2000;182:3423-3428.

464 [10] Kulkarni G, Kridelbaugh DM, Guss AM, Metcalf WW. Hydrogen is a preferred intermediate
465 in the energy-conserving electron transport chain of *Methanosarcina barkeri*. Proc Natl Acad Sci
466 USA 2009;115:15915-15920.

467 [11] Axley MJ, Grahame DA, Stadtman TC. *Escherichia coli* formate-hydrogen lyase. J Biol
468 Chem 1990;265:18213-18218.

469 [12] Sawers G. The hydrogenases and formate dehydrogenases of *Escherichia coli*. Antonie van
470 Leeuwenhoek 1994;66:57-88.

471 [13] Sawers RG. Formate and its role in hydrogen production in *Escherichia coli*. Biochem Soc
472 Trans 2005;33:42-46.

473 [14] Skibinski DAG, Golby P, Chang YS, Sargent F, Hoffman R, Harper R, Guest JR, Attwood
474 MM, Berks BC. Regulation of the hydrogenase-4 operon of *Escherichia coli* by the σ
475 54-dependent transcriptional activators FhlA and HyfR. J Bacteriol 2002;184:6642-6653.

476 [15] Marreiros, BC, Batista, AP, Duarte, AMS, Pereira, MM. A missing link between complex I
477 and group 4 membrane-bound [NiFe] hydrogenases. Biochem Biophys Acta.

2013;1827:198-2009.

[16] Sawabe, T, Ogura, Y, Matsumura, Y, Feng, G, Amin, AKMR, Mino, S, Nakagawa, S, Sawabe, T, Kumar, R, Fukui, Y, Satomi, M, Matsushima, R, Thompson, FL, Gomez-Gil, B, Christen, R, Maruyama, F, Kurokawa, K, Hayashi, T. Updating the vibrio clades defined by multilocus sequence phylogeny: proposal of eight ne clades, and the description of *Vibrio tritonius* sp. nov. *Frontier Microbiol* 2013;4:414.

[17] Matsumura, Y, Sato, K, Al-saari, N, Nakagawa, S, Sawabe, T. Enhanced hydrogen production by a newly described heterotrophic marine bacterium, *Vibrio tritonius* strain AM2, using seaweed as the feed stock. *Int J Hyd Energy* 2014; 39:7270-7277.

[18] Ruimy R, Breittmayer V, Elbaze P, Lafay B, Boussemart O, Gauthier M, Christen R. Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. *Int J Syst Bacteriol* 1994;44:416-426.

[19] Lux TM, Lee R, Love J. Complete genome sequence of a free-living *Vibrio furnissii* sp. nov. strain (NCTC 11218). *J Bacteriol* 2011;193:1487-1488.

[20] Noguchi H, Taniguchi T, Itoh T. MetaGeneAnnotator: detecting species-specific patterns of ribosomal binding site for precise gene prediction in anonymous prokaryotic and phage genomes. *DNA Res* 2008;15:387-396.

[21] Sugawara H, Ohyama A, Mori H, Kurokawa K. Microbial Genome Annotation Pipeline (MiGAP) for diverse users. *Abstr. 20th Int Conf Genome Inf abstr* 2009;S001:1-2

[22] Delcher AL, Harmon D, Kasif S, White O, Salzberg SL. Improved microbial gene identification with GLIMMER. *Nucl Acids Res* 1999;27:4636-4641.

[23] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673-4680.

[24] Larkin MA, Blackshields G, Brown NP, *et al.* Clustal W and Clustal X version 2.0. *Bioinformatics* 2007;23:2947-2948.

[25] Hunter S, Jones P, Mitchell A, *et al.* InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acids Res* 2011;16:306-312.

[26] Naville M, Ghuillot-Gaudeffroy A, Marchais A, Gautheret D. ARNold: a web tool for the prediction of Rho-independent transcription terminators. *RNA Biol* 2011;8:11-13.

[27] Hirokawa T, Chieng SB, Mitaku S. SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* 1998;14:378-379.

[28] Yu NY, Wagner JR, Laird MR, *et al.* PSORTb 3.0: improved protein subcellular localization

512 prediction with refined localization subcategories and predictive capabilities for all prokaryotes.
 513 Bioinformatics 2010;26:1608-1615.

514 [29] Gao F, Zhang C-T. Ori-Finder: a web-based system for finding *oriCs* in unannotated
 515 bacterial genomes. BMC Bioinformatics 2008;79:1

516 [30] Heidelberg JF, Eisen JA, Nelson WC, *et al.* DNA sequence of both chromosomes of the
 517 cholera pathogen *Vibrio cholera*. Nature 2000;406:477-484.

518 [31] Egan ES, Waldor MK. Distinct replication requirements for the two *Vibrio cholerae*
 519 chromosomes. Cell 2003; 114:521-530.

520 [32] Sauter M, Böhm R, Böck A. Mutational analysis of the operon (*hyc*) determining
 521 hydrogenase 3 formation in *Escherichia coli*. Mol Microbiol 1992;6:1523-1532.

522 [33] Rossmann R, Sauter M, Lottspeich F, Böck A. Maturation of the large subunit (HYCE) of
 523 *Escherichia coli* hydrogenase 3 requires nickel incorporation followed by C-terminal processing
 524 at Arg537. Eur J Biochem 1994;220:377-384.

525 [34] Rossmann R, Maier T, Lottspeich F, Böck A. Characterisation of a protease from
 526 *Escherichia coli* involved in hydrogenase maturation. Eur J Biochem 1995;227:545-550.

527 [35] Casalot L, Rousset M. Maturation of the [NiFe] hydrogenases. Trends Microbiol
 528 2001;9:228-237.

529 [36] Self WT, Hasona A, Shanmugam KT. Expression and regulation of a silent operon, *hyf*,
 530 coding for hydrogenase 4 isoenzyme in *Escherichia coli*. J Bacteriol 2004;186:580-587.

531 [37] Coppi MV. The hydrogenases of *Geobacter sulfurreducens*: a comparative genomic
 532 perspective. Microbiol 2005;151:1239-1254.

533 [38] Treberg JR, Brand MD. A model of the proton translocation mechanism of complex I. J Biol
 534 Chem 2011;286:17579-17584.

535 [39] Ma K, Schicho RN, Kelly RM, Adams MWW. Hydrogenase of the hyperthermophile
 536 *Pyrococcus furiosus* is an elemental sulfur reductase or sulfhydrogenase: evidence for a
 537 sulfur-reducing hydrogenase ancestor. Proc Natl Acad Sci USA 1993;90:5341-5344.

538 [40] Kunkel A, Vorholt JA, Thauer RK, Hedderich R. An *Escherichia coli* hydrogenase-3-type
 539 hydrogenase in methanogenic archaea. Eur J Biochem 1998;252:467-476.

540 [41] Meuer J, Bartoschek S, Koch J, Kunkel A, Hedderich R. Purification and catalytic
 541 properties of Ech hydrogenase from *Methanosarcina barkeri*. Eur J Biochem 1999;265:325-335.

542 [42] Fox JD, Kerby RL, Roberts GP, Ludden PW. Characterization of the CO-induced,
 543 CO-tolerant hydrogenase from *Rhodospirillum rubrum* and the gene encoding the large subunit
 544 of the enzyme. J Bacteriol 1996;178:1515-1524.

545 [43] Soboh B, Linder D, Hedderich R. Purification and catalytic properties of a

CO-oxidizing:H₂-evolving enzyme complex from *Carboxythermus hydrogenoformans*. *Eur J Biochem* 2002;269:5712-5721.

[44] Sapra R, Bagramyan K, Adams MWW. A simple energy-conserving system: proton reduction coupled to proton translocation. *Proc Natl Acad Sci USA* 2003;100:7545-7550.

[45] Trchounian K, Trchounian A. *Escherichia coli* hydrogenase 4 (*hyf*) and hydrogenase 2 (*hyb*) contribution in H₂ production during mixed carbon (glucose and glycerol) fermentation at pH 7.5 and pH 5.5. *Int J Hyd Energy* 2014;38:3921-3929.

[46] Trchounian K, Trchounian A. Hydrogen producing activity by *Escherichia coli* hydrogenase 4 (*hyf*) depends on glucose concentration. *Int J Hyd Energy* 2014;39:16914-16918.

[47] Sybirna K, Bottin H. New insights into the systems for heterologous synthesis and maturation of hydrogenases, the most efficient biohydrogen producers. *Int J Hyd Energy* 2013;38:13164-13171.

[48] Sawers RG, Blokesch M, Böck A. Anaerobic formate and hydrogen metabolism. In Curtiss R, III, Kaper JB, Squires CL, Karp PD, Neidhardt FC, Slauch JM, eds. *Escherichia coli* and *Salmonella*. ASM Press, Washington DC. www.ecosal.org. doi: 10.1128/ecosal.3.5.4. ASM Press. 2004.

[49] Popov VO, Lamzin VS. NAD⁺-dependent formate dehydrogenase. *Biochem J* 1994;301:625-643.

Figure Legends

Fig. 1. The gene organization (A) and the transcriptional unit analysis of *Vibrio tritonius* FHL-Hyp gene cluster (B). (A) The location of a predicted terminator was indicated by a hairpin loop symbol. (B) cDNA was synthesized by fhlRT1 and hypBRT primer. Primer pairs for amplifying intergenic regions are as follows: hyf5F and hyfCR in lanes 1, 2 and 3; hyfAF and hyfDR in lanes 4, 5, and 6; hyfDF and hyfHR in lanes 7, 8 and 9; hyfGF and fdhFR in lanes 10, 11 and 12; fhlAF and fdhDR in lanes 13, 14 and 15; hypFF and hypDR in lanes 16, 17 and 18; hypEF and hypBR in lanes 19, 20 and 21; hypDF and hyp3R in lanes 22, 23 and 24. Positive PCR control reactions using the genome DNA template, the PCR reactions using cDNA as template, and negative control reactions using the total RNA appeared in lanes 1, 4, 7, 10, 13, 16, 19 and 22, in lanes 2, 5, 8, 11, 14, 17, 20 and 23, and in lanes 3, 6, 9, 12, 15, 18, 21 and 24, respectively. PCR cycle was set to 40 cycles in some reactions corresponding to the lanes 13-21. Before applying samples, some samples was diluted in the ratios as follow: 1/15 for the PCR samples in lanes 1, 4, 7, 10 and 22; 1/3 for those in lanes 5, 8 and 11; 1/20 for those in lanes 13, 16, 17, 19 and 20, and 1/25 for that in lane 14.

580

581 Fig. 2. Multiple amino acid sequence alignments of *Vt*-HyfG (A) and *Vt*-HyfH (B) encoding the

582 large and small subunit of the [NiFe]-hydrogenases, respectively: (A) Conserved amino acids in

583 [NiFe(CO)(CN)₂] cluster and C-terminal cleavage site are indicated by numbers 1 and 2, and 3,

584 respectively. The amino acid sequences used in the alignment are as follows: *V. furnissii* HyfG

585 (ADT87208, large subunits of hydrogenase complex); *E. coli* HycE (P16431, large subunit of

586 Hyc complex); *E. coli* HyfG (P77329, large subunit of Hyf complex); *C. hydrogenoformans*

587 CooH (ABB14885, CO-induced hydrogenase large subunits); *R. rubrum* CooH (AAC45121,

588 CO-induced hydrogenase large subunits); *E. coli* NuoD (BAA16115, NuoD subunit of Nuo

589 complex). (B) Conserved amino acids in [4Fe4S] ligand and possible additional iron site are

590 indicated by numbers 1 and 2, respectively. The amino acid sequences used in the alignment are

591 as follows: *V. furnissii* HyfI (ADT87206, small subunits of hydrogenase complex); *E. coli* HycG

592 (P16433, small subunit of Hyc complex); *E. coli* HyfI (P77668, small subunit of Hyf complex);

593 *C. hydrogenoformans* CooL (Q3AB34, CO-induced hydrogenase small subunits); *R. rubrum*

594 CooL (AAC45118, CO-induced hydrogenase small subunits); *E. coli* NuoB (BAA16121, Nuo

595 subunit of Nuo complex).

596

597 Fig. 3. Effects of pH of the medium on the H₂ production (A) and the expression of *hyfG* gene

598 (B) of *Vibrio tritonius* strain AM2. *V. tritonius* strain AM2 was batch cultured at 37°C for 12

599 hours using the synthetic media adjusted at three pH conditions. (A) The cumulative H₂

600 production after 12 hour culture was measured in triplicate samples (error bars represent SD).

601 Statistical significance ($P < 0.05$) is indicated by alphabet. (B) The expression folds of the *hyfG*

602 gene were calculated comparing those obtained under pH 7 conditions after normalizing with the

603 expression levels of two reference genes (*proS* and the *pyrH*) ($n=1$).

604

Fig. 1. Matsumura et al.

E.coli HycE
E.coli HyfG

[illegible]

(B) Hvfl

Rubrum

C.hydrogenofmans Cool.

V.tritonus Hyfl

V.furnissii Hyfl

E.coli HycG

E.coli Hyfl

E.coli NuoB

Rubrum

C.hydrogenofmans Cool.

V.tritonus Hyfl

V.furnissii Hyfl

E.coli HycG

E.coli Hyfl

E.coli NuoB

Rubrum

C.hydrogenofmans Cool.

V.tritonus Hyfl

V.furnissii Hyfl

E.coli HycG

E.coli Hyfl

E.coli NuoB

Rubrum

C.hydrogenofmans Cool.

V.tritonus Hyfl

V.furnissii Hyfl

E.coli HycG

E.coli Hyfl

E.coli NuoB

Fig. 2. Matsumura et al.

Fig. 3. Matsumura et al.

Table 1. Predicted function and characteristics of gene products encoded by FHL-Hyp gene cluster of *Vibrio tritonius*.

Gene product	Molecular Mass	Predicted function	Predicted membrane helices	Amino acid sequence identity		Predicted localization
<i>Vt</i>	<i>Vt</i> / <i>Ec</i> (kDa)		<i>Vt</i> / <i>Ec</i>	<i>Vt</i> / <i>Ec</i> (%)	<i>Vt</i> / <i>Vf</i> (%)	<i>Vt</i> / <i>Ec</i>
HyfA	21.6 / 22.2	Electron carrier activity	nd	50	78	Ct / Ct
HyfB	72.6 / 72.6	ATP synthesis coupled electron transport	17 / 16	53	50	CM / CM
HyfC	34.4 / 34.4	Oxidoreductase activity	8 / 8	57	69	CM / CM
HyfD	51.2 / 51.8	ATP synthesis coupled electron transport	12 / 13	62	nd	CM / CM
HyfE	23.7 / 23.4	Oxidoreductase activity	5 / 6	52	nd	CM / CM
HyfF	56.6 / 56.8	ATP synthesis coupled electron transport	13 / 12	53	nd	CM / CM
HyfG	66.0 / 63.4	Hydrogenase activity (Large subunit)	nd	68	84	Ct / Ct
HyfH	21.6 / 20.1	Electron carrier activity	nd	55	80	Ct / CM
HyfI	29.6 / 28.1	Hydrogenase activity (Small subunit)	nd	64	82	- / -
HyfJ	16.5 / 15.6	Formate hydrogenlyase maturation protein	nd	45	74	- / CM
HycI	16.8 / 17.1	Processing of C-terminal end of HycE (and HyfG?*)	nd	53	70	- / -
HydN	19.3 / 19.0	Electron transport protein***	nd	54	78	CM / Ct
FdhH	79.6 / 79.4	Foemate dehydrogenase	nd	66	78	Ct /
FhlA	76.0 / 78.5	Transcriptional activator	nd	41	58	Ct / Ct
FdhD	30.5 / 30.6	Required for formate dehydrogenase activity	nd	52	59	Ct / Ct
HypF	92.0 / 82.1	CN/CO delivery**	nd	38	55	CM / Ct
HypC	9.3 / 9.7	Chaperone/maturation**	nd	35	64	- / -
HypD	41.4 / 41.4	Ni incorporation/maturation**	nd	46	74	Ct / Ct
HypE	35.5 / 33.7	Purine derivative binding ? **	nd	46	67	Ct / Ct
HypA	12.2 / 13.2	Ni incorporation/maturation**	nd	32	60	Ct / Ct
HypB	43.3 / 31.6	Nickelin/Ni insertion**	nd	67	79	Ct / Ct

Vt: *Vibrio tritonius*, *Ec*: *Escherichia coli*, *Vf*: *Vibrio furnissii*. Methods for predictions of protein function and characteristics were described in Materials and Methods (see section 2.3).