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RESEARCH ARTICLE

Effect of expansion of Shine-Dalgarno sequence for expression of malate- and aldehyde-dehydrogenase genes from *Deinococcus geothermalis* in *Escherichia coli*

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Abstract

Thermostable enzymes have applications in biosensors and bioreactors. Overexpression of thermostable enzyme-encoding genes from thermophiles in Escherichia coli is necessary to produce sufficient quantities of such enzymes. Deinococcus gerthermalis is a moderately thermophilic bacterium and has moderately thermostable enzymes. Nicotinamide adenine dinucleotide (NADH) is an important coenzyme for various dehydrogenases. NADH oxidase (Nox) catalyze the oxidation of NADH. Therefore, Nox is a versatile enzyme that can be used in combination with many types of biosensors. This study reveals the effect of expansion of the SD sequence in the leader sequence of NADH oxidase gene from D. geothermalis for overexpression in E. coli, and shows that while 1- or 2-bases expansion of the Shine-Dalgarno sequence is effective. This study should be contributed to the expression of many genes in E. coli.

Introduction

Biosensors are widely used in medical and healthcare fields such as a blood glucose sensor.[1,2] In this fields, thermostable enzymes have wide applications. Biosensors that use thermostable enzymes are successively used to measure concentrations of various substances. To create such biosensors, it is important to supply sufficient amounts of stable enzyme from a thermophile. Nicotinamide adenine dinucleotide (NADH) is important in cellular respiration and acts as a coenzyme for various dehydrogenase such as glucose dehydrogenase or aldehyde dehydrogenase. NADH oxidase (Nox) is a member of the flavonoprotein disulfide reductase family, and catalyze the oxidation of NADH to NAD^{+}[3] Therefore, Nox is a versatile enzyme that can be used in combination with many types of biosensors.[4,5]

We previously successfully overexpressed the Nox gene (*Nox*) from an extremely thermophile, *Thermus thermophilus* HB8 in *Escherichia coli* by modifying 10 nucleotides upstream of the start codon of the gene: GAAATTAACT was replaces with AACACAATTC.[6] The obtained Nox was stable and active at 80℃, but showed low activity at room temperature. Therefore, we attempted to use Nox from another thermophile, *Deinococcus geothermalis*, which survives at temperatures of 40-50 ℃.[7] The enzymes from this thermophile are expected to be both stable and active at room temperature. Overexpression of this *Nox* in *E. coli* was successfully achieved through the same modification of the 10 nucleotides upstream of the start codon of *Nox*.[8] Following up on these results, in the current study, the aim was to determine the most important nucleotide in the 10-base sequence GAAATTAACT. It was observed that only the first base, G, of the 10 bases is essential for the overexpression of *Nox* from *D. geothermalis* in *E. coli*. The importance of this first G was attributed to its complementarity to the 3'-terminal sequence of 16S rRNA in the 30S ribosome subunit. The modification of this first G base actually leads to an expansion of the Shine-Dalgarno (SD) sequence of the *Nox* mRNA. The SD sequence is a ribosome binding site in leader sequence of mRNA and controls the translation level of the gene.[9,10] In this study, the effect of expansion of the SD sequence in the leader sequence of *Nox* from *D. geothermalis* for its overexpression in *E. coli* is discussed. This study should be contributed to the expression of many genes in *E. coli*.

Methods

The pKK223-3 plasmid was used for cloning and as an expression vector. The leader sequence of *Nox* from *D. geothermalis* was modified using polymerase chain reaction with primers ordered and purchased from FASMAC, Japan. Expression vectors were constructed as previously reported.[6] *E. coli* JM109 competent cell was purchased from TaKaRa-Bio, Japan and transformed using these expression vectors, the transformants were inoculated in 20 mL of Luria -Bertani (LB) broth with 0.1 mg/mL ampicillin and incubated at 37 °C with agitation to obtain an optimal density $(OD)_{600}$ of 0.4-0.6.

Isopropyl-*β*-D-thiogalactopyranoside (IPTG) was subsequently added to a final concentration of 10 μM and grown for another 15 h. The lysis method employed was based on a previous report[11] with modifications. Harvested cells were washed in 10 mM Tris-HCl(pH 8.0) and re-centrifuged then suspended in 10 mM Tris-HCl (250 μL per 0.1 g *E. coli*). Cell lysis was performed using lysozyme (150 μg per 0.1 g *E. coli*) at room temperature for 1 h; then 4% sodium deoxycholate solution (5 μL per 0.1 g *E. coli*) and DNase I were added prior to incubation at 37 °C for 1 h. The final extract was obtained as the supernatant by centrifugation at 15,000 rpm (20,000 *g*) at 4 °C for 15 min. The soluble fraction obtained from *E. coli* JM109 containing the appropriate expression vector was analyzed using 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Nox was purified from the soluble fraction by His-tag technology[12] with the HisLink TM Spin Protein Purification System (Promega, USA). The enzyme assay was carried out as previously reported.[6] The prediction of secondary structure of mRNA transcribed from the *Nox* was analyzed using GENETYX (Genetyx, Japan).

Results

Determination of the important sequence in the 10 bases modification

To determine the important bases in the 10-bases sequence for modification upstream of the start codon of the *Nox* in pDNox[P] for overexpression of the gene in *E. coli*, new expression vectors were constructed- pDNox[P1N9], pDNox[P3N7] and pDNox[P5N5] in which the leader sequence of *Nox* is modified (Fig. 1). [P1N9] means the first base is the same as that in [P], remaining nine bases are the same as those in [N], and so on.

	5′SD sequence	Start codon	His-tag	Stop codon
pDNox[N]	AGGA AACACAATTC	ATG *CATCACCACCATCACCAC*	gene-TAG	3′
pDNox[P]	AGGA **GAAATTAACT** (10 bases modification)	ATG *CATCACCACCATCACCAC*	gene-TAG	
pDNox[P1N9]]	AGGA **G**ACACAATTC	ATG *CATCACCACCATCACCAC*	gene-TAG	
pDNox[P3N7]	AGGA **GAA**ACAATTC	ATG *CATCACCACCATCACCAC*	gene-TAG	
pDNox[P5N5]	AGGA **GAAA**TAATTC	ATG *CATCACCACCATCACCAC*	gene-TAG	

Fig. 1 Modification of the leader sequence of *Nox* in the variant expression vectors

E. coli JM109 was transformed using these expression vectors and the transformants cultured in 20 mL LB medium with 10 mol/L IPTG to induce expression of pKK223-3. The produced Nox was extracted from *E. coli* as a soluble fraction and analyzed using SDS-PAGE (Fig. 2-a). The Nox protein band was detected at approximately 40 kDa, and the expression levels of *Nox* of [P1N9], [P3N7] and [P5N5] were the same as that of [P]. It was observed that only the first G base in the modified 10-bases sequence of [P] was important for overexpression of *Nox* in *E. coli*. SDS-PAGE analysis after His-tag purification revealed that the expression levels of *Nox* of [P1N9], [P3N7] and [P5N5]

were the same as that in [P] and similar to those before His-tag purification. However, the protein band of Nox in [N] was hardly detected (Fig. 2-b).

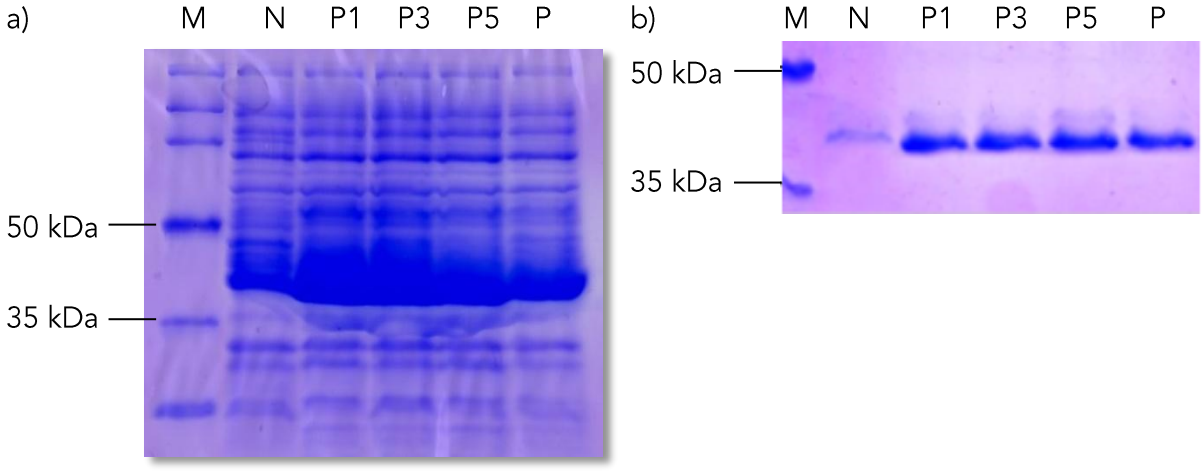

Fig. 2 SDS-PAGE analysis of extracts of *E. coli* containing the variant expression vectors,
a) before His-tag purification, b) after His-tag purification
Lane M: Perfect Protein Marker 15-150 kDa (TaKaRa-Bio, Japan),
Lane N: pDNox[N], Lane P1: pDNox[P1N9], Lane P3: pDNox[P3N7],
Lane P5: pDNox[P5N5], Lane P: pDNox[P]

Expansion of SD sequence in leader sequence

The importance of the first G in the 10-base sequence in [P] was attributed to its complementarity to the 3′-terminal sequence of 16S rRNA in 30S ribosome subunit. The modification of the first G base leads to an expansion of the SD sequence of the *Nox* mRNA. To confirm the effect of expansion of SD sequence for expression of *Nox*, new expression vectors- the pDNox[SD2] to pDNox[SD5] series-were constructed. These have the leader sequence modified and the SD sequence expanded in a stepwise fashion (Fig. 3). pDNox[SD1] is the same as pNox[P1N9].

	$^{5'}$SD sequence	Start codon	His-tag	Stop codon $^{3'}$
pDNox[N]	AGGA AACACAATTC	ATG *CATCACCACCATCACCAC*	gene-TAG	
pDNox[SD1] = [P1N9]	AGGA GACACAATTC	ATG *CATCACCACCATCACCAC*	gene-TAG	
pDNox[SD2]	AGGA GGCACAATTC	ATG *CATCACCACCATCACCAC*	gene-TAG	
pDNox[SD3]	AGGA GGTACAATTC	ATG *CATCACCACCATCACCAC*	gene-TAG	
pDNox[SD4]	AGGA GGTGCAATTC	ATG *CATCACCACCATCACCAC*	gene-TAG	
pDNox[SD5]	AGGA GGTGAAATTC	ATG *CATCACCACCATCACCAC*	gene-TAG	
3′-turminus of 16S rRNA	$^{3'}$AU UCCU CCACUAGGUU			

Fig. 3 Expansion of the SD sequence of *Nox* in the variant expression vectors

SDS-PAGE analysis of *E. coli* extracts containing the various expression vectors without His-tag purification (Fig. 4) showed that the expression level of *Nox* was increased in [SD1] and [SD2] compared with that in [N]. In contrast, the expression levels of *Nox* in [SD3] to [SD5] did not increase compared with that in [N].

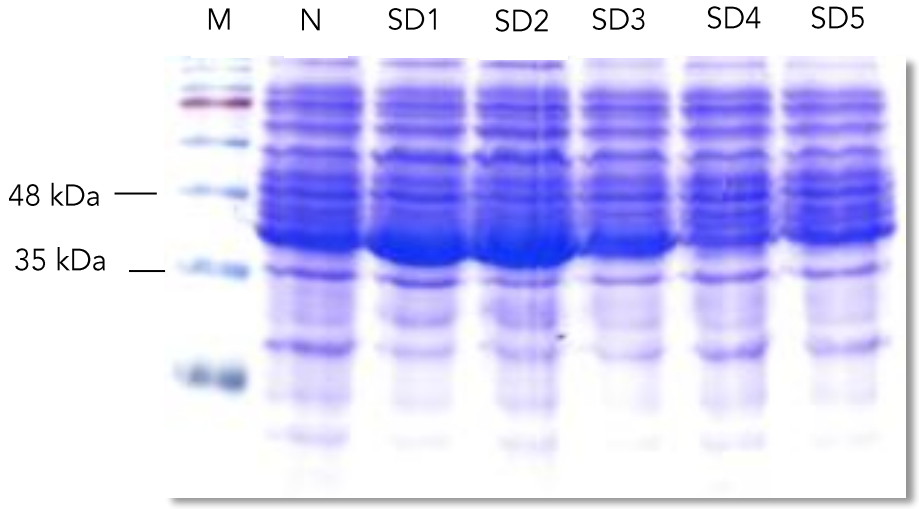

Fig. 4 SDS-PAGE analysis of extracts of *E. coli* containing the variant expression vectors
Lane M: BlueStar Prestained Protein Marker (Nippon Genetics, Japan)
Lane N: pDNox[N], Lane SD1: pDNox[SD1], Lane SD2: pDNox[SD2],
Lane SD3: pDNox[SD3], Lane SD4: pDNox[SD4], Lane SD5: pDNox[SD5],

Activities of Nox in the extracts of *E. coli* containing various expression vectors without His-tag purification were measured base on the decrease in the absorption at 340 nm wavelength, λ_{max}, of NADH (Fig. 5). The activities of Nox from [N] to [SD5] were consistent with the SDS-PAGE results

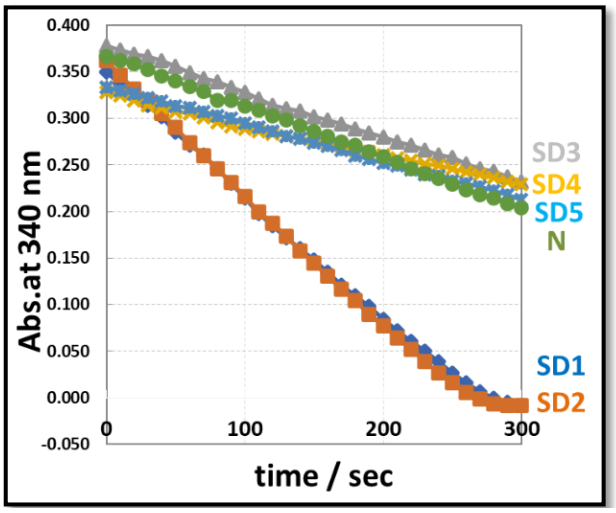

Fig. 5 Nox activity in the extracts of *E. coli* containing the variant expression vectors

It is expected that the expression levels of *Nox* in [SD1] and [SD2] will increase by expansion of SD sequence, but the effect of expansion of SD sequence is limited to two bases. More than three bases of expansion of the SD sequence were not effective for *Nox* overexpression. The secondary structures of the *Nox* mRNA with various SD sequence expansion lengths were analyzed using GENETYX, as the secondary structure around the SD sequence in an mRNA transcribed from a heterogeneous gene strongly influences the level of expression of the heterologous protein.[13,14] The predicted secondary structures of *Nox* mRNA are shown in Fig. 6. The expression level and the secondary structure of *Nox* mRNA were not positively related.

It is not revealed that the effect of expansion of SD sequence of *Nox* for overexpression is limited to two bases. I research the effect of expansion of SD sequence for overexpression of other genes such as malate dehydrogenase or aldehyde dehydrogenase from *D. geothermalis*.

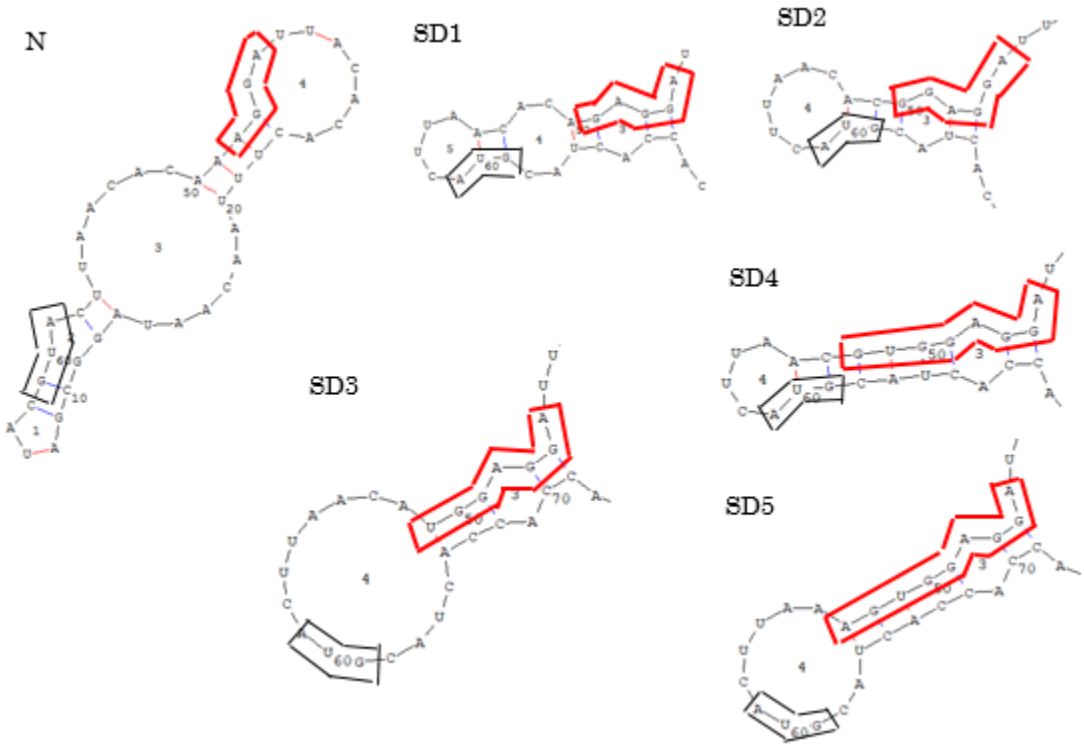

Fig. 6 Secondary structures of mRNA transcribed from the variant expression vectors.
N: pDNox[N], SD1: pDnox[SD1], SD2: pNox[SD2], SD3: pDnox[SD3],
SD4: pNox[SD4], SD5: pNox[SD5], red square indicates the SD sequence.

CONCLUSION.

It is revealed that 1- or 2-bases expansion of SD sequence is effective for the overexpression of the *Nox* from *D. geothermalis* in *E. coli*. On the other hand, the expansions of SD sequence from 3- to 5-bases are not effective for the overexpression.

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