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Genetic analyses of the functions of [NiFe]-hydrogenase maturation endopeptidases in the hyperthermophilic archaeon *Thermococcus kodakarensis*

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Abstract The maturation of [NiFe]-hydrogenases requires a number of accessory proteins, which include hydrogenase-specific endopeptidases. The endopeptidases carry out the final cleavage reaction of the C-terminal regions of [NiFe]-hydrogenase large subunit precursors. The hyperthermophilic archaeon *Thermococcus kodakarensis* harbors two [NiFe]-hydrogenases, a cytoplasmic Hyh and a membrane-bound Mbh, along with two putative hydrogenase-specific endopeptidase genes. In this study, we carried out a genetic examination on the two endopeptidase genes, TK2004 and TK2066. Disruption of TK2004 resulted in a strain that could not grow under conditions requiring hydrogen evolution. The Mbh large subunit precursor (pre-MbhL) in this strain was not processed at all whereas Hyh cleavage was not affected. On the other hand, disruption of TK2066 did not affect the growth of *T. kodakarensis* under the conditions examined. Cleavage of the Hyh large subunit precursor (pre-HyhL) was impaired, but could be observed to some extent. In a strain lacking both TK2004 and TK2066, cleavage of pre-HyhL could not be observed. Our results indicate that pre-MbhL cleavage is carried out solely by the endopeptidase encoded by TK2004. Pre-HyhL cleavage is mainly carried out by TK2066, but TK2004 can also play a minor role in this cleavage.

Keywords Hydrogenase maturation endopeptidase · Hydrogenase maturation · Hydrogenase · *Thermococcus* · Archaea

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Introduction

Hydrogenase catalyzes the reversible oxidation of molecular hydrogen (H_2), and plays a central role in the biological utilization/production of H_2. There are two major classes of hydrogenases, the [NiFe]-hydrogenase and the [FeFe]-hydrogenase, along with the [Fe]-hydrogenase found in some methanogens (Vignais et al. 2001; Vignais and Billoud 2007; Thauer et al. 2010). [NiFe]-hydrogenases vary in subunit composition depending on the species and function of the enzyme, but commonly include two subunits, designated the large and the small subunit. The large subunit contains a binuclear catalytic center consisting of Ni and Fe atoms (Volbeda et al. 1995). The Ni atom is coordinated with four cysteine residues, and two of them also coordinate the Fe atom. The Fe atom also carries three diatomic ligands, two molecules of cyanides (CN) and one molecule of carbon monoxide (CO). This unique catalytic center in the large subunit is formed post-translationally through the functions of several accessory proteins called Hyp proteins (Lutz et al. 1991; Dernedde et al. 1996; Casalot and

Rousset 2001). This 'maturation' process includes binding with specific chaperones that assist in the insertion of Ni and Fe cations and formation and coordination of diatomic ligands (Casalot and Rousset 2001; Watanabe et al. 2012). After formation of the catalytic center, the carboxy-terminal region of the large subunit is cleaved by hydrogenase-specific maturation endopeptidases (Rossmann et al. 1995). This proteolytic cleavage triggers conformational change of the large subunit by which the newly synthesized catalytic center is buried in the protein.

Most of our knowledge on these hydrogenase-specific maturation endopeptidases is based on studies on the enzymes from *Escherichia coli*. *E. coli* has three [NiFe]-hydrogenases (hydrogenases 1–3) along with hydrogenase 4, whose physiological function is not fully understood (Noguchi et al. 2010). Hydrogenase 1 and hydrogenase 2, both of which are located at the periplasmic side of the plasma membrane, function in H_2 oxidation. On the other hand, hydrogenase 3, which is located at the cytoplasmic side of the plasma membrane and is a component of formate hydrogen lyase, functions in the reduction of H^+ to produce H_2 (Vignais and Billoud 2007). Each hydrogenase operon contains one ORF encoding a maturation endopeptidase (*hyaD*, *hybD* and *hycI* for the operons of hydrogenase 1, 2 and 3, respectively). These enzymes belong to the group of aspartic peptidases and are classified into the family A31, according to the MEROPS peptidase database (http://merops.sanger.ac.uk/index.shtml). More specifically, *Ec*-HyaD, *Ec*-HybD and *Ec*-HycI peptidases fall into the sub-groups A31.002, A31.001 and A31.003, respectively. *Ec*-HyaD and *Ec*-HybD display relatively higher similarity to each other (41 % identical) than the other pairs that compare *Ec*-HycI with *Ec*-HyaD or *Ec*-HybD (less than 27 % identical). Biochemical characterization of the hydrogenase 3 maturation processes indicated that the C-terminal processing of pre-HycE (precursor of the large subunit of hydrogenase 3) requires the insertion of the nickel ion to the catalytic center, suggesting that the maturation endopeptidase recognizes the presence of the nickel ion in the catalytic center prior to cleavage (Theodoratou et al. 2000). Structural analyses also support this view: the crystal structure of *Ec*-HybD identified three amino acid residues (Glu^{16}, Asp^{62} and His^{93}) that were suggested to coordinate to the nickel ion (Volbeda et al. 1995). These amino acids are conserved in *Ec*-HyaD (Glu^{19}, Asp^{65} and His^{96}) and *Ec*-HycI (Asp^{16}, Asp^{62} and His^{90}), as well as in the maturation endopeptidases of other organisms, suggesting that this process of nickel recognition is common to this family.

Thermococcus kodakarensis (previously reported as *T. kodakaraensis*) is a hyperthermophilic archaeon isolated from Kodakara Island, Kagoshima, Japan (Morikawa et al. 1994; Atomi et al. 2004). This organism belongs to the euryarchaeal order of Thermococcales, along with members of *Pyrococcus* and *Palaeococcus*. *T. kodakarensis* is an anaerobic heterotroph that grows well in the presence of proteinaceous substrates coupled with the reduction of elemental sulfur (S^0), forming H_2S. *T. kodakarensis* can also grow in the absence of S^0 when pyruvate or carbohydrates such as starch or maltodextrin is present. In these cases, protons are the terminal electron acceptor, resulting in the generation of H_2. The relatively high potential of this organism for H_2 production has been suggested (Kanai et al. 2005). Like other members of Thermococcales, there are three types of [NiFe]-hydrogenase orthologs in *T. kodakarensis*. Among them, two orthologs, the cytosolic Hyh (encoded by *hyhBGSL*: TK2072–TK2069) (Kanai et al. 2003) and the membrane-bound Mbh (encoded by *mbhABCDEFGHIJKLMN*: TK2080–TK2093) are involved in H_2 consumption and evolution processes, respectively, and exhibit hydrogenase activities, while the membrane-bound Mbx (encoded by *mbxABCDFGHH'MJKLN*: TK1226–TK1214) is involved in sulfur reduction, and seems to have no hydrogenase activity (Kanai et al. 2011). As in the case of pre-HycE, cleavage of the Mbh large subunit precursor in *T. kodakarensis* is also dependent on the insertion of the nickel cation (Sasaki et al. 2013). In *T. kodakarensis*, HypA (encoded by TK2008) and a novel Mrp/MinD family ATPase-type HypB (TK2007) constitute an ATP-dependent nickel cation acquisition cycle for [NiFe]-hydrogenase maturation (Sasaki et al. 2013; Watanabe et al. 2015).

In the genome of *T. kodakarensis*, there are two orthologs of [NiFe]-hydrogenase-specific maturation endopeptidases, TK2004 and TK2066, which are 20.3 % identical to each other in terms of primary structure. A previous study from the group of Michael Adams described the heterologous expression of a cytosolic hydrogenase from *Pyrococcus furiosus* (SHI) in *E. coli* (Sun et al. 2010). This enzyme can be considered the counterpart of Hyh in *T. kodakarensis*. In the study, they found that co-expression of PF0975 (a TK2066 homolog) resulted in higher levels of hydrogenase activity in the *E. coli* cell-free extracts, compared to those observed when co-expressing PF0617 (a TK2004 homolog), suggesting that the endopeptidase encoded by PF0975 better recognizes the SHI precursor, at least in *E. coli*.

To obtain genetic evidence to clarify the physiological roles of the two [NiFe]-hydrogenase-specific maturation endopeptidases in *T. kodakarensis*, here we constructed gene knockout mutants of either one or both enzymes in *T. kodakarensis*. The results obtained in this study allow us to propose the physiological roles of the two endopeptidases in this organism and other members of the Thermococcales.

Table 1 *T. kodakarensis* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
T. kodakarensis strains		
KOD1	Wild type	Morikawa et al. (1994), Atomi et al. (2004)
KU216	KOD1 Δ*pyrF*	Sato et al. (2005)
KU216-ST	KU216 *hyhL-ST*	This study
ΔHYD4-ST	KU216 *hyhL-ST* ΔTK2004	This study
ΔHYD6-ST	KU216 *hyhL-ST* ΔTK2066	This study
ΔHYD46-ST	KU216 *hyhL-ST* ΔTK2004 ΔTK2066	This study
Plasmids		
pUC118	Amp^r general cloning vector	Takara Bio (Otsu, Japan)
pUD3	pUC118 derivative; *pyrF* marker cassette	Yokooji et al. (2009)
pUD3_2069ST	pUD3 derivative; *hyhL-ST*	This study
pUD3_2004D	pUD3 derivative; ΔTK2004	This study
pUD3_2066D	pUD3 derivative; ΔTK2066	This study
pLC70	*E. coli–T. kodakarensis* shuttle vector	Santangelo et al. (2008)
pLC2004	pLC70 derivative; P_{TK2008}-TK2004	This study
pLC2066	pLC70 derivative; P_{TK2072}-TK2066	This study

Materials and methods

Microorganisms and culture conditions

Escherichia coli DH5α was used for general DNA manipulation and sequencing. *E. coli* strains were cultivated in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl) at 37 °C. Ampicillin was added to the medium at a concentration of 100 μg mL^{-1}.

Thermococcus kodakarensis strains and plasmids used in this study are listed in Table 1. *T. kodakarensis* strains were cultivated under strictly anaerobic conditions at 85 °C in a nutrient-rich medium (ASW-YT) or a synthetic medium (ASW-AA). ASW-YT medium, composed of 0.8 × artificial seawater (0.8 × ASW), 5.0 g L^{-1} yeast extract and 5.0 g L^{-1} tryptone, was supplemented with 2 g L^{-1} elemental sulfur or 5 g L^{-1} sodium pyruvate prior to inoculation (ASW-YT-S^0 medium or ASW-YT-Pyr medium, respectively). ASW-AA medium consisted of 0.8 × ASW, a mixture of 20 amino acids, modified Wolfe's trace minerals, a vitamin mixture, and 2.0 g L^{-1} of elemental sulfur (Robb and Place 1995; Sato et al. 2003). In all cases, resazurin was added at a concentration of 0.8 mg L^{-1}, and, prior to inoculation, Na_2S $9H_2O$ was added to the medium until it became colorless. Unless mentioned otherwise, all chemicals were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Construction of a plasmid to introduce Strep-tag II at the carboxy-terminus of HyhL

A *T. kodakarensis* strain that expresses a modified HyhL (encoded by TK2069) that contains two Strep-tag II sequences in tandem flanked with flexible linkers (GGGSWSHPQFEK-GGGSGGGSGGSAWSHPQFEK, underlines indicate Strep-tag II sequences) at its carboxy-terminus was constructed. Plasmid construction was carried out as follows. A DNA region (~2.0 kbp) containing the stop codon of TK2069 was amplified with the primers 2069_F/2069_R. All primer sequences used in this study are listed in Table 2. The amplified fragment, after digestion with BamHI and SalI, was inserted into the respective sites of pUD3, that contains a *pyrF* marker gene cassette inserted in the ApaI site of pUC118 (Yokooji et al. 2009), and designated pUD3_2069. To introduce the C-terminal peptide sequence, inverse PCR was performed with pUD3_2069 (as a template) and the primer set HyhL-Strept-f/HyhL-Strept-r. The amplified fragment was self-ligated to generate pUD3_2069ST. The absence of unintended mutations was confirmed by nucleotide sequencing.

Construction of disruption plasmids for TK2004 and TK2066

To construct gene disruption plasmids for TK2004 or TK2066, each gene was amplified with its 5′- and 3′-flanking regions (~1 kbp) using the primer set 2004_F/2004_R

Table 2 Sequences of primers used in this study

Name	Sequences (5' -> 3')
2069_F	AAAAGGATCCCCACGCACTCCATCTGTATC
2069_R	AAAAGTCGACCTCTCTTCAGCCTCTCATCG
HyhL-Strept-r	ACTTCCTCCTCCTTTTTCAAATTGTGGATGGCTCCAACTTCCTCCTCCAAGCCTCGCCACGTGCACTG
HyhL-Strept-f	GGAGGAGGAAGCGGAGGAAGCGCATGGAGTCACCCACAGTTCGAGAAGTAGCCTCCCAACTTTTTTAAGT
TK2069_S_3F	CCGCGTGGAGTGCTCGTTTA
TK2069_S_4R	AGGACAGAACTGTCAATCAA
2004_F	AAAAGGATCCTCGCCGAGGAGTTCGGCGTT
2004_R	AAAAGTCGACCCAGGAACTGCAAGGCACAT
2004_I_R	TATTCAATACCCCCTGAAAATTCC
2004_I_F	GAAAGCTATTTAAAACTCCG
TK2004_out_F	TCCTCAAAGAGGAAGGACAC
TK2004_out_R	GGCTTGGTATCTGGCATTAG
TK2004_in_F	CCTTGAAGATGTCTTCTCCG
TK2004_in_R	TCAGAATCTCCGCAATCAGC
TK2004-A (NotI)	AAGGAATTCAGCGGCCGCTTTCATCCCCTCATTTCGAT
TK2004-B	GGACGCTCATGGTATCACCGAGTTCCTATT
TK2004-C	CGGTGATACCATGAGCGTCCTTGAAGATGT
TK2004-D (SalI)	ACGCGTCGACGATCAAAAATTTTCACGGAG
2066_F	AAAAGGATCCGGCGATAGACCTGATTGACG
2066_R	AAAAGTCGACTCCGATGTCAAAACCGTAGC
2066_I_R	GGCTAACCACCTATGATTTC
2066_I_F	CCCAAAGGTTTATATACTCC
TK2066_out_F	TGAGATCCACCAATCCGTTC
TK2066_out_R	CGAGGAACTTTATCACGTGG
TK2066_in_F	GCAACGAGCTGATGAAAGAC
TK2066_in_R	AGTTTTTCGGCGATCTCGAC
TK2066_A (NotI)	AAGGAATTCAGCGGCCGCAGATATCACCTGCGAGCCCG
TK2066_B	GTACTCTCACCCTTACCACCCCCAATGAAC
TK2066_C	GGTGGTAAGGGTGAGAGTACTCATCCTCGC
TK2066_D (SalI)	ACGCGTCGACACCTAACACCAACTCTTTCA

or 2066_F/2066_R, respectively. The fragments after BamHI and SalI digestion were inserted into the respective sites of pUD3. Inverse PCR was performed to remove the respective coding regions using 2004_I_F/2004_I_R (for the TK2004 disruption vector) or 2066_I_F/2066_I_R (for the TK2066 disruption vector), and the amplified fragments were self-ligated to generate pUD3_2004D or pUD3_2066D. The absence of unintended mutations was confirmed by nucleotide sequencing.

Construction of complementation vectors for TK2004 and TK2066

For re-introduction of TK2004 into gene disruption strains, the TK2004 gene and its promoter region (5′-flanking region of TK2008, 117 bp) were amplified from genomic DNA with TK2004-A/TK2004-B and TK2004-C/TK2004-D, respectively. The amplified fragments were connected by fusion PCR using TK2004-A/TK2004-D. The amplified product and pLC70, a *T. kodakarensis–E. coli* shuttle vector (Santangelo et al. 2008), were cleaved with NotI/SalI and ligated to one another, to generate pLC2004.

For re-introduction of TK2066 into gene disruption strains, the same strategy was taken. The TK2066 gene and its promoter region (5′-flanking of TK2072, 385 bp) were amplified with TK2066-A/TK2066-B, and TK2066-C/TK2066-D, respectively. Fusion PCR was performed with TK2066-A/TK2066-D using the two fragments as templates. The amplified product and pLC70 were cleaved with NotI/SalI and ligated to one another, generating pLC2066.

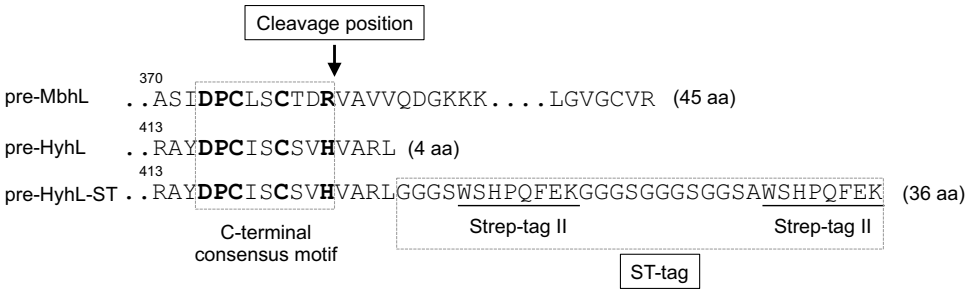


Fig. 1 Amino acid sequences of the carboxy-terminal regions of pre-MbhL (*top*) and pre-HyhL (*middle*). A genetically engineered pre-HyhL derivative (pre-HyhL-ST) that consists of pre-HyhL fused to a tag region that contains two Strep-tag II sequences (ST-tag) is also shown (*bottom*). The C-terminal consensus motif and predicted cleavage positions are indicated. Predicted numbers of amino acid residues that are released following cleavage by maturation endopeptidases are shown in parenthesis

Transformation of *T. kodakarensis*

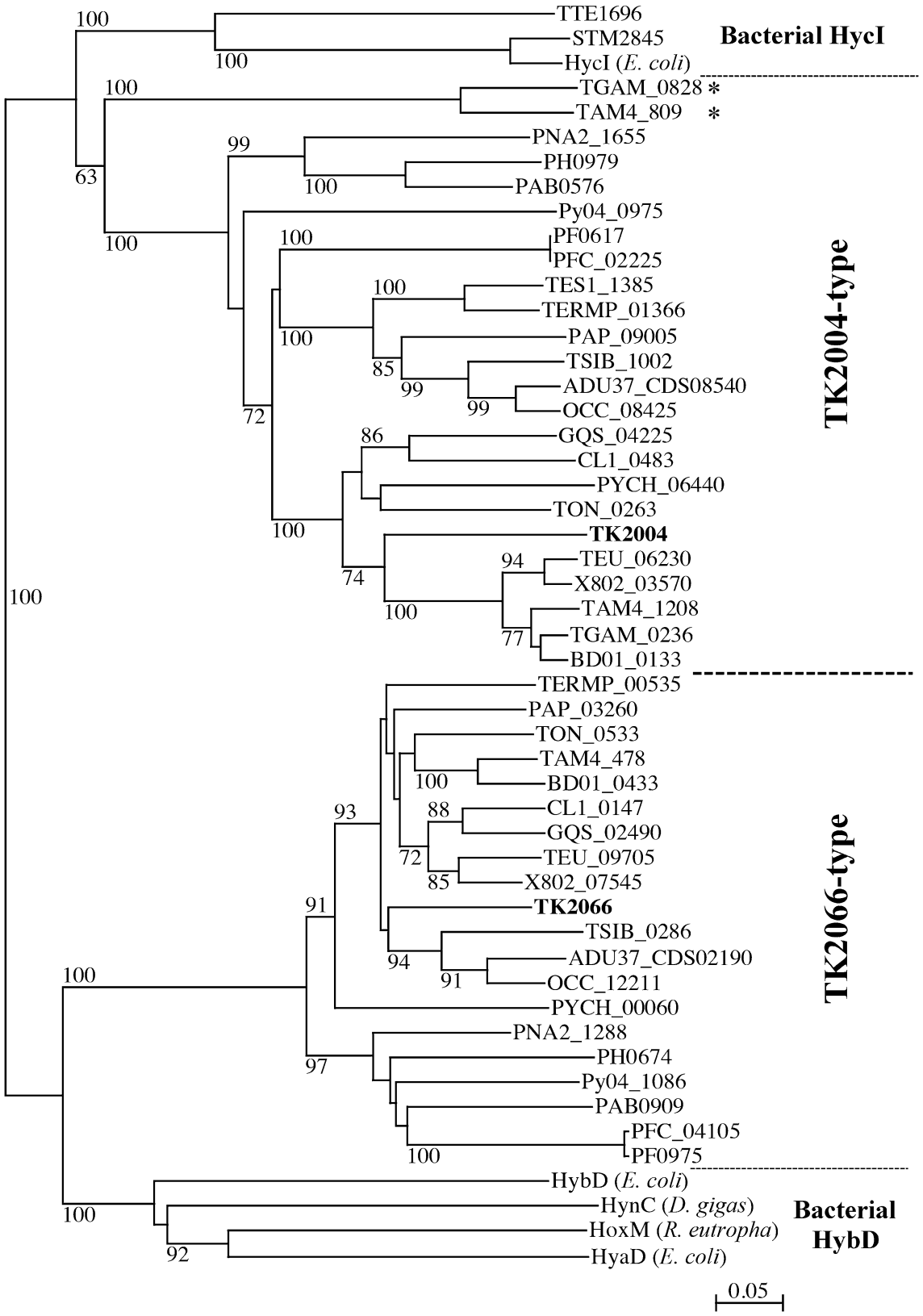
Disruption of specific genes in *T. kodakarensis* was performed by single-crossover homologous recombination. *T. kodakarensis* KU216 (Δ*pyrF*) (Sato et al. 2005) was used as the host to develop a strain that expresses an HyhL protein with Strep-tag II-containing peptides at its carboxy-terminus. For selection and isolation of recombinant strains, ASW-AA was used to enrich transformants that display uracil prototrophy owing to the integration of the plasmid pUD3_2069ST by single crossover recombination. Solid media used in the second step to isolate gene disruption strains were based on ASW-YT medium containing 1.0 g L^{-1} gelrite, 0.4 g L^{-1} polysulfide and 7.5 g L^{-1} 5-fluoroorotic acid (5FOA). Transformation procedures and selection methods based on uracil prototrophy and resistance towards 5FOA are described elsewhere (Sato et al. 2005; Hirata et al. 2008). Genotypes of the isolated transformants were analyzed by PCR with the primer set TK2069_S_3F/TK2069_S_4R. Transformants whose amplified DNA products displayed the expected size were chosen, and relevant sequences were confirmed by direct sequencing. From the transformants, one strain, named as KU216-ST (Δ*pyrF*), was used as the host to further disrupt TK2004 and/or TK2066 by the same method described above. A ΔTK2004 strain deleted of TK2004 (ΔHYD4-ST) using pUD3_2004D, a ΔTK2066 strain deleted of TK2066 (ΔHYD6-ST) using pUD3_2066D, and a double-knockout strain (ΔTK2004/ΔTK2066, ΔHYD46-ST) were constructed. Gene disruption at the TK2004 locus was checked by PCR using two sets of primers; one set (TK2004_out_F/TK2004_out_R) consisted of primers that anneal outside of the recombination regions and the other set (TK2004_in_F/TK2004_in_R) consisted of primers that anneal within TK2004. Similarly, gene disruption at the TK2066 locus was checked by PCR using two sets of primers, TK2066_out_F/TK2066_out_R and TK2066_in_F/TK2066_in_R. Correct gene disruptions were finally confirmed by direct sequencing of the respective regions. To isolate transformants harboring pLC70, pLC2004 or pLC2066, cells were selected based on their resistance towards simvastatin by methods shown elsewhere (Matsumi et al. 2007).

Growth measurements

Growth characteristics of host cells (strain KU216-ST) and mutant cells (ΔHYD4-ST, ΔHYD6-ST and ΔHYD46-ST) were measured as follows. Each strain was precultured in 20 mL of ASW-YT medium with 0.5 % (w/v) S^0 at 85 °C. After preculture, cells were inoculated into 20 mL of ASW-YT-S^0 or ASW-YT-Pyr, and cultured at 85 °C. Cell density was monitored by measuring turbidity at 660 nm at appropriate intervals with a spectrophotometer Miniphoto518R (Taitec, Koshigaya, Japan).

Western blot analysis

The maturation of HyhL and MbhL was analyzed by western blot analysis. KU216-ST, ΔHYD4-ST, ΔHYD6-ST and ΔHYD46-ST cells were grown in ASW-YT-S^0 medium at 85 °C. Cells were harvested by centrifugation (20,000×*g*, 20 min, 4 °C), suspended in 50 mM Tris–HCl (pH 8.0) supplemented with 0.1 % (v/v) Triton X-100, and vigorously shaken by a vortex for 30 min. The mixture was centrifuged (20,000×*g*, 20 min, 4 °C) and the supernatant was used as cell extracts. Protein concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as the standard. The cell extracts (3 μg protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blot analysis using rabbit polyclonal antibodies against recombinant HyhL or MbhL. For the detection of Strep-Tag II, a peroxidase-conjugated Strep-tag II monoclonal antibody, Strep-Tag II antibody HRP conjugate (Merck, Darmstadt, Germany), was used.


TTE1696
STM2845
HycI (E. coli)
Bacterial HycI
TGAM_0828 *
TAM4_809 *
PNA2_1655
PH0979
PAB0576
Py04_0975
PF0617
PFC_02225
TES1_1385
TERMP_01366
PAP_09005
TSIB_1002
ADU37_CDS08540
OCC_08425
GQS_04225
CL1_0483
PYCH_06440
TON_0263
TK2004
TEU_06230
X802_03570
TAM4_1208
TGAM_0236
BD01_0133
TK2004-type
TERMP_00535
PAP_03260
TON_0533
TAM4_478
BD01_0433
CL1_0147
GQS_02490
TEU_09705
X802_07545
TK2066
TSIB_0286
ADU37_CDS02190
OCC_12211
PYCH_00060
PNA2_1288
PH0674
Py04_1086
PAB0909
PFC_04105
PF0975
TK2066-type
HybD (E. coli)
HynC (D. gigas)
HoxM (R. eutropha)
HyaD (E. coli)
Bacterial HybD
0.05

◂**Fig. 2** A phylogenetic tree of [NiFe]-hydrogenase maturation endopeptidase homolog sequences from members of Thermococcales and selected bacterial species. The tree was constructed by the neighbor-joining method. The proteins used were those from *E. coli* (HyaD, HybD and HycI), *Ralstonia eutropha* H16 (HoxM encoded by PHG005), *Desulfovibrio gigas* (HynC encoded by DGI_2260), *Thermoanaerobacter tengcongensis* (TTE1696), *Salmonella enterica* subsp. enterica serovar Typhimurium LT2 (STM2845), *Thermococcus kodakarensis* (TK2004 and TK2066), *Thermococcus onnurineus* (TON_0263 and TON_0533), *Thermococcus gammatolerans* (TGAM_0236 and TGAM_0828), *Thermococcus sibiricus* (TSIB_0286 and TSIB_1002), *Thermococcus barophilus* (TERMP_00535 and TERMP_01366), *Thermococcus* sp. 4557 (GQS_02490 and GQS_04225), *Thermococcus* sp. AM4 (TAM4_478, TAM4_809 and TAM4_1208), *Thermococcus* sp. CL1 (CL1_0147 and CL1_0483), *Thermococcus litoralis* (OCC_08425 and OCC_12211), *Thermococcus* sp. ES1 (TES1_1385), *Thermococcus nautili* (BD01_0133 and BD01_0433), *Thermococcus eurythermalis* (TEU_06230 and TEU_09705), *Thermococcus guaymasensis* (X802_03570 and X802_07545), *Thermococcus* sp. 2319 x 1 (ADU37_CDS08540 and ADU37_CDS02190), *Pyrococcus horikoshii* (PH0674 and PH0979), *Pyrococcus abyssi* (PAB0576 and PAB0909), *Pyrococcus furiosus* DSM 3638 (PF0617 and PF0975), *P. furiosus* COM1 (PFC_02225 and PFC_04105), *Pyrococcus* sp. NA2 (PNA2_1288 and PNA2_1655), *Pyrococcus yayanosii* (PYCH_00060 and PYCH_06440), *Pyrococcus* sp. ST04 (Py04_0975 and Py04_1086) and *Palaeococcus pacificus* (PAP_03260 and PAP_09005). Only bootstrap values above 60 % are indicated. The proteins from *T. gammatolerans* (TGAM_0828) and *Thermococcus* sp. AM4 (TAM4_809) that are discussed in the text are indicated with *asterisks*

Phylogenetic analysis

Sequences of hydrogenase-specific endopeptidases from various sources were collected and aligned by using the ClustalW program provided by GenomeNet (http://www.genome.jp/). Multiple-sequence alignment was performed with the following default parameters: protein weight matrix, BLOSUM; gap open, 10; gap extension, 0.05; clustering, NJ. The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987). Bootstrap resampling was performed 1000 times.

Results

Endopeptidase recognition sites of the [NiFe]-hydrogenase large subunits in *T. kodakarensis*

There are two hydrogenase gene clusters on the *T. kodakarensis* genome that have been biochemically or genetically confirmed to encode active hydrogenases (Kanai et al. 2003, 2011). One is the cytosolic Hyh (encoded by *hyhBGSL*: TK2072–TK2069) and the other is the membrane-bound Mbh (encoded by *mbhABCDEFGHIJKLMN*: TK2080–TK2093). The large subunits of these hydrogenases are encoded by *hyhL* (TK2069) and *mbhL* (TK2091), respectively. The C-terminal regions of both proteins harbor the C-terminal consensus motif DPCXXCXXH or DPCXXCXXR (Fig. 1) found in many [NiFe]-hydrogenase large subunits (Rossmann et al. 1994, 1995; Fritsche et al. 1999). The two cysteine residues were first shown to bind to the Ni atom in the [NiFe]-hydrogenase from *Desulfovibrio gigas* (Volbeda et al. 1995). The C-terminal amide bond of the conserved basic residue in the *E. coli* hydrogenase 3 large subunit precursor (Arg^{537}) was shown to be cleaved by the *Ec*-HycI endopeptidase (Rossmann et al. 1994). We can therefore presume that HyhL and MbhL precursor proteins, as in the case of other [NiFe]-hydrogenase large subunit proteins, are cleaved at these conserved basic residues by specific endopeptidases.

Hydrogenase-specific endopeptidase homologs in *T. kodakarensis* and other members of Thermococcales

On the *T. kodakarensis* genome, there are two homologs of [NiFe]-hydrogenase-specific maturation endopeptidases, TK2004 and TK2066, encoding proteins of 17.0 and 16.3 kDa, respectively. Although TK2066 is located nearby the *hyhBGSL* locus (TK2072–TK2069), two functionally unknown genes (TK2068: conserved hypothetical protein; TK2067: predicted nucleic acid-binding protein, containing PIN domain) are present in between, and these genes have been reported to be transcribed independently, based on RNA seq analysis (Jäger et al. 2014). TK2004 resides in an 11 kbp region in which hydrogenase maturation genes are present, including *hypA* (TK2008), *hypB* (TK2007), *hypC* (TK2001), *hypD* (TK2000), *hypE* (TK1993) and *hypF* (TK1997), but is apart from both of the [NiFe]-hydrogenase structural genes. When the sequences of the two endopeptidases from *T. kodakarensis* are compared with those from *E. coli*, TK2004 is more similar to *Ec*-HycI (27 % identical), while TK2066 displays relatively higher similarity to *Ec*-HybD (26 % identical) (Fig. 2).

At present, complete genome sequences have been reported for 22 members of the Thermococcales. A BLAST search with the sequences of the two endopeptidases from *T. kodakarensis* revealed that TK2004 homologs are present in all members (Fig. 2). On the other hand, TK2066 homologs were found in 20 of the 22 members of the Thermococcales. The two species in which they were absent were *Thermococcus gammatolerans* and *Thermococcus* sp. ES1. In the case of *T. gammatolerans*, this organism does not harbor an HyhL homolog (Zivanovic et al. 2009), which might provide an explanation for the absence of a TK2066 homolog. In contrast, this is not the case in *Thermococcus* sp. ES1, which harbors multiple homologs of HyhL, MbhL and coenzyme F_{420} hydrogenase large subunits (Jung et al. 2014). The TK2004 and TK2066 homologs from the Thermococcales are more closely related to the

Ec-HycI and *Ec*-HybD homologs from bacteria, respectively, than to each other, suggesting that the TK2004/HycI and TK2066/HybD families diverged earlier than the divergence of the archaeal/bacterial domains.

Tag insertion into the Hyh large subunit

To determine the physiological role of the two endopeptidase homologs in *T. kodakarensis*, we set out to disrupt each gene and examine the effects on the maturation of the large subunits of Hyh and Mbh. However, we anticipated that it would be difficult to judge whether the Hyh large subunit precursor (pre-HyhL) was cleaved or not, as cleavage of the native protein results in the release of only four amino acid residues. To alleviate this difficulty, we modified the C-terminal region of pre-HyhL and added a peptide sequence that contains two Strep-tag II sequences in tandem flanked with flexible linkers (Fig. 1). *T. kodakarensis* KU216 was used as the host strain and the DNA sequence corresponding to the peptide was inserted into the native HyhL gene. PCR analysis and DNA sequencing confirmed the insertion in one transformant, designated as strain KU216-ST (Fig. 3a). Cleavage of the modified Hyh large subunit precursor (pre-HyhL-ST) would release a peptide of 36 residues (Fig. 1). Strain KU216-ST exhibited growth characteristics similar to that of strain KU216 under both H_2S-producing (ASW-YT-S^0) and H_2-producing (ASW-YT-Pyr) conditions (Fig. 3b). KU216-ST was thus used as a host strain for further genetic modification of the endopeptidases.

Disruption of endopeptidase genes in *T. kodakarensis* KU216-ST

Plasmids designed to disrupt TK2004 and TK2066 were constructed as described in the Materials and Methods section. *T. kodakarensis* KU216-ST was individually transformed with each plasmid, and gene disruption strains were selected based on uracil prototrophy, followed by resistance towards 5-FOA. A double disruption strain was also constructed. The genotypes of the transformants were examined by PCR, and the results are shown in Fig. 4. The mutants, all of which harbored the modified HyhL-ST (Fig. 3), are designated as ΔHYD4-ST (ΔTK2004), ΔHYD6-ST (ΔTK2066), and ΔHYD46-ST (ΔTK2004/ΔTK2066).

Growth characteristics of the gene disruption strains

It has previously been reported that the function of Mbh is essential for growth of *T. kodakarensis* under H_2-evolving conditions, such as in media with sodium pyruvate or maltodextrins without S^0 (Kanai et al. 2011; Santangelo et al. 2011). Deletion of Mbh genes does not affect growth on amino acids coupled to S^0 reduction. By contrast, deletion of Hyh does not affect growth under these conditions. Similar results have also been reported for the closely related *P. furiosus* (Lipscomb et al. 2011; Schut et al. 2012, 2013). When we examined growth of the endopeptidase gene disruption strains (Fig. 5), we observed that gene deletion had no effect when cells were grown in the presence of S^0. Under H_2-evolving conditions, the deletion of TK2066 did not affect growth (strain ΔHYD6-ST). However, we observed that cells devoid of TK2004 (strains ΔHYD4-ST, ΔHYD46-ST) could not grow at all under these conditions. This suggests that TK2004 is at least involved in the maturation process of Mbh.

Cleavage of the large subunit precursor in the gene disruption strains

We directly examined the cleavage of the Mbh and Hyh large subunit precursors in the gene disruption strains by western blot analyses, using specific polyclonal antibodies raised against the purified, recombinant large subunit proteins. Cells were grown in medium with S^0, as this allows growth of all disruption strains. The results concerning the Mbh large subunit are shown in Fig. 6a. Almost complete cleavage of the Mbh precursor protein (pre-MbhL) can be observed in the host strain KU216-ST. Similar results were observed in the TK2066 disruption strain (strain ΔHYD6-ST). However, in ΔTK2004 (strain ΔHYD4-ST), no cleavage could be detected, and an accumulation of the precursor protein pre-MbhL was observed. The results indicate that TK2004 is essential for pre-MbhL processing, and agrees well with the growth defects observed in this strain under H_2-evolving conditions.

Similar analyses were performed for pre-HyhL-ST (Fig. 6b). In this case, complete cleavage was observed in the host strain and in the ΔTK2004 strain (ΔHYD4-ST). In the TK2066 disruption strain (ΔHYD6-ST), cleavage was incomplete and accumulation of pre-HyhL-ST was clearly detected. When both endopeptidase genes were disrupted (strain ΔHYD46-ST), cleavage could not be observed. The presence of the intact precursor was confirmed by western blot analysis with anti-Strep-tag II antibodies. The results indicate that TK2066 is the major endopeptidase responsible for pre-HyhL cleavage. They also suggest that the partial cleavage observed in the ΔTK2066 strain was dependent on the function of TK2004.

Complementation experiments on the gene disruption strains

To confirm that the effects observed with each gene disruption were solely due to the absence of the targeted gene,

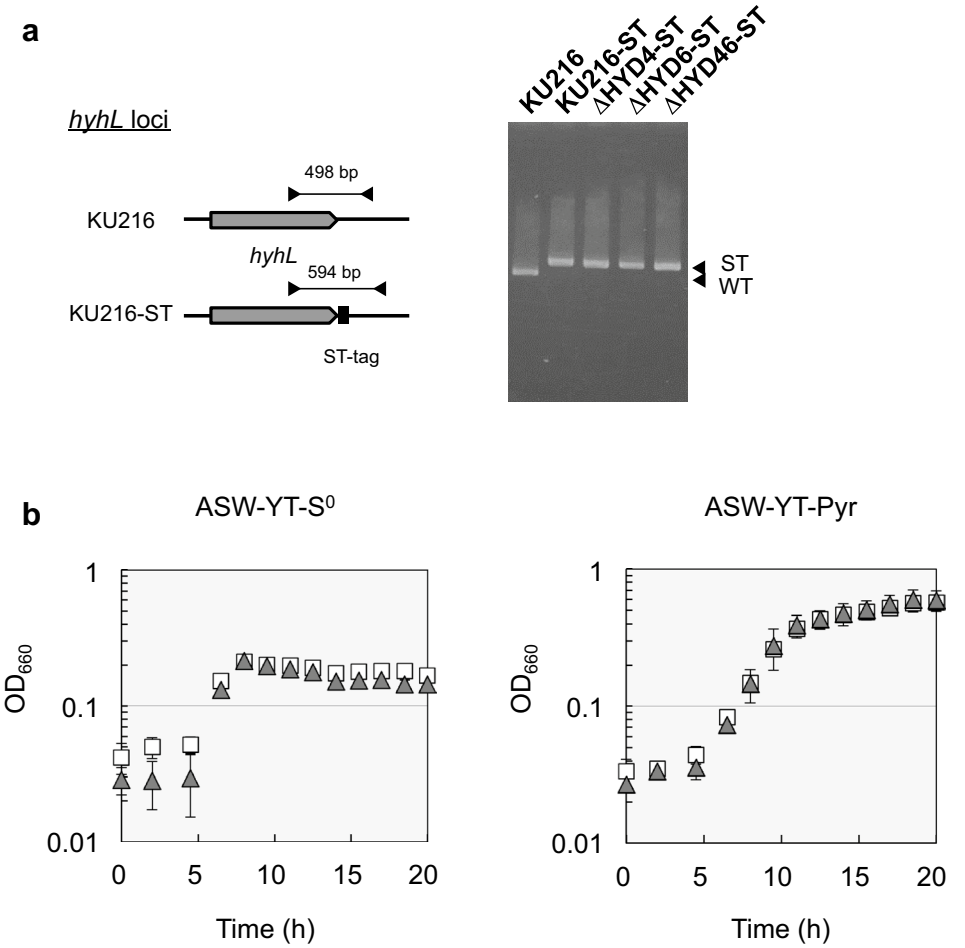


Fig. 3 **a** Construction of strain KU216-ST that expresses a genetically engineered pre-HyhL derivative, pre-HyhL-ST. A graphic representation of the *hyhL* locus before and after the ST-tag sequence insertion is shown on the *left*. The approximate positions of the primers (TK2069_S_3F/TK2069_S_4R) used for analyzing the genetic rearrangement, and the expected lengths of the amplified fragments are also shown. The *right panel* shows the result of PCR using the primers. DNA bands with lengths corresponding to loci with the ST-tag sequences are indicated with "ST", while that corresponding to the wild-type locus is indicated with "WT". **b** Growth properties of *T. kodakarensis* KU216 and KU216-ST in ASW-YT-based medium supplemented with elemental sulfur (ASW-YT-S^0) (*left panel*) or sodium pyruvate (ASW-YT-Pyr) (*right p*anel). Symbols: *open squares* KU216; and *closed triangles* KU216-ST. OD_{660}, optical density at 660 nm

we carried out complementation experiments on all disruption strains. Intact TK2004 or TK2066 were introduced into the disruption strains on a self-replicating plasmid, derived from pLC70 (Santangelo et al. 2008). In the case of the ΔTK2004 strain (ΔHYD4-ST), reintroduction of the gene completely restored the ability of the strain to cleave pre-MbhL [Fig. 7a, ΔHYD4-ST(pLC2004)]. The pLC70 plasmid without the gene did not have any effect. Likewise, the incomplete cleavage of pre-HyhL-ST observed in both the TK2066 disruption strain (ΔHYD6-ST) and the double gene disruption strain (ΔHYD46-ST) was complemented by the reintroduction of the TK2066 gene [Fig. 7b, ΔHYD6-ST(pLC2066) and ΔHYD46-ST(pLC2066)]. Intriguingly, we also observed a complete digestion of pre-HyhL-ST to HyhL by the introduction of the TK2004 gene in the double gene disruption strain [Fig. 7b, ΔHYD46-ST(pLC2004)], which seems inconsistent with the incomplete digestion of pre-HyhL-ST in the TK2066-disrupted strain. This is most likely due to an increased amount of intracellular TK2004 protein compared to the TK2066-disrupted strain. The copy number of pLC70 in *T. kodakarensis* was shown to be ~3 per chromosome (Santangelo et al. 2008), resulting in higher expression of TK2004 proteins in the complemented strain.

Discussion

The genetic evidence provided in this study clearly demonstrates that the cleavage of pre-MbhL occurs by the function of TK2004, and not TK2066 (Fig. 8). TK2066 encodes the endopeptidase mainly responsible for the processing of pre-HyhL. However, although to a lower extent, we found that TK2004 can also take part in the cleavage of pre-HyhL. According to Vignais and co-workers (Vignais et al. 2001; Vignais and Billoud 2007), MbhL from *T. kodakarensis* is classified into group 4 among the [NiFe]-hydrogenases, together with *Ec*-HycE, the large subunit of hydrogenase 3 in *E. coli*. On the other hand, HyhL is classified into group 3b while the large subunits of *E. coli* hydrogenase 1 (*Ec*-HyaB) and hydrogenase 2 (*Ec*-HybC) are classified in group 1. In *E. coli*, as *Ec*-HycI is responsible for the cleavage of *Ec*-HycE, it seems reasonable that, among the two endopeptidases in *T. kodakarensis*, the one

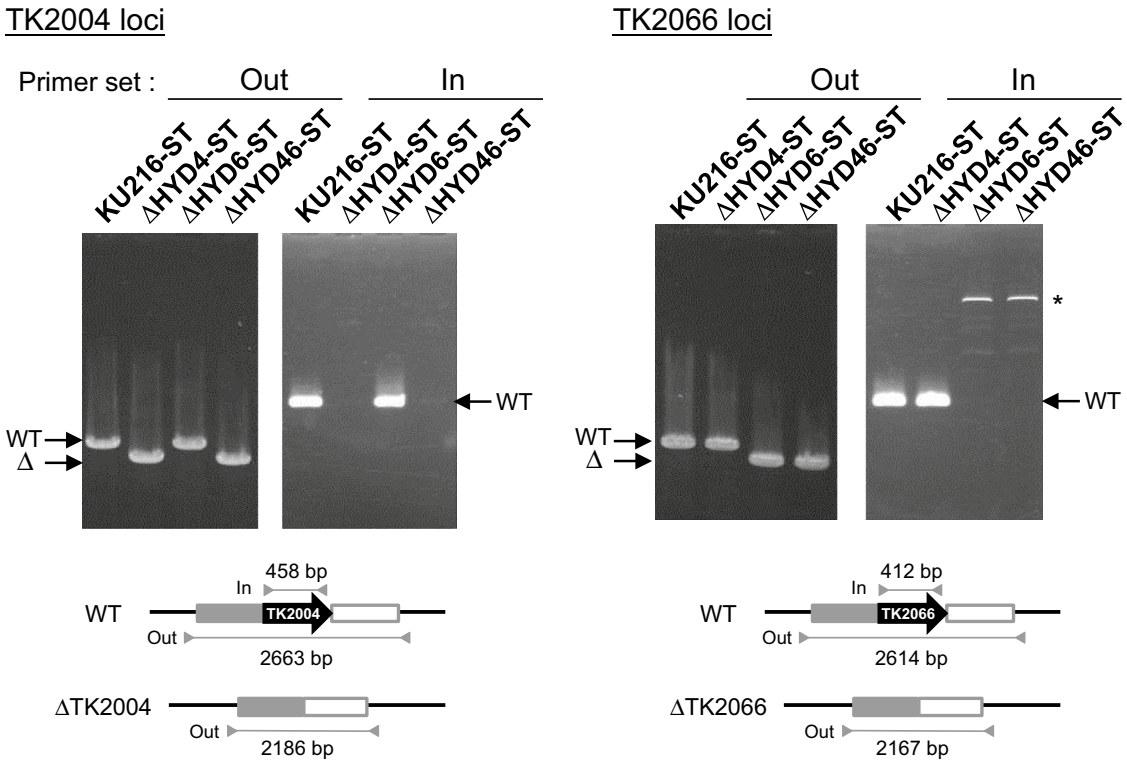


Fig. 4 Construction of *T. kodakarensis* ΔHYD4-ST (Δ*pyrF, hyhL-ST,* ΔTK2004), ΔHYD6-ST (Δ*pyrF, hyhL-ST,* ΔTK2066), and ΔHYD46-ST (Δ*pyrF, hyhL-ST,* ΔTK2004, ΔTK2066). Graphic representations of the TK2004 (*left*) and TK2066 (*right*) loci before and after gene disruption are shown below the gels. The approximate positions of the primers used for genotype analyses, and the expected lengths of the amplified fragments are also shown. Amplified products of the TK2004 (*left*) and TK2066 (*right*) loci are shown. Primers used were TK2004_out_F/TK2004_out_R (primer set: Out), TK2004_in_F/TK2004_in_R (primer set: In), TK2066_out_F/TK2066_out_R (primer set: Out), and TK2066_in_F/TK2066_in_R (primer set: In). DNA bands with lengths corresponding to the wild-type and disrupted loci are indicated with "WT" and "Δ", respectively. Non-specific amplification is shown by an *asterisk*

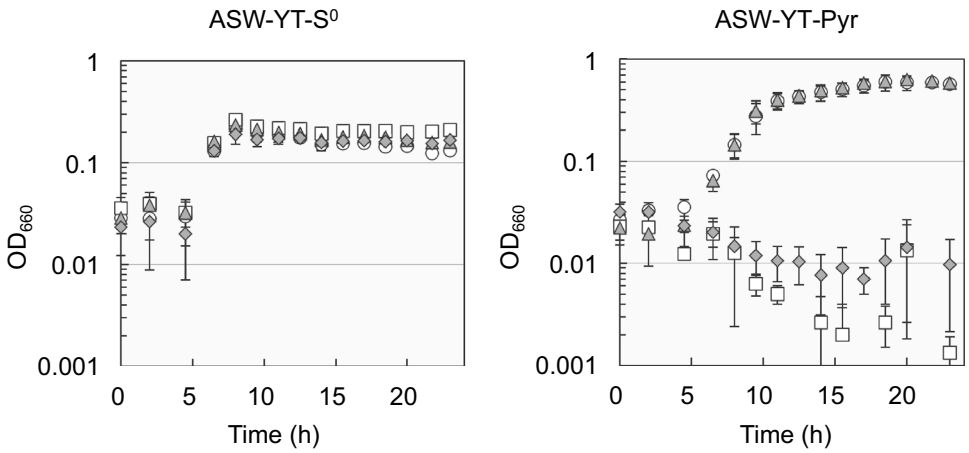


Fig. 5 Growth properties of *T. kodakarensis* KU216-ST, ΔHYD4-ST, ΔHYD6-ST and ΔHYD46-ST in ASW-YT-based medium supplemented with elemental sulfur (ASW-YT-S^0) (*left panel*) or with sodium pyruvate (ASW-YT-Pyr) (*right panel*). Symbols: *open circles* KU216; *open squares* ΔHYD4-ST; *closed triangles* ΔHYD6-ST; and *closed diamonds* ΔHYD46-ST. OD_{660}, Optical density at 660 nm

that more resembles *Ec*-HycI, TK2004, is responsible for the cleavage of pre-MbhL.

The specific sequences on the hydrogenase large subunit precursors that are recognized by the different endopeptidases are still not known. One clear difference between HyhL and MbhL precursor proteins from members of the Thermococcales is that the C-terminal extensions in the precursor protein are much shorter in the case HyhL proteins (4 or 5 residues) compared to those from MbhL proteins (45 residues or longer). However, the length of the extensions alone cannot be responsible for the discrimination, as TK2066 was still able to cleave pre-HyhL-ST,

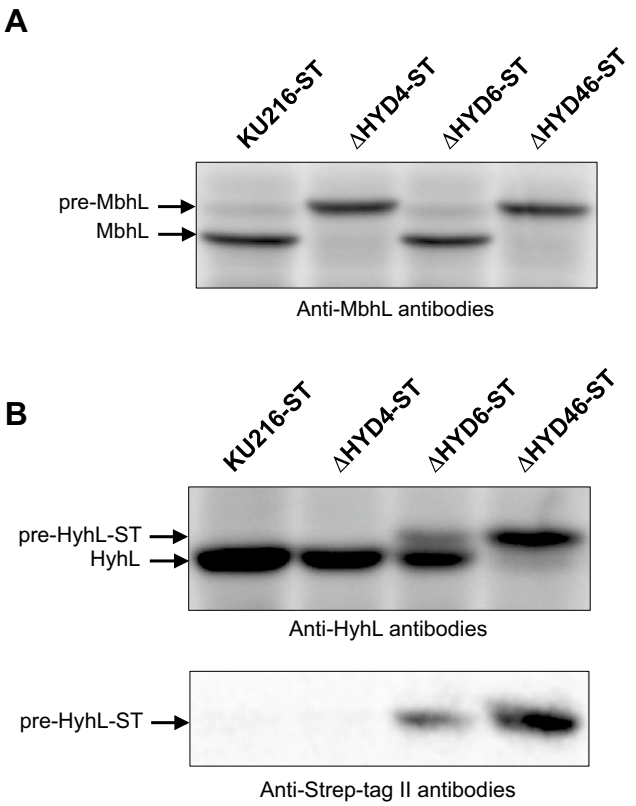


Fig. 6 **a** Maturation of MbhL in the mutant strains devoid of maturation endopeptidase(s). Cells were grown in ASW-YT medium with 0.5 % (w/v) elemental sulfur. Cell extracts (3 μg) were subjected to SDS-PAGE, followed by western blot analysis with anti-MbhL antibodies. The precursor and the mature forms are indicated as pre-MbhL and MbhL, respectively. **b** Maturation of HyhL in the mutant strains devoid of maturation endopeptidase(s). Samples were prepared as in **a**, and western blot analysis was performed with anti-HyhL polyclonal antibodies (*upper*) or anti-Strep-tag II monoclonal antibodies (*lower*). The precursor and the mature forms are indicated as pre-HyhL-ST and HyhL, respectively. In both **a**, **b**, the names of the strains used are indicated above the membranes

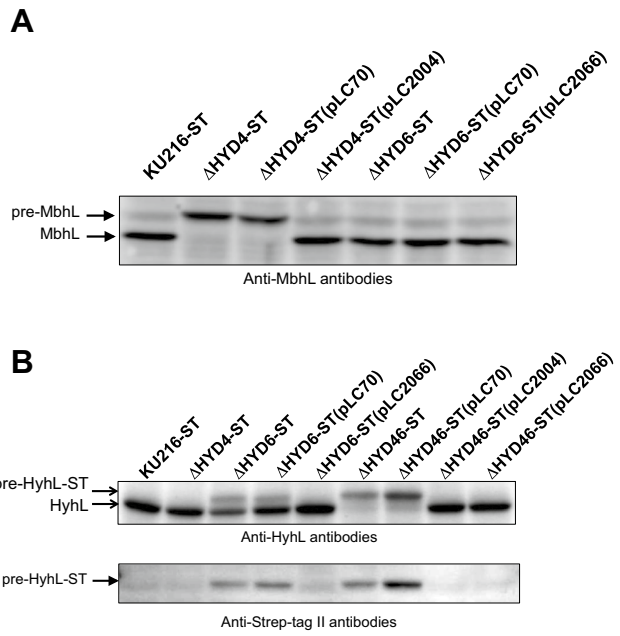


Fig. 7 **a** Maturation of MbhL in disruption and complementation strains of TK2004 or TK2066. Samples were prepared as in Fig. 6, followed by western blot analysis with anti-MbhL antibodies. **b** Maturation of HyhL in disruption and complementation strains of TK2004 and/or TK2066. Samples were prepared as in **a**, followed by western blot analysis with anti-HyhL antibodies. Plasmids and the genotypes of the strains are shown in Table 1. Plasmids introduced into specific strains are indicated in parentheses

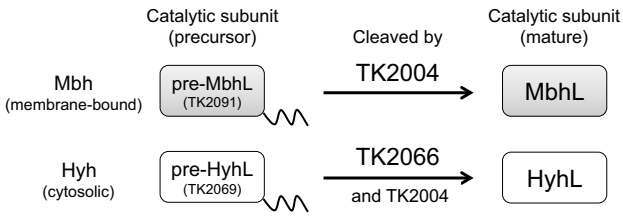


Fig. 8 A diagram illustrating the roles of the two hydrogenase-specific endopeptidases (TK2004 and TK2066) in *T. kodakarensis*

which has a C-terminal peptide of 36 residues (4 native residues + 32 artificial residues). Another tendency when comparing the HyhL and MbhL homologs is that all of the HyhL homolog precursors harbor a His as the basic residue at the cleavage site, whereas all MbhL homolog precursors harbor an Arg residue at the corresponding site. As the crystal structure of TK2066 has recently been determined (Kwon et al. 2016), further structure analysis of TK2004 may reveal differences that govern the substrate specificities of these two endopeptidases.

When we searched the archaeal genomes for homologs of TK2004 and TK2066, we found, as stated above, that closely related homologs are present in the majority of Thermococcales species (Figs. 2, S1). Almost all of the other homologs from archaea derived from methanogens, along with one homolog found in *Archaeoglobus*. We carried out a phylogenetic analysis on these sequences with the addition of several representative sequences deriving from bacteria (Fig. S1). Although diverging at an early stage, we were able to identify a clade that included the TK2004 and HycI homologs. The clade included three major branches consisting of (1) TK2004 homologs from the Thermococcales, (2) homologs from several methanogens (designated here as Type III), and (3) HycI homologs from bacteria. On the other hand, the TK2066 homologs and the HybD homologs formed a clade along with several homologs from methanogens (Type II). There was also another large group of homologs from methanogens (Type I). In Fig. S1, we included this branch in the HybD-type group, but we cannot rule out that this branch represents

a third group of hydrogenase-specific endopeptidases. In many cases, the genes encoding the Type I endopeptidases from methanogens lie within operons that encode the subunits of F_{420}-reducing hydrogenases, suggesting that the enzymes are involved in the cleavage of F_{420}-reducing hydrogenase precursors. The homologs from *Methanopyrus kandleri* (MK1499) and *Archaeoglobus fulgidus* (AF_1378) do not fall into any group.

In the TK2004 homolog group from Thermococcales, we noticed that *T. gammatolerans* and *Thermococcus* sp. AM4 harbor a second TK2004-type hydrogenase-specific endopeptidase, TGAM_0828 and TAM4_809, respectively, in addition to TGAM_0236 and TAM4_1208 (Figs. 2, S1). TGAM_0828 and TAM4_809 may represent an independent group of endopeptidases in Thermococcales. In *T. gammatolerans*, there are three additional hydrogenases besides Mbh, two presumed to be components of formate hydrogenlyase (TGAM_0244, TGAM_0059) and another, that resembles F_{420}-reducing hydrogenase (TGAM_0068). *Thermococcus* sp. AM4 harbors two hydrogenases presumed to be components of formate hydrogenlyase (TAM4_1173) and carbon monoxide dehydrogenase-hydrogenase complex (TAM4_1098). As the precursors of these hydrogenases harbor the conserved C-terminal motif (DPCXXCXXH/R) in their large subunits, it is tempting to speculate that TGAM_0828 and TAM4_809 are responsible for their processing. However, this must await experimental confirmation, as we observe that although *Thermococcus onnurineus* (Lee et al. 2008; Kim et al. 2013) and a number of other members of Thermococcales also harbor these additional hydrogenases, a third endopeptidase homolog cannot be identified in these organisms.

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