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Development of halophilic protein expression system capable of creating a productive enzyme

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Research aims

Haloarchaea are microorganisms that grow under extremely high saline conditions. Most of the proteins and enzymes in the haloarchaea requires a high concentration of salt for stability and catalytic activity[1]. This halophilic property of the enzymes makes them useful for industrial applications, such as food, drug, and fermentation products. Recently, we have been trying to improve gene manipulation techniques in haloarchaea[2]. In this study, the regulatory system of haloarchaeal denitrification was analyzed using the genetic method that we have been developing.

Haloferax volcanii grows by denitrification in an anaerobic environment[3–5]. The denitrifying enzymes of haloarchaea are induced only under the anaerobic condition, but the mechanism of induction control is still unknown. As shown in Fig. 1, a novel DNA-binding protein is encoded by the *narR* gene which is adjacent to the nitrate reductase gene operon *narBCGHD*[6]. To elucidate the role of NarR in the genetic control of denitrification in haloarchaea, we performed expression experiments using recombinant NarR in an *narR*-deletion mutant of *Hfx. volcanii*.

Methods

Deletion of the *narR* gene was performed by a double-crossover method using the *ΔpyrE2* mutant of *Hfx. volcanii* (strain H26)[7]. A pTA*narR* plasmid for gene recombination was constructed by insertion of the upstream (USR) and downstream (DSR) regions of the *narR* gene into a pTA131 vector as shown in Fig. 2A. The pTA*narR* plasmid was introduced into *Hfx. volcanii* H26, then a two-step selection of homologous recombinants was performed based on the nonrequirement for uracil (1st step) and resistance to 5-fluoroorotate (2nd step). Deletion of the *narR* gene was demonstrated in strain SH01 by PCR amplification using USR-f and DSR-r primers (Fig. 2B). Expression vectors for NarR were constructed as follows: the *narR* gene was joined to a *Haloarcula marismortui katG* gene promoter or a *Hfx. volcanii nirK* gene promoter. Here it is notable that KatG is a constitutive enzyme while NirK is induced under the anaerobic condition[2,4]. The resulting DNA fragments were inserted into the *Escherichia coli-Hfx. volcanii* shuttle plasmid pMLH32S to construct expression vectors pHKNR and pHNNR, respectively (Fig. 2C)[2,8]. The pMLH32S, pHKNR, and pHNNR vectors were introduced into SH01 to obtain SH02, SH03, and SH09, respectively. The mutant NarRs were also expressed by using the *katG* gene promoter.

Fig. 1. Gene structure of *narR/narBCGHD*. The novel DNA-binding protein NarR is encoded next to the nitrate reductase gene *narBCGHD* in *Hfx. volcanii* genome.

Results

The effects of the NarR deletion on the growth of *Hfx. volcanii* were analyzed using strains H26, SH01, SH02, SH03, and SH09 as shown in panels A, B, C, D, and E, respectively, in Fig. 3. Under the aerobic condition, all five strains grew quickly and the growth rates were almost uniform. The strains that lacked NarR did not grow anaerobically in the presence of nitrate (B and C), whereas anaerobic growth was recovered by expression of the recombinant NarR (D and E). The result clearly demonstrated that NarR is essential for the denitrification-dependent anaerobic growth of *Hfx. volcanii*.

NarR contains a helix-turn-helix DNA-binding motif in the C-terminal end of the sequence. A novel Cys-rich motif that involves four or five conserved cysteines was identified in the NarR homologues (Fig. 4A). Point-mutagenesis of the *narR* gene was carried out by substitution of the four conserved cysteines (Cys17, Cys81, Cys83, Cys91) and one nonconserved cysteine (Cys100) with serine residues. The five mutant NarRs were expressed in SH01 cells, and then the growth rate of each strain was measured. The result, shown in Fig. 4B, indicated that all four conserved cysteines in the Cys-rich motif were necessary for the regulatory function of NarR in denitrification.

Conclusion

The novel DNA-binding protein NarR, which is characteristic of haloarchaea, controls denitrifying growth of the microbe. It has been known that bacterial denitrification is controlled by the two-component FixLJ and/or FNR system[9], although these regulatory systems are not present

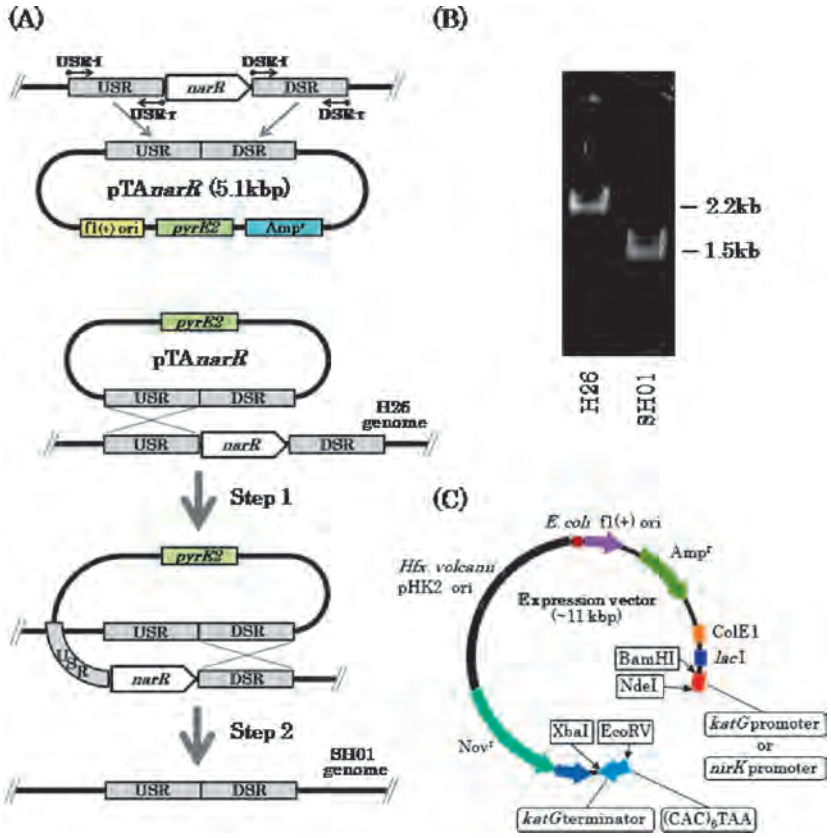

Fig. 2. Experimental protocols. Deletion of the *narR* gene was performed by a double-crossover method (A, B). *Haloarchaeal katG* or *nirK* promoters were utilized for expression of the recombinant NarR (C).

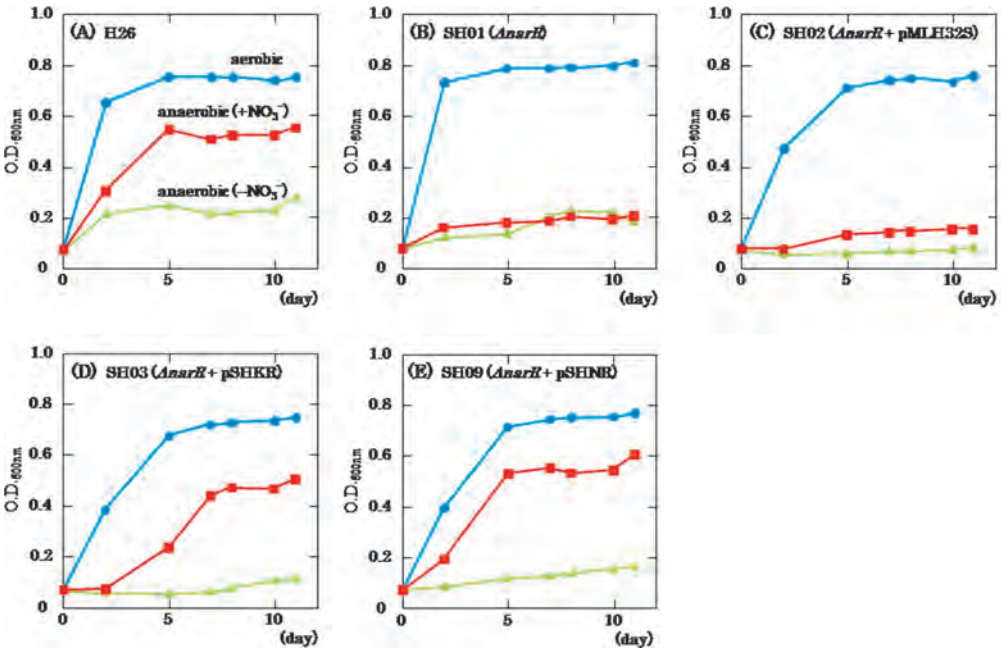

Fig. 3. Effect of NarR deletion on the growth of *Hfx. volcanii*. Growth rates of H26(A), SH01(B), SH02(C), SH03(D), and SH09(E) in the presence of 21% O_2 (blue), 0.2% O_2 supplemented with 50 mM KNO_3 (red), or 0.2% O_2 without KNO_3 (green) were monitored.

in the archaea. Therefore, it is probable that the control of denitrification by the NarR system originally evolved in haloarchaea. NarR homologues are present in six of the nine haloarchaeal species for which the total genome sequence is available. NarR-type DNA-binding proteins play a significant role in the regulation of several anaerobic metabolisms including denitrification in the haloarchaea[10].

In this study, it was demonstrated that the four conserved cysteines in the novel Cys-rich motif were essential for the NarR function. A proposed mechanism of denitrification control by NarR is illustrated in Fig. 5. It is probable that the Cys-rich motif functions as an O_2 or redox sensor. Further investigations of the NarR system (purification of the recombinant NarR, binding assays, etc.) will be performed

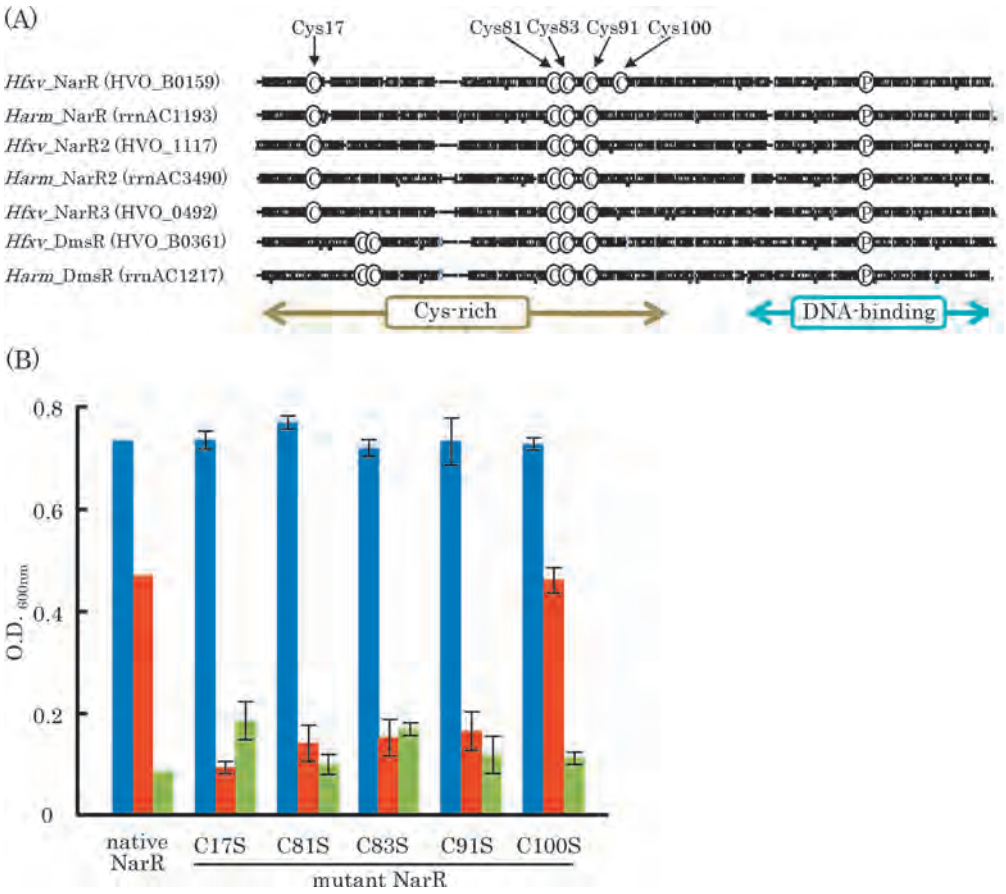

Fig. 4. The Cys-rich motif of NarR. The putative amino acid sequence of the *Hfx. Volcanii* NarR (HVO_B0159) was aligned with those of the homologous proteins(A). Mutant NarRs, in which four conserved cysteines (Cys17, Cys81, Cys83, Cys91) and one nonconserved cysteine (Cys100) were replaced with serine and expressed in SH01(B). Optical densities of each culture were measured after eight days cultivation (n=3). Growth condition: 21% O_2 (blue), 0.2% O_2 supplemented with 50 mM KNO_3 (red), and 0.2% O_2 without KNO_3 (green).

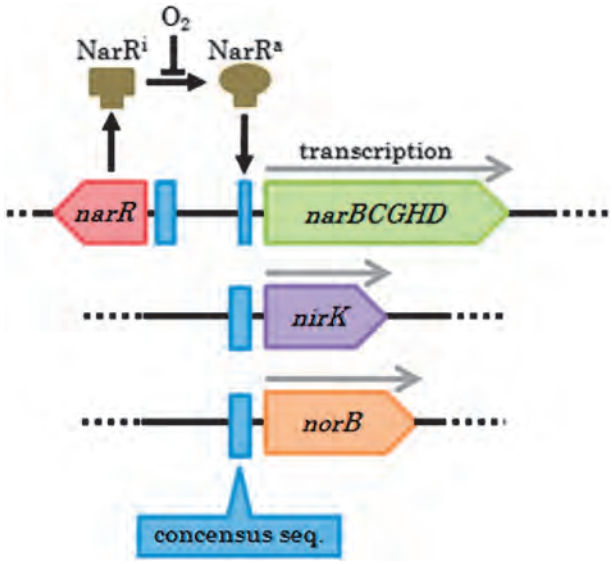

Fig. 5. Proposed mechanism of denitrification control by NarR. NarR is activated in the anaerobic condition. Binding of the activated NarR with the consensus sequence (CGAA(c/g)A(c/t)GTTC(a/g)) triggers transcription of the genes encoding denitrifying enzymes.

in the future.

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