THE EUROPEAN SOCIETY OF MEDICINE
Medical Research Archives, Volume 12 Issue 7

RESEARCH ARTICLE

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

Masahide Ishikawa[1], Natsumi Ishikawa[1], Tomomi Oya-Iwasaki[1]

[1] Department of Life Science and Green Chemistry, Graduate School of Engineering, Saitama Institute of Technology, Fusaiji, Fukaya 3690293, Japan

OPEN ACCESS

PUBLISHED
31 July 2024

CITATION
Ishikawa, M., Ishikawa, N., et al., 2024. Effect of expansion of Shine-Dalgarno sequence for expression of malate- and aldehyde-dehydrogenase genes from *Deinococcus geothermalis* in *Escherichia coli*. Medical Research Archives, [online] 12(7). https://doi.org/10.18103/mra.v12i7. 5729

DOI
https://doi.org/10.18103/mra.v12i7. 5729

ISSN
2375-1924

ABSTRACT

The thermostability of thermophilic enzymes makes them ideal for use in biotechnology and medical science research. The overexpression of heterogeneous genes from thermophiles, such as the mesophilic *Deinococcus geothermalis*, in *Escherichia coli* is a genetic engineering technique for producing stable and useful proteins. In our previous study, 1- or 2-base expansion of the Shine-Dalgarno sequence, which is a ribosome-binding site in mRNA, was effective for the overexpression of the nicotinamide adenine dinucleotide oxidase gene from *Deinococcus. geothermalis* in *Escherichia coli*. In the present study, we examined the effect of expanding the Shine-Dalgarno sequence of the malate dehydrogenase gene and three genes from the aldehyde dehydrogenase family. Our results revealed that a 1- or 2-base expansion of the Shine-Dalgarno sequence was effective for the overexpression of all genes from *Deinococcus geothermalis* in *Escherichia coli*. However, the effects of the expansion of Shine-Dalgarno sequence from 3 to 5 bases were different between these genes.

Introduction

Thermophilic enzymes are widely used in biotechnology and industry. For example, biosensors, such as blood glucose sensors, are widely used in the medical and healthcare fields such as a blood glucose sensor.[1,2] Thermostable enzymes are widely applicable to measure concentrations of various substances. To create such biosensors, sufficient amounts of stable enzymes from thermophiles must be supplied.

In our previous report, we revealed the expansion of Shine-Dalgarno (SD) sequence was effective to expression of the nicotinamide adenine dinucleotide (NADH) oxidase gene (*nox*) from *Deinococcus geothermalis* in *Escherichia. coli*.[3] *D. geothermalis* is a thermophile, which can survive at temperatures of 40-50 °C.[4] The SD sequence is a ribosome binding site complementary to the 3'-terminal sequence of 16S ribosomal RNA (rRNA) in the 30S ribosome subunit in leader sequence of mRNA and controls the translation level of the gene.[5-7] 1- or 2-base expansion of SD sequence is effective for the overexpression, but the expansions of SD sequence from 3- to 5-bases are not effective.

In this study, we aimed to verify the effect of expanding the SD sequence on the expression of the malate dehydrogenase (MDH) gene, *mdh* (locus tag Dgeo_2161) from *D. geothermalis* in *E. coli*. MDH is an enzyme that catalyzes the oxidation of L-malate to oxaloacetate as part of the citric acid cycle. This enzyme is useful for the determination of L-*malate* and glutamate oxaloacetate transaminase in clinical analysis.[8,9] We also examined the effect of the expanding the SD sequence on other aldehyde dehydrogenase (ALDH) family genes: *aldh1* (locus tag Dgeo_1120), *aldh2* (locus tag Dgeo_1174), and *p5cdh* (locus tag Dgeo_0850). *aldh1* is the gene that encodes ALDH1 and is annotated as a succinic semialdehyde dehydrogenase. *aldh2* and *p5cdh* are genes that encode ALDH2 and P5CDH annotated as 1-pyrroline-5-carboxylate dehydrogenase and delta-1-pyrroline-5-carboxylate dehydrogenase 2, respectively. The ALDH family forms an enzyme superfamily that metabolizes endogenous and exogenous aldehydes and is classified substrate specificity.[10]

Methods

Deinococcus geothermalis DSM 11300 obtained from DSMZ (Braunschweig, Germany) was cultured in 1.5 mL TGY broth (1% tryptone, 0.5% yeast extract, 0.5% glucose, and 0.5% NaCl) at 42 °C overnight. The genomic DNA of *D. geothermalis* was extracted using the Gentra Puregene Yeast/Bact. kit (Qiagen, the Netherlands). The gene expression systems were used of pKK223-3 and *E. coli* JM109 as the expression vector and host cell, respectively. The leader sequence of *mdh*, *aldh1*, *aldh2*, and *p5cdh* from *D. geothermalis* were modified using polymerase chain reaction (PCR) with primers ordered and purchased from Sigma-Aldrich Japan or FASMAC (Tokyo, Japan). The forward primer sequences were modified between the SD sequence and start codon of the target gene to expand SD sequence stepwise. Expression vectors were constructed as previously reported.[11,12] The PCR product was digested with *Eco*RI and *Hin*dIII purchased from TaKaRa Bio (Tokyo, Japan) then purified using Wizard® SV Gel and the PCR Clean-Up System from Promega (Tokyo, Japan). The expression vector pKK223-3 was digested using the same restriction enzymes and subsequently dephosphorylated with alkaline phosphatase (calf intestine) and purified with Wizard® SV Gel and the PCR Clean-Up System. For *p5cdh*, *Pst* I was used as restriction enzyme instead of *Hin*dIII. The purified digested genes and expression vectors were ligated using a ligation kit, and competent cells of *E. coli* JM109 were transformed with each expression vector. The transformed *E. coli* JM109 cells were screened on Luria-Bertani broth plates containing ampicillin. The obtained transformant was inoculated in 20 mL of Luria-Bertani 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[13] with modifications. The harvested cells were washed in 10 mM Tris-HCl (pH 8.0), re-centrifuged, and suspended in 10 mM Tris-HCl (2.5 mL per 1 g of *E. coli*). Cell lysis was performed using lysozyme (1.5 mg per 1 g of *E. coli*) at 25 °C for 1 h. Then, 4% sodium deoxycholate solution (50 µL per 1 g of *E. coli*) and DNase I were added prior to incubation at 37 °C for 1 h. The final extract was obtained as a supernatant through 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). MDH was purified from the soluble fraction using His-tag technology[14] with the HisLink™ Spin Protein Purification System (Promega).

The enzyme assay of ALDH1, ALDH2 and P5CDH was carried out according to the literature.[15] The standard enzyme reaction mixture totaled 3 mL and contained 50 mM potassium phosphate (pH 7.4), 1.15 mM $MgCl_2$, 0.5 mM NAD^+ as a cofactor, and 50 mM acetaldehyde as a substrate. The enzyme reaction was performed at 25 °C and was started by adding NAD^+ solution and a preliminary 100 µL of the enzyme solution. ALDH activity was measured as the increase in absorption at 340 nm (A_{340}) of NADH, the product of the enzymatic reaction, using a UV spectrometer, SmartSpec Plus Spectrophotometer of Bio-Rad (Hercules, CA, USA). The enzyme solution was diluted from extracts of *E. coli* so that A_{340} increased linearly to calculate relative activity of the enzyme. The dilution rates of enzyme solution were 10 times for ALDH1 and 4 times for ALDH2 and P5CDH. The relative activity per 1 g of *E. coli* was calculated using the following formula. (A_{340} at 90 sec – A_{340} at 30 sec) indicates an increase in A_{340} for 1 min, 3.00 (mL) is the volume of rection mixture, 6.22 ($mmol^{-1}$ mL cm^{-1}) means millimolar extinction coefficient of NADH, 1.00 (cm) is the optical path length, and 0.100 (mL) is the volume of the enzyme solution. The dilution rate of enzyme solution was 10-fold for ALDH1 and 4-fold for ALDH2 and P5CDH. 2.5 mL per 1 g of *E. coli* means the volume of 10 mM Tris-HCl (pH 8.0) added to the *E. coli* pellet. The relative activity per 1 g of *E. coli* is shown as average of three independent experiments.

$$\text{relative activity (U/mL)} = \frac{(A_{340} \text{ at 90 sec} - A_{340} \text{ at 30 sec}) \times 3.00 \text{ (mL)} \text{ (mL)}}{6.22 \text{ (mmol}^{-1} \text{ mL cm}^{-1}) \times 1.00 \text{ (cm)} \times 0.100 \text{ (mL)}}$$

relative activity per 1 g of *E. coli* =
relative activity (U/mL) × dilution rate of enzyme solution × 2.5 mL per 1 g of *E. coli*

Results

EFFECT OF EXPANSION OF SHINE-DALGARNO SEQUENCE IN LEADER SEQUENCE OF *mdh*

In our previous report,[3] we revealed that expansion of SD sequence affected the expression of *nox* from *D. geothermalis* in *E. coli*. The 1- or 2-base expansion of SD sequence was effective for the overexpression, but the expansion of SD sequence from 3- to 5-bases was not effective. To confirm the effect of expansion of SD sequence on the expression of another gene, *mdh* from *D. geothermalis* in *E. coli*, expression vectors with a modified leader sequence, pDgMDH[N] and pDgMDH[SD1]-[SD5] were constructed as shown in Fig. 1.

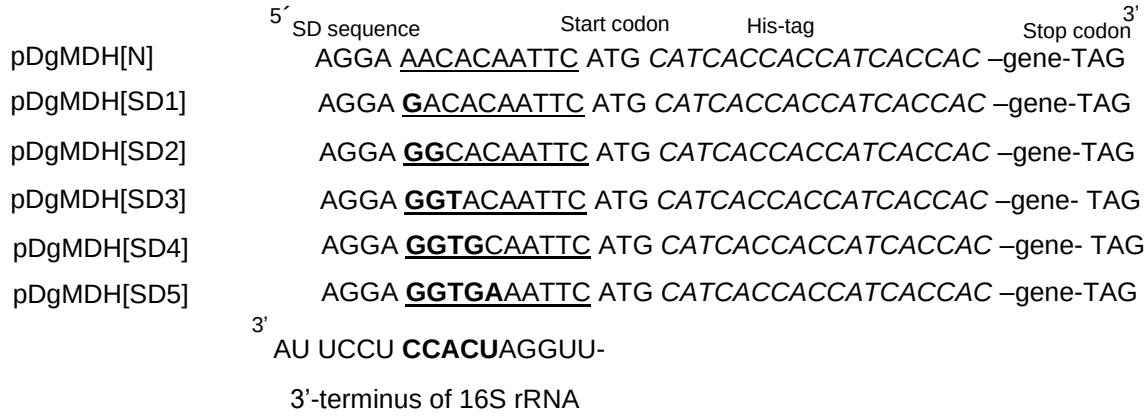

Fig.1 Expansion of SD sequence in the leader sequence of *mdh* in the variant expression vectors

Using these expression vectors, *E. coli* JM109 was transformed, and the transformants were cultured in 20 mL of Luria -Bertani medium with 10 □mol/L IPTG as an inducer. The MDH was extracted from *E. coli* as a soluble fraction and analyzed through SDS-PAGE (Fig. 2a). The expression levels of *mdh* gradually increased in [SD1], [SD2], and [SD3] compared to that in [N], but the expression levels of *mdh* in [SD4] did not increase. The expression of *mdh* in [SD5] increased slightly. These results were similar to those of *nox* from *D. geothermalis* in our previous report.[3] However, *mdh* was different from *nox* in that the expression level of [SD3] was increased considerably and that of [SD5] was increased slightly. SDS-PAGE following His-tag purification revealed that the trend in the expression of *mdh* of [N] and [SD1]-[SD4] was similar to that before His-tag purification. However, the protein band of MDH in [SD5] was hardly detected (Fig. 2b). Therefore, the expression was compared without His-tag purification in subsequent experiments.

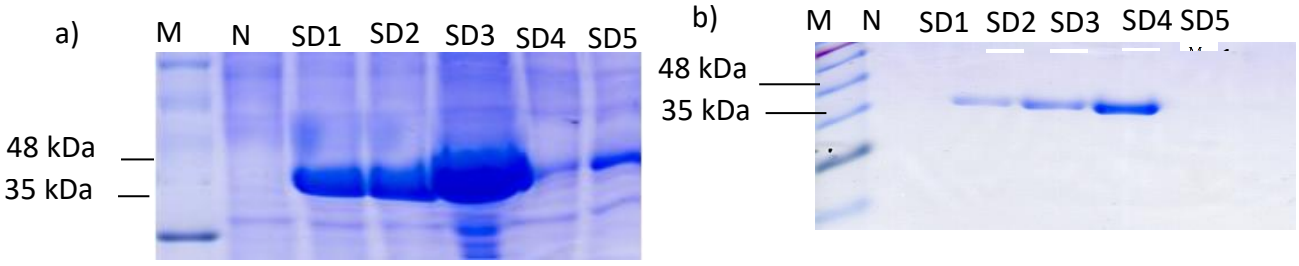

Fig. 2 SDS-PAGE of extracts of *E. coli* containing variant expression vectors of *mdh*.
a) Before His-tag purification, b) after His-tag purification
Lane M: Perfect protein marker 15–150 kDa (TaKaRa-Bio, Japan),
Lane N: pDgMDH[N], Lane SD1: pDgMDH[SD1],
Lane SD2: pDgMDH[SD2], Lane SD3: pDgMDH[SD3],
Lane SD4: pDgMDH[SD4], and Lane SD5: pDgMDH[SD5]

IMPORTANCE OF COMPLEMENTARITY TO THE 3'-TERMINAL SEQUENCE OF 16S RRNA

Similar results were observed between *mdh* and *nox*, where the expression level increased in [SD1], a 1-base expansion of SD sequence, but not increased in [SD4], 4-base expansion of the SD sequence. To confirm the effects of expanding the SD sequence for gene expression regarding the complementarity to the 3'-terminus sequence of 16S rRNA, new expression vectors- pDgMDH[SD1T], pDgMDH[SD1C], pDgMDH[SD4T], and pDgMDH[SD4C]- were constructed (Fig. 3). These were modified so that they had no complementarity to the 3'-terminus sequence of 16S rRNA at the first or the fourth bases of the expanded SD sequence. pDgMDH[SD1A], pDgMDH[SD1G], pDgMDH[SD4A], and pDgMDH[SD4G] were identical to pDgMDH[N], pDgMDH[SD1], pDgMDH[SD3] and pDgMDH[SD4], respectively.

	5´ Start codon His-tag Stop codon 3'
pDgMDH[N] = [SD1A]	AGGA **A**ACACAATTC ATG *CATCACCACCATCACCAC* –gene-TAG
pDgMDH[SD1] = [SD1G]	AGGA **G**ACACAATTC ATG *CATCACCACCATCACCAC* –gene-TAG
pDgMDH[SD1T]	AGGA **T**ACACAATTC ATG *CATCACCACCATCACCAC* –gene-TAG
pDgMDH[SD1C]	AGGA **C**ACACAATTC ATG *CATCACCACCATCACCAC* –gene- TAG
pDgMDH[SD4]= [SD4G]	AGGA **GGTG**CAATTC ATG *CATCACCACCATCACCAC* –gene- TAG
pDgMDH[SD3] = [SD4A]	AGGA **GGTA**CAATTC ATG *CATCACCACCATCACCAC* –gene-TAG
pDgMDH[SD4T]	AGGA **GGTT**CAATTC ATG *CATCACCACCATCACCAC* –gene-TAG
pDgMDH[SD4C]	AGGA **GGTC**CAATTC ATG *CATCACCACCATCACCAC* –gene-TAG
3'-terminus of 16S rRNA	3' AU UCCU **C**CA**C**UAGGUU-

Fig. 3 Exchanges at the first base of [SD1] and the fourth base of [SD4] in *mdh* expression vectors.

The SDS-PAGE of extracts of *E. coli* containing the various expression vectors without His-tag purification (Fig. 4a and b) showed that the expression of *mdh* was increased only in [SD1] (=[SD1G]) in SD1 series and did not increase in [SD4] (=[SD4G]) in SD4 series. These results suggested that the complementarity to the 3'-terminus of 16S rRNA is important for the effect of SD sequence expansion on expression of *mdh*.

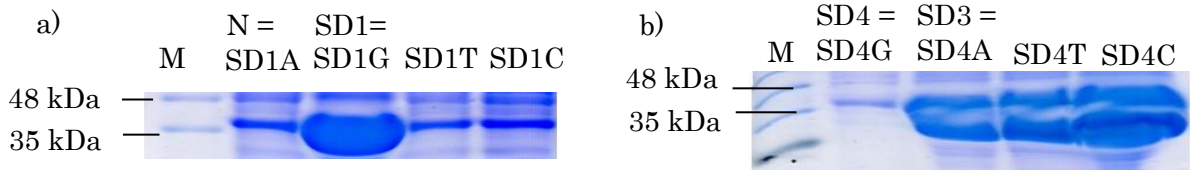

Fig. 4 SDS-PAGE of extracts of E. coli containing the variant expression vectors of *mdh* shown in Fig.3. a) SD1 series, b) SD4 series.

EFFECT OF THE EXPANSION OF SHINE-DALGARNO SEQUENCE IN ALDEHYDE DEHYDROGENASE GENE *ALDH1 ALDH2* AND *P5CDH*

The effect of expanding the SD sequence of *mdh* was similar to that of *nox* in [N], [SD2] and [SD4]; however, it was different from that of *nox* in [SD3] and [SD5]. Therefore, to examine the effect of the expanding the SD sequence in other gene, *aldh1*, we constructed new expression vectors, pDgALDH1[N], [SD1]-[SD5], similar to those constructed for *mdh*, and pDgALDH1[P] with a modified leader sequence, GAAATTAACT, which was effective for overexpression of genes, as our previous reports,[11,12] The SDS-PAGE of *E. coli* extracts containing the various expression vectors without His-tag purification (Fig. 5a) showed that expression of *aldh1* increased in [SD1] and [SD2], but not in [SD4] and [SD5], similar to that of *nox*. In contrast, the expression of *aldh1* in [SD3] increased similar to that of *mdh*.

We also examined the effect of the expanding the SD sequence in *aldh2* and *p5cdh* which are genes of aldehyde dehydrogenase family as same as *aldh1*. In the case of *aldh2* and *p5cdh*, the His-tag sequence was not present downstream of the ATG initiation codon. As the SDS-PAGE results shown in Fig. 5b, the expression of *aldh2* increased in [SD1] and [SD2], but not in [SD3] and [SD4], similar to that of *nox*. However, the expression of *aldh2* in [SD5] was slightly increased similar to that of *mdh*. In the case of *p5cdh*, as shown in Fig. 5c, the expression increased in [SD1]-[SD3] similar to that of *mdh*, but not in [SD5] similar to that of *nox*. In contrast, the expression of *p5cdh* in [SD4] increased, unlike that of other genes.

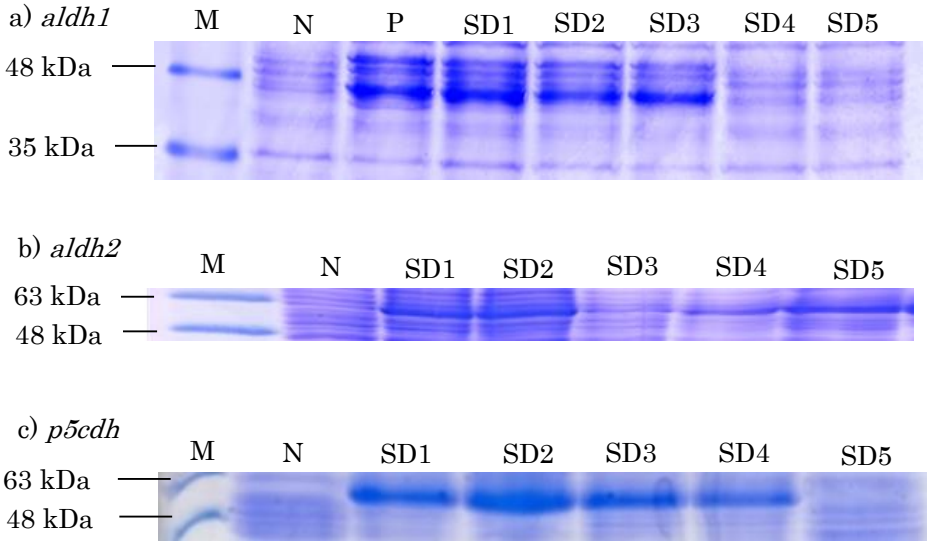

Fig. 5 SDS-PAGE of extracts of *E. coli* containing the variant expression vectors of ALDH family genes, a) Lane M: BlueStar prestained protein parker (Nippon Genetics, Japan), Lane N: pDgALDH1[N], Lane P: pDgALDH1[P], Lane SD1: pDgALDH1[SD1], Lane SD2: pDgALDH1[SD2], Lane SD3: pDgALDH1[SD3], Lane SD4: pDgALDH1[SD4], Lane SD5: pDgALDH1[SD5] for *aldh1*, b) and the pDgALDH2 series for *aldh2*, and c) the pDgP5CDH series for *p5cdh*.

ALDEHYDE DEHYDROGENASE ACTIVITY IN THE EXTRACTS OF *ESCHERICHIA COLI* CONTAINING THE VARIANT EXPRESSION VECTORS OF *ALDH1 ALDH2* AND *P5CDH*

The activities of ALDH1 in the extracts of *E. coli* containing various expression vectors for *aldh1* without His-tag purification were measured based on the increase in the absorption at 340 nm (A_{340}) of NADH (Fig. 6a). The activities of ALDH1 from [N] to [SD5] were consistent with the results of SDS-PAGE (Fig. 5a).

To compare the expression levels of the extracts of *E. coli* containing various expression vectors quantitively, we calculated the relative activity of aldehyde dehydrogenase per 1 g of *E. coli*. The results are shown in Fig. 6b-d for *aldh1*, aldh2, *p5cdh*, respectively. The expression of *aldh1* in [SD1], [SD2] and [SD3] increased about 6-, 7-, and 9-fold, respectively, compared to that of [N] respectively. However, the values in [SD4] and [SD5] did not increased. The expression of *aldh2* in [SD1], [SD2] and [SD5] increased by approximately 3-fold compared to that in [N], and the values in [SD3] and [SD4] increased by approximately 1.5-fold. The expression of *p5cdh* in [SD1], [SD2], [SD3] and [SD4] increased about 4-, 8-, 5- and 4-fold, respectively, compared to that in [N], but not increased in [SD5].

These results were almost matched those of SDS-PAGE, as shown in Fig. 5.

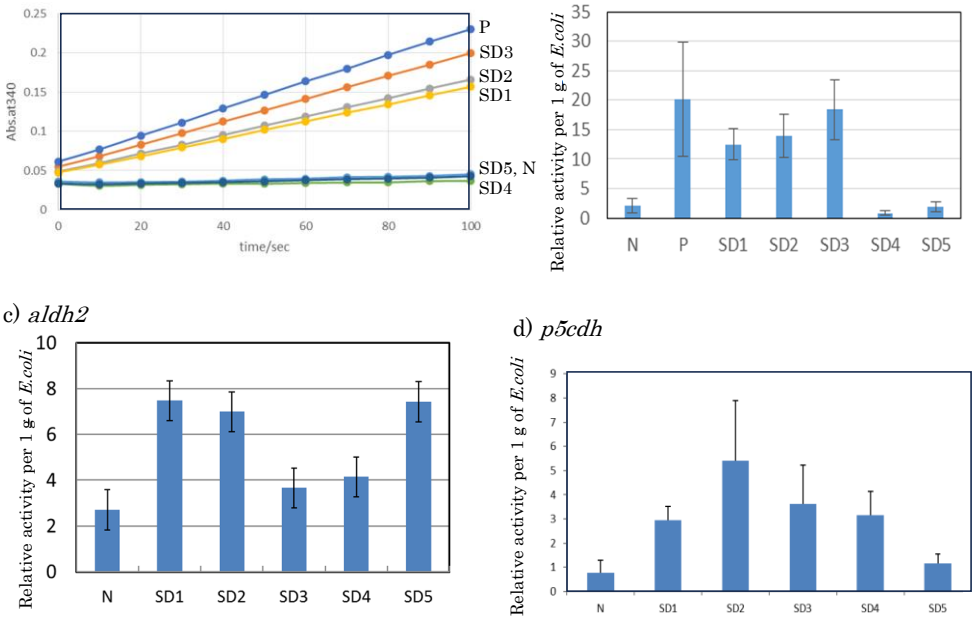

Fig. 6 ALDH activity in the extracts of *E. coli* containing variant expression vectors of *aldh1* a), and relative activity per 1 g of *E. coli* containing variant expression vectors of *aldh1* b), *aldh2* c), and *p5cdh* d). Error bars are based on three independent experiments.

Discussion

We examined the effect of expanding the SD sequence on the expression of *mdh*, *aldh1*, *aldh2,* and *p5cdh* and compared them with the effect on *nox* reported in our previous study.[3] Our results suggest that the expression of all genes in [SD1] and [SD2] increased following 1- and 2-base expansion of the SD sequence. In our results in *mdh*, the expression was increased only in [SD1] = [SD1G], which has a complementary base to the 3'-terminus of 16S rRNA at the expanded base of SD sequence. In contrast, the expression did not increase in [N] = [SD1A], [SD1C], or [SD1T], which had no complementary base at the expanded base. Similar results were observed for *p5cdh* (data not shown). These results indicate that the effect of 1-base expansion of SD sequence causes complementarity to the 3'-terminus of 16S rRNA. We hypothesize that the effect of 2-base expansion also causes complementarity. In contrast, the effects of the expanding the SD sequence from 3 to 5 bases was different among the five genes. In [SD3], i.e., the 3-base expansion, the expression of *mdh*, *aldh1* and *p5cdh* increased but not that of *aldh2* and *nox*. In the case of [SD4], i.e., the 4-base expansion, the expression increased only for *p5cdh*. In [SD5], i.e., 5-base expansion, the expression of *aldh2* significantly increased and that of *mdh* slightly increased, but *aldh1*, *p5cdh,* and *nox* expression did not increase. In our results of SD4 series in *mdh*, the expression was not increased only in [SD4] = [SD4G], which has a complementary base to the 3'-terminus of 16S rRNA at the expanded base of SD sequence. In contrast, the expression increased in [SD3] = [SD4A], [SD4C], or [SD4T], which didn't have a complementary base at the expanded base. These results indicate that the effect of 4-base expansion of SD sequence in [SD4] causes complementarity to the 3'-terminus of 16S rRNA as the same as that of [SD1] in *mdh*. However, the reason the effect of 4-base expansion of the SD sequence being different among the five genes remains unclear. The secondary structure around the SD sequence in an mRNA transcribed from a heterogeneous gene strongly influences the expression of the heterologous protein.[16,17] However, in our previous report, we showed the expression level was not positively related to the secondary structure of *nox* mRNA.[3] In the case of other genes, the secondary structure of these mRNA were not positively correlated with the expression of these genes (data not shown).

Although we present some interesting results regarding this system, the reason the effect of 3- to 5- base expansion of the SD sequence being different among the five genes is not clear. This aspect should be further investigated in future studies.

Conclusions

A 1- or 2-base expansion of the SD sequence was effective for the overexpression of *mdh*, *aldh1*, *aldh2*, *p5cdh,* and *nox* from *D. geothermalis* in *E. coli*. However, the effects of the expanding the SD sequence from 3 to 5 bases was different among the five genes. A 3-base expansion of the SD sequence was effective for *mdh*, *aldh1* and *p5cdh* but not for *nox* and *aldh2*. A 4-base expansion of the SD sequence was effective for *aldh2* and *p5cdh*, but not for *mdh*, *aldh1,* and *nox*. Finally, a 5-base expansion of the SD sequence was effective for *aldh2* only. Our results suggested that the effect of 1-base expansion of SD sequence causes complementarity to 3'-terminus of 16S rRNA in *mdh* and *p5cdh*. However, the reason for the of difference in the effects of the 3- to 5-base expansion of SD sequence is not clear.

References

1. Updike SJ, Hicks GP. The enzyme electrode. *Nature*. 1967;214:986-988. doi: 10.1038/214986a0
2. Schchiri M, Kawamori R, Yamasaki Y, Hakui N, Abe H. Wearable artificial endocrine pancreas with needle-type glucose sensor. *Lancet*. 1982;320:129-1131. doi: 10.1016/s0140-6736(82)92788-x
3. Ishikawa M. Effect of expansion of Shine-Dalgarno sequence for overexpression of nicotinamide adenine dinucleotide oxidase gene from *Deinococcus geothermalis* in *Escherichia coli*. *Med. Res. Arch.*. July 2023;11(7.2). doi: 10.1016/S1001-0742(14)60650-1
4. Ferreira AC, Nobre MF, Rainey FA et al. *Deinococcus geothermalis* sp. nov. and *Deinococcus murrayi* sp. nov., two exttremely radiation-resistant and slightly thermophilic species from hot springs. *Int. J. Syst. Bacteriol.* 1997;47(4):939–947. doi: 10.1099/00207713-47-4-939
5. Shine D, Dalgarno L. The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. *Proc. Natl. Acad. Sci. USA* 1974;71:1342-1346. doi: 10.1073/pnas.7.1.4.1342
6. Steitz JA, Jakes K. How ribosomes select initiator regions in mRNA: base pair formation between the 3'-terminus of 16S rRNA and the mRNA during initiation of protein synthesis in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 1975;72(12):4734-4738. Doi: 10.1073/pnas.72.12.4734
7. Lee K, Holland-Staley CA, Cunningham PR. Genetic analysis of the Shine-Dalgarno interaction: selection of alternative functional mRNA-rRNA combinations. *RNA* 1996;2(12):1270-1285. PMID:8972775
8. Musrati RA, Kollarova M, Mernik N, Mikulasova D. Malate dehydrogenase: distribution, function and properties. *Gen. Physol. Biophys*.1998;17(3):193-210. PMID:9834842
9. Minarik P, Tomaskova N, Kollarova M. Antalik M. Malate dehydrogenase--structure and function. *Gen. Physiol. Biophys*. 2000;21(3):257-265. PMID:12537350
10. Sophos NA, Vasiliou V. Aldehyde dehydrogenase gene superfamily: the 2002 update. *Chem Biol Interact*. 2003;143–144:5–22. doi: 10.1016/s0009-2797(02)00163-1
11. Suzuki S, Matsumura N, Ohoka T, Sakuma S, Nakamura T, Ishikawa M. Important sequence for overexpression of NADH oxidase gene from *Thermus thermophilus* HB8 in *Escherichia coli*. *J. Environ. Sci.* 2009(Suppl 1):S105-107. doi: 10.1016/S1001-0742(09)60049-8
12. Sase K, Iwasaki T, Karasawa H, Ishikawa M. Overexpression of NADH oxidase gene from *Deinococcus geothermalis* in *Escherichia coli*. *J. Environ. Sci.* 2013; 25(Suppl.): S169-171. doi: 10.1016/S1001-0742(14)60650-1
13. Leberman R, Antonsson B, Giovanelli R, Guariguata R, Schumann R, Wittinghofer A. A simplified procedure for the isolation of bacterial polypeptide elongation factor EF-Tu. *Anal. Biochem.* 1980;104:29–36. doi: 10.1016/0003-2697(80)90272-9
14. Hengen P. Purification of His-Tag fusion proteins from *Escherichia coli*. *Trends biochem. Sci.* 1995;20(7):285--286. doi: 10.1016/s0968-0004(00)89045-3
15. Lebsack ME, Gordon ER, Lieber CS. Effect of chronic ethanol consumption on aldehyde dehydrogenase activity in the baboon, *Biochem. Pharmacol.* 1981;30:2273–2277. doi: 10.1016/0006-2952(81)90098-8
16. de Smit, MH, van Duin, J. Secondary structure of the ribosome binding site determines translational efficiency: a quantitative analysis. *Proc. Natl. Acad. Sci. USA*. 1990;87:7668–7672. doi: 10.1073/pnas.87.19.7668
17. Yin J, Bao L, Tian H, Gao X, Yao W. Quantitative relationship between the mRNA secondary structure of translational initiation region and the expression level of heterologous protein in *Eschrichia coli*. *J. Ind. Microbiol. Biotechnol.* 2016;43(1):97-102. doi: 10.1007/s10295-015-1699-1