Nitrate reductases in *Hydrogenobacter thermophilus* with evolutionarily ancient features: distinctive localization and electron transfer

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Running title: Nitrate reductases of *Hydrogenobacter thermophilus*

Keywords: Nitrogen metabolism; denitrification; nitrate assimilation; electron transfer; ferredoxin; membrane enzyme

This is the accepted version of the following article:
Kameya, M., Kanbe, H., Igarashi, Y., Arai, H. & Ishii, M. (2017) Nitrate reductases in *Hydrogenobacter thermophilus* with evolutionarily ancient features: distinctive localization and electron transfer. *Molecular Microbiology* (2017), which has been published in final form at http://dx.doi.org/10.1111/mmi.13756 .

Summary

Dissimilatory nitrate reductase (NAR) and assimilatory nitrate reductase (NAS) serve as key enzymes for nitrogen catabolism and anabolism in many organisms. We purified NAR and NAS from *H. thermophilus*, a hydrogen-oxidizing chemolithoautotroph belonging to the phylogenetically deepest branch in the *Bacteria* domain. Physiological contribution of these enzymes to nitrate respiration and assimilation was clarified by transcriptomic analysis and gene disruption experiments. These enzymes showed several features unreported in bacteria, such as the periplasmic orientation of NAR anchored with a putative transmembrane subunit and the specific electron transfer from a [4Fe-4S]-type ferredoxin to NAS. While some of their enzymatic properties are shared with NARs from archaea and with NASs from phototrophs, phylogenetic analysis indicated that *H. thermophilus* NAR and NAS have deep evolutionary origins that cannot be explained by a recent horizontal gene transfer event from archaea and phototrophs. These findings revealed the diversity of NAR and NAS in nonphotosynthetic bacteria, and they also implied that the outward orientation of NAR and the ferredoxin-dependent electron transfer of NAS are evolutionarily ancient features preserved in *H. thermophilus*.

Introduction

Nitrogen is an essential element in cellular components along with carbon, hydrogen, and oxygen; thus, oxidoreduction of nitrogen compounds is involved in a diverse range of cellular anabolic and catabolic pathways. Nitrate is an abundant form of nitrogen in natural environments, and nitrate-reducing pathways operate in many microbes as the first reaction of their central metabolism, namely, nitrate assimilation and respiration.

During nitrate respiration, membrane-bound dissimilatory nitrate reductase (NAR; EC 1.7.99.4) reduces nitrate to nitrite via a transfer of electrons from quinol. NAR is classified into two subtypes depending on its localization, nNAR and pNAR, whose catalytic centers are located on the cytoplasmic and the exterior sides of cell membrane, respectively (Richardson *et al.*, 2001). Known nNAR and pNAR are specifically distributed in bacteria and archaea, respectively, and no bacterial pNAR has been found and characterized although many bacterial NAR genes possess remnant signal peptide regions in their N termini (Martinez-Espinosa *et al.*, 2007, Ize *et al.*, 2009). nNAR consists of the following heterotrimeric subunits: a catalytic subunit containing a molybdenum cofactor (NarG), a ferredoxin (Fd)-like electron transfer subunit containing Fe-S clusters (NarH), and the membrane-integral subunit with transmembrane helices (NarI). Archaeal pNAR shares homologous NarG and NarH with nNAR, but contains no NarI homolog. pNARs have been purified as a two-subunit (Hochstein & Lang, 1991, Yoshimatsu *et al.*, 2000, Yoshimatsu *et al.*, 2002) or three-subunit (de Vries *et al.*, 2010) heteromer, and the latter contains a membrane-anchored subunit, NarM, in addition to NarGH. The nitrite produced by nNAR is excreted to the periplasmic space by a transporter and is further reduced to nitric oxide by dissimilatory nitrite reductase (dNIR; EC 1.7.2.1) in the periplasm. Nitrate reduction by nNAR accompanies formation of a transmembrane proton gradient, which allows for energy conservation and ATP synthesis under anaerobic conditions with nitrate serving as the terminal electron acceptor instead of oxygen.

In contrast to nitrate respiration, nitrate assimilation is catalyzed by cytosolic soluble enzymes—assimilatory nitrate reductase (NAS) and assimilatory nitrite reductase—which reduce nitrate to nitrite and nitrite to ammonium, respectively. The resulting ammonium is incorporated into organic compounds during anabolic synthesis of various nitrogenous metabolites. Bacterial NAS is classified into two groups that use different kinds of electron donors (Moreno-Vivian *et al.*, 1999, Richardson *et al.*, 2001). One group reacts with NADH or NADPH and is called NAD(P)H-dependent NAS (NAD[P]H-NAS; EC 1.7.1.2). The other group uses Fd, a small metalloprotein known as an electron carrier involved in photosynthesis and various redox metabolic pathways. This group is called Fd-dependent NAS (Fd-NAS; EC 1.7.7.2) and has been found in cyanobacteria, whereas NAD(P)H-NAS is the type of enzyme predominant among nonphotosynthetic bacteria. As an exception, one study reported *Clostridium perfringens* NAS as Fd-NAS (Chiba & Ishimoto, 1973), but later analyses suggested that this NAS is most likely not Fd-NAS but NAD(P)H-NAS (Fujinaga *et al.*, 1999). NAD(P)H-NAS is a heterodimeric enzyme composed of a catalytic subunit and an NAD(P)H-binding subunit, whereas Fd-NAS is a monomer of a subunit homologous to catalytic subunits of NAD(P)H-NAS and NAR (NarG).

Hydrogenobacter thermophilus TK-6 is a hydrogen-oxidizing chemolithoautotroph belonging to *Aquificales*, the phylogenetically deepest branch in the *Bacteria* domain. This species' unique central metabolism has been subjected to intensive biochemical studies as a model organism for research on bacteria

Table 1. Purification of NAR from *H. thermophilus*

Fraction	Activity [a] (U)	Protein (mg)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Solubilized fraction	2.6	15	0.17	1	100
Butyl-Toyopearl	3.8	1.9	2.0	12	147
CM-Toyopearl	2.7	0.86	3.1	19	104
MonoS	2.3	0.18	13	75	88

a, Assayed by using MV as the electron donor.

with outstanding evolutionary lineages. *H. thermophilus* optimally grows on ammonium as the assimilatory source of nitrogen, but can also use nitrate as an alternative nitrogen source (Kawasumi *et al.*, 1984). Although *H. thermophilus* is tolerant to oxidative stresses and able to grow by aerobic respiration (Sato *et al.*, 2014, Sato *et al.*, 2012a), this bacterium can also grow by nitrate respiration under anaerobic conditions, by switching the terminal electron acceptor from oxygen to nitrate and producing dinitrogen as a final product (Suzuki *et al.*, 2001). A number of novel characteristics have been found in the nitrogen metabolism of this bacterium (Suzuki *et al.*, 2006b, Kameya *et al.*, 2006, Kameya *et al.*, 2010, Chiba *et al.*, 2012a, Chiba *et al.*, 2012b), but neither NAR nor NAS of *H. thermophilus* has been analyzed biochemically.

In the present study, NAR and NAS were purified from *H. thermophilus*, and their physiological function was determined by transcriptomic analysis and gene disruption experiments. These enzymes were found to have several unexpected features that were previously assumed to be specific to archaea or photosynthetic organisms. We also discuss the networks of electron transfer and energy synthesis in this bacterium.

Results

NAR purification

To detect *H. thermophilus* NAR, the cells were anaerobically cultivated with nitrate as the terminal electron acceptor, and NAR activity was assayed in the solubilized membrane fraction. When an artificial electron carrier, methyl viologen (MV), was used as the electron donor in assay mixtures, the membrane fraction showed significant activity to reduce nitrate to nitrite. This result suggested the presence of a membrane-bound nitrate reductase.

An enzyme responsible for this activity was purified (Table 1). The purified protein yielded three bands on SDS-PAGE analysis (Fig. 1A). N-terminal sequences of the large and middle subunits (designated as NarG and NarH, respectively below) were determined to be FRVMEPV and AKAFN, respectively. The former sequence is identical to residues 27–33 of a putative molybdoenzyme encoded by HTH_1717 (*narG*), suggesting cleavage of its N-terminal 26 residues. The latter sequence is identical to residues 2–6 of the protein encoded by HTH_1716 (*narH*). The N terminus of the small subunit (designated as NarI below) could not be sequenced, probably due to a post-translational modification. Chemical cleavage by CNBr and subsequent N-terminal sequencing identified the terminus as GDKDHRV; this sequence is identical to the internal sequence of the protein encoded by HTH_1715 (*narI*). The three genes constitute a gene cluster in the genome (Fig. 2A). The molecular masses of the products of these genes were calculated to be 132, 42, and 32 kDa, respectively. These values are consistent with those estimated from the

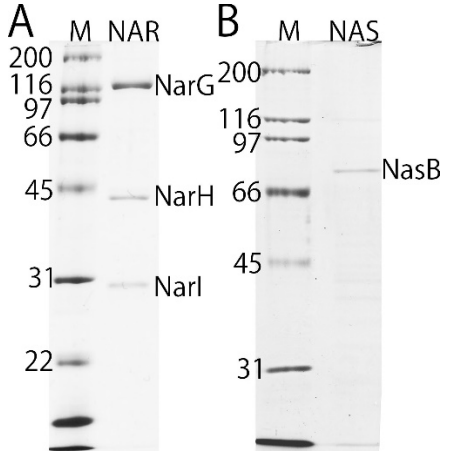

Figure 1. SDS-PAGE analysis of the NAR (A) and NAS (B) purified from *H. thermophilus*. For separation, 13% (A) or 10% (B) acrylamide gels were used. Lanes M: molecular mass markers.

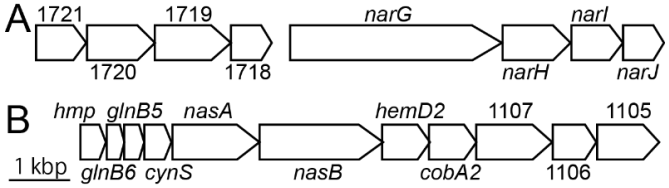

Figure 2. Physical maps of the gene clusters of NAR (A) and NAS (B) in the *H. thermophilus* genome. Numbers represent the HTH_ locus tags of genes that do not have an assigned name.

band positions on SDS-PAGE.

The amino acid sequences of these subunits were compared with those of known bacterial NARs. The proteins encoded by HTH_1717 and *E. coli narG* (NCBI GI 16129187) share similarity (25% identity and E-value of 3×10^{-43}), suggesting that HTH_1717 encodes the catalytic subunit of the purified enzyme. Unexpectedly, this similarity is significantly lower than that between *E. coli* and archaeal NarGs (e.g., 33% identity and the E-value of 3×10^{-127} between *E. coli* and *Haloferax mediterranei* NarGs). This low similarity indicates that HTH_1717 is phylogenetically distant not only from archaeal but also from known bacterial NarGs. HTH_1716 showed similarity with *E. coli* NarH (NCBI GI 16129188) with 29% identity and the E-value of 1×10^{-51}. On the other hand, HTH_1715 shares no significant similarity with bacterial *narI*.

HTH_1715 showed low similarity to NarM of archaeal pNAR, for example, 25% identity and the E-value of 8×10^{-20} in relation to *H. mediterranei* NarM (NCBI GI 41349583). In addition, its downstream gene, HTH_1714, is homologous to archaeal *narJ* that encodes a chaperone for NAR maturation. The presence of genes homologous to archaeal *narM* and *narJ* suggested that the NAR of *H. thermophilus* has architectural and biosynthetic features similar to those of NARs purified from archaea.

The SOSUI (Mitaku *et al.*, 2002) and TMAP (Milpetz *et al.*, 1995) software packages predicted the existence of a single transmembrane helix in the N terminus of the *H. thermophilus* NarI. Meanwhile, known bacterial NarIs and archaeal NarMs possess five and two transmembrane helices, respectively (de Vries *et al.*, 2010), suggesting that the *H. thermophilus* NarI is more loosely associated with the membrane than these anchor subunits. No additional transmembrane helix was found in the other region of *H. thermophilus* NarGHI.

Four genes with an unknown function (HTH_1718 through HTH_1721) were adjacent to the region upstream of *narG*. Although the NAR gene clusters in many bacteria contain *narK* encoding a nitrate/nitrate transporter, which imports nitrate and exports nitrites for periplasmic dNIR, no *narK* homolog is present in the gene clusters encoding dNIR (Suzuki *et al.*, 2006b) and NAR in *H. thermophilus*.

Known bacterial NarG homologs possess a remnant twin-arginine motif at their N termini (Ize *et al.*, 2009, Martinez-Espinosa *et al.*, 2007). While the canonical twin-arginine motif causes a protein export across the cell membrane followed by the cleavage of the signal peptide, the remnant motif is responsible to NAR maturation without accompanying the protein translocation and the peptide cleavage. *H. thermophilus* NarG possesses the twin-arginine motif in the N terminus region, and this region shows a higher degree of conservation of this motif in comparison with other bacterial NarGs (Fig. 3). This N-terminal sequence pointed to the possibility that the highly conserved twin-arginine motif allows *H. thermophilus* NAR to be translocated into the periplasmic side of the cell membrane as reported only in archaea (Yoshimatsu *et al.*, 2002, Martinez-Espinosa *et al.*, 2007). In addition, it should be noted that this N-terminal region was cleaved from the NarG purified from *H. thermophilus*. While similar truncation has been reported in NarG of archaeal pNAR, such truncation has not been reported in bacterial nNAR (Fig. 3). The conserved motif sequence and its cleavage strongly suggest that this region functions as a secretion signal peptide and that NAR is translocated to the periplasmic side in *H. thermophilus*.

The whole-cell assay of NAR

To identify the localization of NAR, the whole-cell NAR activity was assayed with MV or benzylviologen (BV) serving as the electron donor. Although MV and BV are artificial electron carriers with similar structure, the reduced form of the latter can penetrate the cell membrane whereas that of the former cannot (Carter *et al.*, 1995, Jones & Garland, 1977). The intact cells showed a significant NAR activity when each electron carrier was used (Table 2), as is the case for archaeal pNARs (Martinez-Espinosa *et al.*, 2007, Yoshimatsu *et al.*, 2002, de Vries *et al.*, 2010). These activities were approximately constant before and after the sonication of the cell suspension, indicating that *H. thermophilus* NAR is located on the periplas-

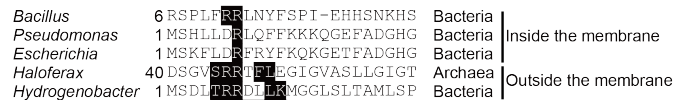

Figure 3. Alignment of N-terminal regions of NarG homologs and the conserved sequence of the twin-arginine motif, [S/T]RRxFLK. The strains and NCBI GI numbers of each NarG homolog are as follows: *Pseudomonas fluorescens*, 11344601; *E. coli* MG1655, 16129187; *B. subtilis* 168, 255767790; *H. marismortui*, 18447796; *H. thermophilus* TK-6, 288788417. Lower case letters represent the residues cleaved from mature NarG, which have been clarified by N-terminal amino acid sequencing in this study and previous studies (McPherson *et al.*, 1984, Philippot *et al.*, 1997, Yoshimatsu *et al.*, 2002). Regions where the twin-arginine motif is conserved are shown as black boxes.

Table 2. Enzymatic activity of intact and sonicated cells

	NAR (%) [a]			Malate dehydrogenase (%) [c]
	MV	BV	— [b]	
Intact cells[d]	75±1	87±6	<2	2±3
Disrupted cells[e]	100±1	91±6	<2	100±9

Relative activities of NAR and malate dehydrogenase are shown with standard deviations (n = 3).
a, All assay mixtures contained 5 mM dithionite and the specified electron carrier. Activity of the disrupted cells with MV was set to 100%.
b, No electron carrier was added.
c, All assay mixtures contained 0.2 mM NADH as the electron donor for malate dehydrogenase. Activity of the disrupted cells with MV was set to 100%.
d, A suspension of intact cells without sonication was used for the assays.
e, A suspension of intact cells was sonicated and then used for the assays.

Table 3. Purification of NAS from *H. thermophilus*

Fraction	Activity [a] (U)	Protein (mg)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude extract	18	962	0.019	1	100
Butyl-Toyopearl	16	65	0.24	13	88
DEAE-Toyopearl	5.8	21	0.28	15	32
Hydroxyapatite	4.1	2.9	1.4	76	23
MonoQ	0.45	0.032	14	758	3

a, Assayed by using Fd1 as the electron donor

mic and not the cytosolic side. The successful preparation of the intact cells without significant contamination with disrupted cells was confirmed by the absence of malate dehydrogenase activity in the intact cell suspension (Table 2).

NAS purification

To identify *H. thermophilus* NAS, we cultivated the cells under aerobic conditions in the presence of nitrate as the sole nitrogen source and prepared the cytosolic crude extract. No significant nitrate-reducing activity was detected when NADH or NADPH was used as the electron donor in the assay. This result suggested that *H. thermophilus* possesses no NAD(P)H-NAS, which is the dominant type of NAS among non-photosynthetic bacteria. When reduced MV was used in the assay as the electron donor, a nitrate-reducing activity was detected, and the enzyme responsible for this activity was purified from the crude extract. The purified enzyme, however, was identical to NarGHI, indicating that the MV-dependent activity (that we detected in the cytosolic fraction) came not from NAS but from NAR. This result is also suggestive of the leaky expression of NAR under aerobic conditions and its weak binding to the cellular membrane.

Three Fd***s***, Fd1, Fd2, and Fd3, have been found in *H. thermophilus*, and some of them were shown to serve as an electron carrier in the central metabolism of this bacterium (Ikeda *et al.*, 2010, Yamamoto *et al.*, 2010, Kameya *et al.*, 2007). Unexpectedly, a significant nitrate-reducing activity was detected in the crude extract when either reduced Fd1 or Fd2 was used as the electron donor. Because the purified NAR showed no significant activity toward these Fd***s*** (see below), we assumed that another nitrate reductase reactive with the Fd***s*** was present in the crude extract. This Fd-dependent activity was detected in the crude extract of the cells grown with nitrate, but was undetectable in the cells grown with ammonium, suggesting that expression of this enzyme is induced by the culture conditions where nitrate is assimilated as the nitrogen source.

The enzyme responsible for the Fd-dependent nitrate reductase activity was enriched and nearly purified from the cytosolic crude extract (Table 3). The purified fraction yielded a single band on SDS-PAGE (Fig. 1). The N-terminal amino acid sequence was MVPFQ, and this sequence is identical to the N terminus of the protein encoded by HTH_1110 (designated as *nasB*). The molecular mass calculated from the primary sequence was 77 kDa, and this value was in agreement with the molecular weight estimated by SDS-PAGE. NasB is homologous to cyanobacterial Fd-NAS (39% identity and E-value of 2×10^{-172} relative to *Synechococcus elongatus* NAS; NCBI GI 499561846) and the catalytic subunit of NAD(P)H-NAS (42% identity and E-value of 5×10^{-175} in relation to *Pseudomonas aeruginosa* NAS; NCBI GI

740617567). *nasA*, a gene homologous to those encoding a nitrate transporter, is located 2 bp upstream of *nasB* (Fig. 2B). No gene homologous to the NAD(P)H-binding subunit of NAD(P)H-NAS was found in this gene cluster. These genetic observations support our notion that *H. thermophilus* possesses not NAD(P)H-NAS but Fd-NAS encoded by *nasB*.

Phylogenetic analysis of NAR and NAS

Using the amino acid sequences of catalytic subunits of NAR and NAS, we constructed a phylogenetic tree (Fig. 4).

H. thermophilus NAS forms a cluster with homologs from *Aquificales*, suggesting that similar Fd-NASs prevail among this order. While Fd-NAS has been found also in cyanobacteria and haloarchaea, the *H. thermophilus* NAS is located outside the two clusters of the reported Fd-NASs. This phylogenetic distance suggests that the *H. thermophilus* Fd-NAS has evolved independently of the known Fd-NASs, as is the case with the Fd-dependent glutamate synthase (Fd-GltS; EC 1.4.7.1) in this bacterium (Kameya *et al.*, 2007).

Surprisingly, *H. thermophilus* NAR is located outside the large cluster consisting of known NARs along with ethylbenzene dehydrogenase (EC 1.17.99.2) and selenate reductase (EC 1.97.1.9), periplasmic molybdoenzymes serving as the outgroup of known NARs. This distinct phylogenetic position is suggestive of early divergence of *H. thermophilus* NAR before the ancestor of previously reported NARs branched from those of the other molybdoenzymes.

Enzymatic properties of NAR and NAS

Gel filtration estimated the native molecular masses of the purified NAR and NAS to be 107 and 42 kDa, respectively. The latter value is significantly lower than that of the single polypeptide of NasB (77 kDa), indicating that the purified enzyme was monomeric. The reason why this chromatographic estimate of molecular mass is so low might be the compact folding of thermophilic enzymes, as often seen in enzymes of this bacterium (Kameya *et al.*, 2007, Kameya *et al.*, 2010). Likewise, it is conceivable that NAR forms a heterotrimer of NarGHI because the estimated mass was lower than the sum of the masses deduced from sequences (206 kDa).

The purified NAR and NAS showed the highest activity in pH ranges 7.0–8.0 and 7.5–8.4, respectively. Although MV and BV could serve as electron donors

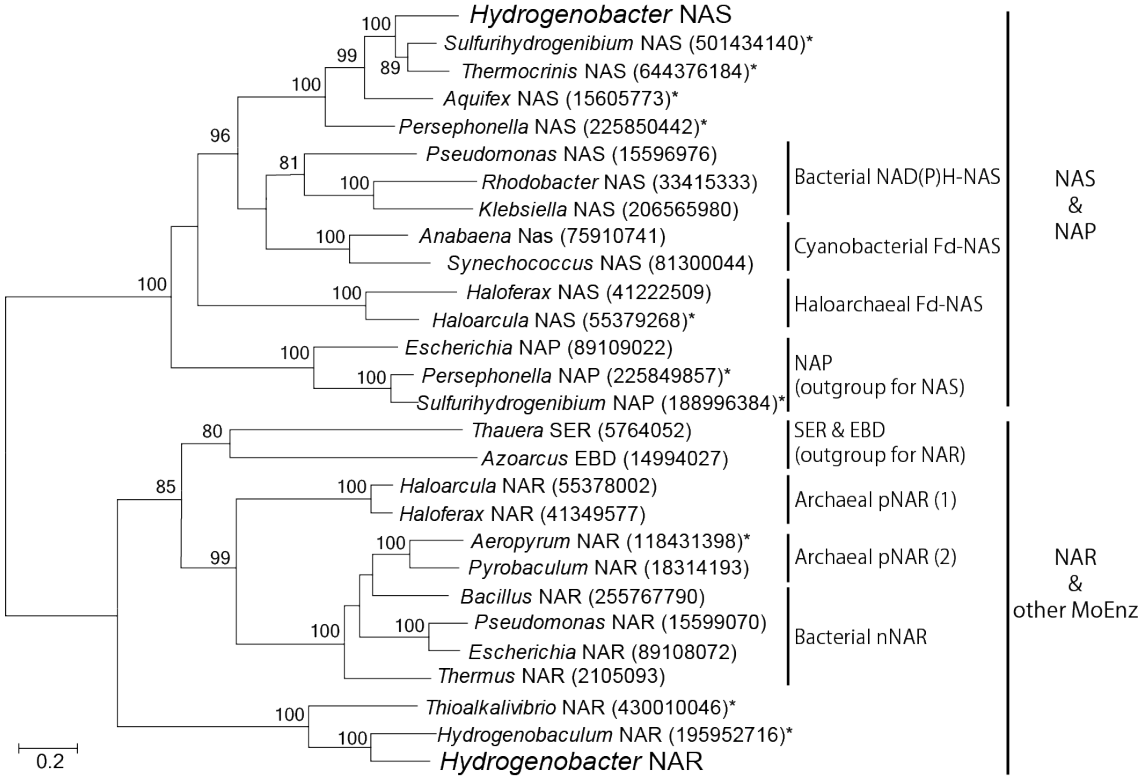

Figure 4. The Phylogenetic tree of nitrate reductases and other molybdoenzymes (MoEnz) constructed by the maximum likelihood method on the basis of the amino acid sequences of the catalytic subunit. NCBI GI numbers of each sequence are shown in parentheses. Bootstrap confidence values (>70) are shown as percentages of bootstrap replicates. Asterisks represent putative proteins that have not been purified and characterized in other studies. Periplasmic nitrate reductase (NAP; EC 1.9.6.1), the third group of nitrate reductase (Richardson *et al.*, 2001, Sparacino-Watkins *et al.*, 2014), serves as the outgroup of NASs. Two molybdoenzymes—ethylbenzene dehydrogenase (EBD) and selenate reductase (SER)—were used as the outgroup of known NARs.

for NAR, no significant NAR activity was observed when NADH, NADPH, or any of the Fd*s* from *H. thermophilus* were used instead. NAS showed comparable reactivity toward Fd1 and Fd2, [4Fe-4S]-type Fd*s* sharing high homology to each other (Ikeda *et al.*, 2005). The K_m value for Fd1 was estimated to be 11 ± 2 μM. Unexpectedly, NAS showed no significant reactivity toward MV, BV, or Fd3, a [2Fe-2S]-type Fd. Fd-NASs that have been found among cyanobacteria and haloarchaea react with [2Fe-2S] Fd*s* as native electron donors and with MV and BV as artificial electron donors (Rubio *et al.*, 1996, Martinez-Espinosa *et al.*, 2001). Thus, the absence of reactivity with these electron donors is unusual among known Fd-NASs.

Transcriptomic analysis

The expression pattern of NAR and NAS was studied by microarray analysis of cells grown in the aerobic ammonium-rich condition (A) as a control, the anaerobic condition where denitrification is active (B), or the aerobic ammonium-limited condition where nitrate assimilation is active (C).

Expression of the genes in the NAR cluster was induced by condition B, whereas that in the NAS cluster was induced by condition C (Fig. 5). These expression patterns indicate that expression of NAR and NAS is induced by an oxygen and ammonium deficiency, respectively. Nitrate respiration is energetically less favorable than aerobic respiration. Likewise, nitrate assimilation is unfavorable compared to ammonium assimilation because the former requires an additional reducing power to convert nitrate to ammonium. Thus, the regulations of nitrate respiration and assimilation observed in this study should enhance the metabolic efficiency of *H. thermophilus* by repressing these unfavored pathways in the presence of oxygen and ammonium, respectively. Significant induction of *narGHI* and *nasAB* expression was not observed in conditions C and B, respectively.

No significant change was observed in the transcriptional activity of HTH_1721–1718 and of HTH_1108 (*cobA2*) through HTH_1105 under the conditions tested. This expression profile suggests that none of these genes is included in the NAR and NAS operons or involved in nitrate respiration and assimilation.

Outside the NAR and NAS gene clusters, the expression of dissimilatory nitrite reductase (*nirS*) and

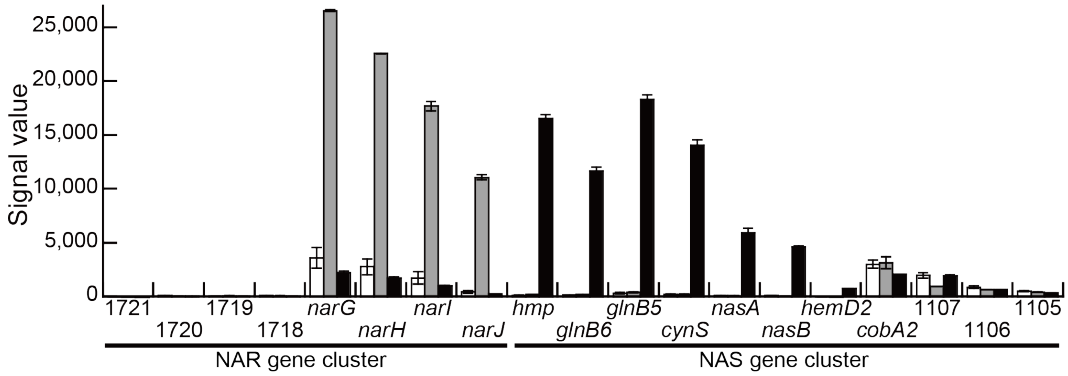

Figure 5. The microarray gene expression profile of the genes in the NAR and NAS gene clusters. The length of the white, gray, and black bars represent the mean of the microarray signal values of each gene under the nitrate-free aerobic (condition A), anaerobic (condition B), and ammonium-free aerobic conditions (condition C), respectively. Error bars represent the standard deviations of the signal values.

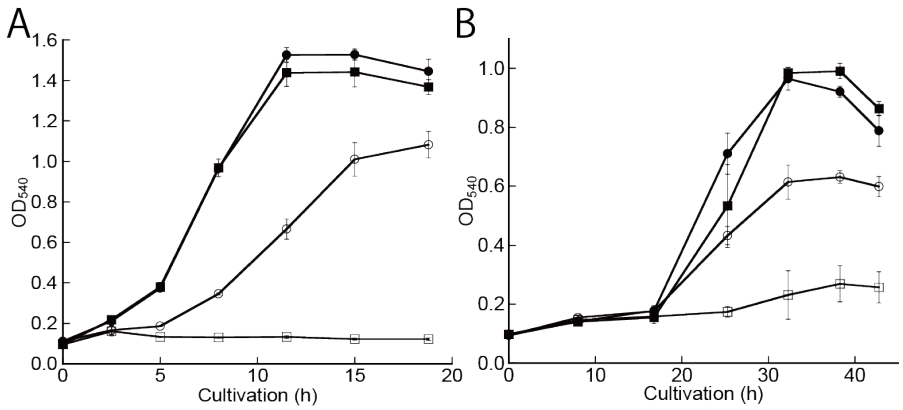

Figure 6. Growth curves of *H. thermophilus* strains under aerobic (A) and anaerobic (B) conditions. Optical density at 540 nm of wild type (circles) and Δ*nasB* (squares) was plotted. The culture medium contained 4 g/l sodium nitrate with (closed symbols) or without (open symbols) 3 g/l ammonium sulfate as a nitrogen source. Error bars represent the standard deviations of the optical density at each time point.

nitric oxide reductase (*norBC*) was significantly up-regulated >10-fold under condition B. These two enzymes are involved in nitrate respiration along with NAR, and the present result demonstrates that the expression of these genes is induced at the transcriptional level under the anaerobic condition. The existence of a unique regulatory system is suggested the upstream region of these genes, where the consensus binding motifs of known expression regulators are absent as described previously (Suzuki *et al.*, 2006a, Suzuki *et al.*, 2006b). On the other hand, the ammonium-limited condition C induced the expression of the genes encoding ammonium transporter (*amtB*) >100-fold, which forms a gene cluster with glutamine synthetase (*glnA*) on the genome. In many organisms, these two proteins are involved in the ammonium assimilation, and their expressions are induced by a transcriptional regulator, NtrC, responsive to ammonium starvation. No NtrC-binding motif (Reitzer & Magasanik, 1986) is found in the upstream regions of the gene clusters harboring *nasB* and *amtB* in the *H. thermophilus* genome, suggesting that another unknown mechanism regulates the expression of these genes in response to nitrogen availability.

Gene disruption

A Δ*nasB* mutant was prepared by insertion of a kanamycin resistance gene into *nasB* according to a previously described method (Yamamoto *et al.*, 2006). We also attempted to prepare a Δ*narG* mutant by the same method, without success.

Under aerobic conditions, the Δ*nasB* mutant did not grow on nitrate in the absence of ammonium, and addition of ammonium restored the growth of this mutant (Fig. 6A). This result demonstrated that NasB is necessary for nitrate assimilation and cannot be compensated by other enzymes. On the other hand, *nasB* was found to be unnecessary for denitrification because the Δ*nasB* mutant showed vigorous growth under anaerobic conditions in the presence of ammonium (Fig. 6B). One study on a ΔNAS mutant of *Bacillus subtilis* showed that anaerobic conditions allowed this mutant to grow well on nitrate as the sole nitrogen source because its nNAR expression was induced and provided nitrite for assimilation in the cytoplasm (Nakano *et al.*, 1998). In contrast to *B. subtilis*, *H. thermophilus* Δ*nasB* showed only faint growth in the anaerobic condition without ammonium, even though NAR expression was expected to be induced. The probable reason for the difference in growth behavior of the ΔNAS mutants is the difference in NAR localization, i.e., either the cytosolic or periplasmic side of the membrane.

Discussion

In this study, NAR and NAS were purified from *H. thermophilus*, and their enzymatic features including the subunit composition, localization, electron donors, expression profile, and physiological role were clarified.

Localization of NAR on the periplasmic side of the membrane is a feature previously unknown among bacteria. The whole-cell assay of NAR activity revealed the outward orientation of this enzyme in *H. thermophilus*. Furthermore, the highly conserved twin-arginine motif and its truncation in the purified NarG confirmed the periplasmic localization of the *H. thermophilus* NAR. Similar outward orientation has been reported for archaeal pNARs from *Haloarcula marismortui* (Yoshimatsu *et al.*, 2002), *H. mediterranei* (Martinez-Espinosa *et al.*, 2007), and *Pyrobaculum aerophilum* (de Vries *et al.*, 2010). Here we clearly demonstrated the presence of pNAR in bacteria, whereas previously this enzyme has only been found in archaea. Genes encoding a component of the twin-arginine translocation machinery are found in the *H. thermophilus* genome (HTH_0502-0505), and their homolog in a related species has been characterized (Rollauer *et al.*, 2012). In addition, twin-arginine motifs are also found in other membrane-bound proteins in *H. thermophilus* (Ueda et al., 2007). These facts are consistent with the NAR export by the twin-arginine translocation system in this organism. pNAR does not require any nitrate/nitrite transporter in principle whereas nNAR does (Fig. 7). The absence of the transporter gene in the NAR gene cluster agrees with the outward orientation of *H. thermophilus* NAR. The poor growth of the ΔNAS mutant on nitrate under anaerobic conditions is also conceivable because pNAR cannot provide intracellular nitrite for assimilation in place of NAS.

H. thermophilus NarI is homologous to EbdC in ethylbenzene dehydrogenase, whose crystal structure has been solved (Kloer *et al.*, 2006). Comparison of the primary sequences revealed that residues involved in the binding of heme *b* in EbdC are conserved in *H. thermophilus* NarI, and thus NarI possibly coordinates heme for the electron transfer from an external donor to NarGH. It is still unclear what electron-transfer component donates electrons to NarI in this bacterium. Although haloarchaeal pNARs are supposed to form a supercomplex with the Rieske protein and diheme cytochrome *b* (Yoshimatsu *et al.*, 2007, Martinez-Espinosa *et al.*, 2007), *H. thermophilus* genome contains

no homologs of these haloarchaeal genes. *H. thermophilus* oxidizes hydrogen as the energy source, and previous studies identified membrane-bound hydrogenases (Ueda *et al.*, 2007, Ishii *et al.*, 2000, Sato *et al.*, 2012b) that transfer electron to methionaquinone in this bacterium (Ishii *et al.*, 1991). In addition, *H. thermophilus* conserves genes encoding the respiratory complex I, and the electron transfer from NADH to ubiquinone was observed in a relative species (Scheide *et al.*, 2002). Thus, it is plausible that the hydrogenases and the complex I serve as indirect electron donors for NAR if NarI can receive electrons from the quinone pool.

From a bioenergetics standpoint, nNAR is advantageous because it can generate a proton electrochemical gradient across the cytoplasmic membrane for ATP synthesis (Fig. 7B). On the other hand, pNAR cannot generate the proton gradient by itself due to the periplasmic localization of its catalytic center (Fig. 7A). Thus, the outward orientation of *H. thermophilus* pNAR seems energetically disadvantageous, although it may contribute to a proton gradient formation if it couples with the complex I or hydrogenases. Meanwhile, a potential advantage of the periplasmic localization is protection of cells from toxic nitrogen oxides at a high growth temperature of *H. thermophilus*, as suggested by a study on pNAR from *P. aerophilum* (de Vries *et al.*, 2010).

Our analysis shows that *H. thermophilus* NAR is phylogenetically distant from the archaeal pNARs (Fig. 4) despite sharing the same localization on the cell membrane. This phylogenetic distance indicates that *H. thermophilus* NAR and archaeal pNARs have not been acquired by horizontal transfer between these taxa but evolved independently. This evolutionary conclusion and the phylogenetically deep origin of *H. thermophilus* suggests that bacterial nNAR evolved from pNAR, and this idea is consistent with a previous presumption that nNAR is derived from an enzyme with the outward orientation (Ize *et al.*, 2009). The deduced origin of nNAR indicates that the energetically unfavored pNAR system, which was originally believed to be specific to archaea, is not a minor derivative but an ancestral form of the energetically favored nNAR system.

The phylogenetic branch of *H. thermophilus* NAR is located not only outside that of known NARs, but also outside the phylogenetic branch of other periplasmic molybdoenzymes such as ethylbenzene dehydrogenase and selenate reductase. The phylogenetic tree suggests that *H. thermophilus* NAR diverged from other NARs at the earliest evolutionary stage and that the above molybdoenzymes have evolved from the probable ancestral pNAR. The latter notion is in agreement with a previously reported hypothesis that archaeal pNARs are evolutionary precursors of the periplasmic molybdoenzymes (de Vries *et al.*, 2010).

We identified *H. thermophilus* NAS as Fd-NAS not NAD(P)H-NAS. Another example of Fd-dependent enzymes involved in nitrogen anabolism of this bacterium is Fd-GltS, which had been believed to be specific to cyanobacteria and plants before its discovery in *H. thermophilus* (Kameya *et al.*, 2007). Thus, the studies revealed that an Fd-dependent metabolic network, which is driven by Fd-NAS and Fd-GltS, is present not only in photosynthetic organisms but also in nonphotosynthetic bacteria including *H. thermophilus*. This finding suggests that Fd may serve as an electron carrier in nitrogen assimilation in a wider range of organisms than previously thought. Indeed, Fd-GltS was recently found in the *Archaea* domain (Pire *et al.*, 2014), and an interesting future question is what range of organism possesses the Fd-dependent network in nitrogen anabolism.

The physiological and evolutionary background for the existence of an Fd-dependent metabolic network

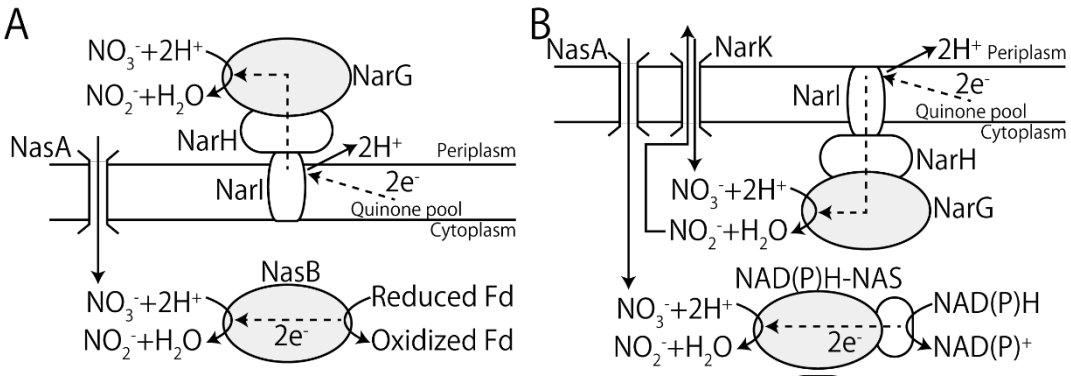

Figure 7. Organization of NAR and NAS in *H. thermophilus* (A) and other nonphotosynthetic bacteria (B). The catalytic subunits of NAR and NAS are shown as gray circles. The other subunits are shown as open rounded squares. Electron transfers are indicated by dotted arrows. *A*, NAR is linked to hydrogenase through a putative electron transfer mediated by a quinone pool. *B*, Assimilatory and dissimilatory nitrate transporters are labeled as NasA and NarK, respectively, which are the typical gene names of these transporters.

is an intriguing issue because the corresponding network in many organisms is NAD(P)H-dependent. It is believed that Fd appeared as a primordial electron carrier at the earliest evolutionary stage of life, and that NAD(P)H and NAD(P)H-dependent oxidoreductases replaced Fd and Fd-dependent enzymes at a later stage (Daniel & Danson, 1995, Fitch & Bruschi, 1987, Eck & Dayhoff, 1966). In cyanobacteria, Fd is an electron carrier indispensable for photosynthesis, the central energy metabolism of phototrophic organisms. Meanwhile, *H. thermophilus* utilizes Fd as an essential electron carrier for the reductive tricarboxylic acid cycle (Ikeda *et al.*, 2010, Yamamoto *et al.*, 2006, Yamamoto *et al.*, 2010), the central carbon metabolic pathway via which this bacterium fixes carbon dioxide as the sole carbon source. This multifaceted physiological necessity of Fd should have forced each organism to maintain a constitutive supply of reduced Fd as a major pathway of intracellular electron flux. Consequently, this constraint might have prevented these organisms from replacing the Fd-dependent enzymes in nitrogen anabolism by NAD(P)H-dependent enzymes.

All of the reported Fd-NASs react with [2Fe-2S]-type Fd***s*** (Rubio *et al.*, 1996, Martinez-Espinosa *et al.*, 2001), which are often called "plant-type Fd." In contrast, *H. thermophilus* NAS showed no significant activity toward [2Fe-2S]-type Fd3 and instead reacted with [4Fe-4S]-type Fd1 and Fd2. While Fd1 and Fd2 are highly homologous to each other, Fd3 shares no significant similarity to these two Fd***s***. Thus, it is conceivable that the difference in the structure of Fd1-3 can differentiate their reactivity with NAS, as often reported in previous studies on Fd-dependent enzymes (Ikeda *et al.*, 2005). In addition, another distinctive feature of *H. thermophilus* NAS is the absence of an activity toward MV and BV, which are commonly used for assays of Fd-dependent enzymes. The reason for this unusual feature is unknown, but it is possible that the binding of Fd may cause a conformational change accompanying activation of the catalytic center. Structural analysis of this enzyme may elucidate the molecular mechanism. The above finding also suggests the possibility that previous studies using MV and BV might have overlooked undiscovered Fd-dependent enzymes.

Our homology search identified homologs of *H. thermophilus* Fd-NAS and pNAR among *Aquificales* species (Fig. 4). Genes highly homologous to *H. thermophilus narG* were found in several ε-proteobacterial species such as *Thioalkalivibrio nitratireducens*. In this bacterium, a soluble monomeric nitrate reductase was identified (Filimonenkov *et al.*, 2010) although the gene encoding this enzyme was not. Further studies on these phylogenetically outstanding enzymes may expand our knowledge about the diversity of nitrate reductases.

Experimental procedures

The bacterial strain and culture conditions

H. thermophilus TK-6 (IAM 12695) was cultivated at 70°C under the following three conditions: (A) the aerobic condition, where the cells were cultivated in an inorganic medium (Shiba *et al.*, 1982) containing 3 g/l ammonium sulfate as the sole nitrogen source under a gas phase of 75% H_2, 10% O_2, and 15% CO_2; (B) the anaerobic condition, where the cells were cultivated under a gas phase of 85% H_2 and 15% CO_2 in a medium that additionally contained 4 g/l sodium nitrate as the terminal electron acceptor; and (C) the aerobic ammonium-limited condition, where the cells were cultivated under the same gas phase as in condition A, in a medium where 4 g/l sodium nitrate was substituted for ammonium sulfate as the sole nitrogen source.

For evaluation of the effects of *nasB* disruption, wild-type and the mutant *H. thermophilus* were precultured in condition A. The cells were harvested and washed to remove the ammonium contamination from the preculture medium and were then inoculated into a medium containing 4 g/l sodium nitrate with or without 4 g/l ammonium sulfate as a nitrogen source. The cells were cultivated under the aerobic or anaerobic gas phase described above (condition A or B).

Escherichia coli JM109 was used as the host strain for construction of plasmids for gene disruption.

*Purification of Fd***s**

Fd1, Fd2, and Fd3 were overexpressed in *E. coli* and purified as described previously (Ikeda *et al.*, 2005, Kameya *et al.*, 2007).

Detection of the nitrate reductase activity

The assay mixtures contained 40 mM sodium phosphate buffer (pH 8.0), 1 mM sodium nitrate, 5 mM MV, 5 mM sodium dithionite, and the enzyme in a total volume of 500 µl. MV was replaced by 5 mM BV or 10 µM Fd1, Fd2, or Fd3 if necessary. The mixtures were anaerobically incubated at 70□C under an Ar gas phase, and the amount of nitrite produced from nitrate was determined by the diazo-coupling reaction (Showe & DeMoss, 1968). The assay mixtures were diluted to 667 µl with deionized water and mixed with 200 µl of a coloring reagent, which was prepared by

mixing 4% sulfanilamide in 25% HCl and 0.08% naphthylethylene-diamine dihydrochloride at a ratio of 2:1. Nitrite concentration was determined by measuring absorbance at 540 nm. One unit was defined as the amount of enzyme that produces 1 μmol of nitrite per minute.

For measurement of NAD(P)H-NAS activity, MV and dithionite in the above assay mixture were replaced by 0.2 mM NADH or NADPH. The decrease in the amount of NAD(P)H was determined by monitoring absorbance at 340 nm. The activity was also measured by nitrite quantification as described above.

NAR purification

To induce NAR expression, we anaerobically cultivated *H. thermophilus* in a medium containing nitrate as the electron acceptor (condition B described above). The harvested cells were resuspended in 20 mM sodium phosphate buffer (pH 6.5) and passed through a French pressure cell, and cell debris was removed by centrifugation at 10,000 × *g* for 10 min. From the supernatant, the membrane fraction was isolated by ultracentrifugation at 100,000 ×*g* for 1 h. After a wash with the buffer to remove the cytosolic proteins, the precipitated membrane fraction was resuspended in 20 mM sodium phosphate buffer (pH 6.5) containing 0.5% Triton X-100 and was stirred for 1.5 h at 4°C. The solubilized proteins were separated by ultracentrifugation at 100,000 ×*g* for 1 h. The supernatant, designated as the solubilized membrane fraction, was applied to a CM-Toyopearl open column (Tosoh, Tokyo, Japan) equilibrated with 20 mM sodium phosphate buffer (pH 6.5) containing 0.5% Triton X-100. The bound proteins were eluted with 20 mM Tris-HCl buffer (pH 6.5) containing 1 M NaCl. Triton X-100 was absent in the eluents used at this and subsequent chromatographic steps because this reagent made no significant difference. After the addition of ammonium sulfate to 30% saturation, the eluted protein solution was applied to a Butyl-Toyopearl column (Tosoh) equilibrated with 20 mM sodium phosphate buffer (pH 6.5) containing ammonium sulfate at 30% saturation. This and subsequent chromatographic steps were conducted on an ÄKTa purifier system (GE Healthcare, Piscataway, NJ, USA). The proteins were eluted with a gradient of ammonium sulfate from 30% to 0%. The active fractions were dialyzed against 20 mM sodium phosphate buffer (pH 6.5) and were applied to a CM-Toyopearl column (Tosoh) equilibrated with 20 mM sodium phosphate buffer (pH 6.5). The proteins were eluted with a gradient of NaCl from 0 to 1 M, and the active fractions were dialyzed against 20 mM sodium phosphate buffer (pH 6.5) and applied to a MonoS HR 5/5 column (GE Healthcare) equilibrated with 20 mM sodium phosphate buffer (pH 6.5). The proteins were eluted with a gradient of NaCl from 0 to 1 M, and active fractions were designated as purified NAR. Throughout the purification procedures, NAR activity was monitored by the assay involving MV as the electron donor.

NAS purification

To induce NAS expression, we cultured *H. thermophilus* aerobically in the presence of nitrate as the sole nitrogen source (condition C described above). The cells were disrupted by sonication, and cell debris and the membrane fraction were removed by ultracentrifugation at 100,000 ×*g* for 1 h. The supernatant, designated as the crude extract, was applied to a DE52 open column (Whatman, Brentford, Middlesex, UK) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$. The bound proteins were eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl, and ammonium sulfate was added to 30% saturation. The protein solution was applied to the Butyl-Toyopearl column equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$ and ammonium sulfate at 30% saturation. This and subsequent chromatographic steps were conducted on the ÄKTa purifier system. The proteins were eluted with a gradient of ammonium sulfate from 30% to 0%. The active fractions were dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$, and were applied to a DEAE-Toyopearl column (Tosoh) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$. The proteins were eluted with a gradient of NaCl from 0 to 1 M. The active fractions were applied to a CHT Ceramic Hydroxyapatite column (Bio-Rad, Hercules, CA, USA) equilibrated with 1 mM potassium phosphate buffer (pH 7.0). The proteins were eluted with a gradient of potassium phosphate buffer from 1 to 400 mM. The active fractions were dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$ and were applied to a MonoQ HR 5/5 column (GE Healthcare) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$. The proteins were eluted with a gradient of NaCl from 0 to 1 M. The active fractions were designated as the purified NAS. Throughout the purification procedures, NAS activity was monitored by the assay involving Fd1 as the electron donor.

The whole-cell assay of the NAR activity

H. thermophilus was anaerobically cultivated in the

presence of nitrate as were the cells used for NAR purification. The harvested cells were resuspended in 20 mM sodium phosphate buffer (pH 7.2). A portion of the intact cell suspension was subjected to sonication for cell disruption, and the resultant suspension containing cell debris was designated as the disrupted-cell suspension.

The intact- and disrupted-cell suspensions were added to nitrate reductase assay mixtures containing 40 mM sodium phosphate buffer (pH 8.0), 1 mM sodium nitrate, 5 mM MV or BV, and 5 mM sodium dithionite. The mixtures were anaerobically incubated at 70°C under an Ar gas phase, and the cells and cell debris were removed by centrifugation (as described above) after the incubation. The amount of nitrite produced in the assay mixtures was determined as described above.

The control samples of cytosolic enzymes were set up as follows: we measured malate dehydrogenase (EC 1.1.1.37) activity in the intact- and disrupted-cell suspensions. The assay mixture containing 20 mM sodium phosphate buffer (pH 8.0), 0.2 mM NADH, 3 mM oxaloacetate, and the suspension was incubated at 70°C, and the decrease in the amount of NADH was monitored by measuring absorbance at 340 nm.

N-terminal amino acid sequencing

For sequencing of the N termini of NarGHI and NasB, the purified enzymes were subjected to SDS-PAGE followed by transfer to a membrane (0.2 μm Sequi-Blot PVDF; Bio-Rad). To determine the internal sequence of NarI, the protein solution was dried under vacuum and treated with 200 μl of 0.9 mg ml^{-1} CNBr in a 70% (v/v) formic acid solution for 16 h at room temperature (Jahnen *et al.*, 1990). After removal of CNBr and formic acid by drying under vacuum, we separated the digested peptides by SDS-PAGE and transferred them to the membrane. A band with approximate molecular mass of 18 kDa was subjected to the subsequent sequencing. The N-terminal amino acid sequences were determined on a Procise 492HT (Applied Biosystems, Foster City, CA, USA).

Gel filtration

For estimation of molecular weights of native NAR and NAS, gel filtration was conducted on a Superose 6 HR 10/30 column (GE Healthcare) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$ and 150 mM NaCl. Gel Filtration Standard (Bio-Rad) was used as a molecular maker for calibration. Each analysis of standards or the enzyme samples was performed in triplicate.

Transcriptomic analysis

H. thermophilus was cultivated under one of the three above-mentioned conditions (A–C), in 10 ml of the medium dispensed in 100-ml vials sealed with rubber caps. Two vials of cells were prepared and independently cultivated in each condition. The cells were harvested when the optical density reached ~0.5. RNA was extracted from the harvested cells, followed by tiling microarray analysis according to NimbleGen Arrays User's Guide for Gene Expression Analysis (Roche NimbleGen, Basel, Switzerland) as described previously (Sato *et al.*, 2012b). All expression data are deposited in the GEO database under the series accession number of GSE70944.

Gene disruption of nitrate reductases

Plasmids for disruption of *nasB* or *narG* were prepared according to a previously described method (Yamamoto *et al.*, 2006). A fragment of *nasB* was amplified from *H. thermophilus* genomic DNA by PCR with primers nasB-F and nasB-R (Table S1) and was inserted into pUC19. A thermostable-kanamycin-resistance gene, *htk*, was amplified from pUC18-*htk* by PCR with primers htk-F-PstI and htk-R-PstI (Table S1) and inserted into the PstI site in the middle of the above-mentioned *nasB* fragment, resulting in a pUC19 containing a *nasB* fragment with an *htk* insertion. Similarly, a pUC19 plasmid harboring a *narG* fragment with an *htk* insertion was prepared by PCR with primer sets narG-F/narG-R and htk-F-ScaI/htk-R-ScaI (Table S1) followed by insertion of *htk* into the ScaI site in the *narG* fragment. Each primer sequence was designed on the basis of the whole-genome sequence of *H. thermophilus* (Arai *et al.*, 2010).

The two plasmids were introduced into *H. thermophilus* by the previously reported method (Yamamoto *et al.*, 2006), and the cells were aerobically cultivated on solid plates of the inorganic medium supplemented with kanamycin. The mutants resulting from double-crossover homologous recombination were selected by kanamycin resistance, and the disruption of each gene was confirmed by PCR.

Phylogenetic analysis

Amino acid sequences of the catalytic subunits of NAR and NAS and their homologs were aligned in the MUSCLE software (Edgar, 2004). Phylogenetic trees were constructed by the maximum likelihood method in MEGA 6 (Tamura *et al.*, 2013).

Acknowledgments

This work was supported in part by Grant-in-Aid for JSPS Fellows 20-6284 to MK and JSPS KAKENHI Grant Number 18380055 to MI.

Conflict of interest

The authors declare that they have no conflicts of interest in relation to the contents of this article.

Author contributions

MK, YI, and MI designed the study. MK, HK, and HA performed the microarray experiments and analyzed the data. MK performed the purification, enzymatic characterization, construction of the mutant, and phylogenetic analysis and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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