Regular article https://metallomicsresearch.brte.org

Gene Encoding for Methyltransferase Contributing to Dimethyl Diselenide Synthesis Among Methylated Selenium from *Stutzerimonas stutzeri* NT-I

Osamu Otsuka[1,2*], Mitsuo Yamashita[1]

[1] Collaborative Research Programs for metal biotechnology, Shibaura Institute of Technology, Tokyo, Japan
[2] KFC Ltd., Saitama, Japan

Abstract

Selenium (Se) is a rare metal refined from the slime byproduct of copper anodes. Selenium circulates globally in various valence states and forms. Soluble selenooxyanions, such as selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) are converted to volatile dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe) and vaporized. Although some microorganisms synthesize volatile Se compounds, and volatile Se compounds is used for resource recovery and soil remediation, the synthesis pathway of DMDSe has not yet been identified.

We hypothesized that a methyltransferase in the *Stutzerimonas stutzeri* NT-I specific contig is involved in the synthesis of methylated Se in *S. stutzeri* NT-I and cloned the gene encoding the enzyme. We carried out qualitative analysis of synthesized volatile Se compounds using the transgenic *E. coli* DH5α pGEM-mdsN. A novel gene involved in DMDSe synthesis was identified and named *mdsN* and found to encode a class I SAM-dependent methyltransferase. When the *mdsN* was introduced into *E. coli* DH5α, the recombinant *E. coli* DH5α pGEM-mdsN acquired the ability to synthesize DMDSe, which corresponds to 62% of the initial Se concentration. In this paper, we report a novel finding that *E. coli* DH5α pGEM-mdsN, in which *mdsN* from *S. stutzeri* NT-I was transformed into *E. coli* DH5α, synthesized DMDSe from SeO_3^{2-} and Bio-Se^0 from *S. stutzeri* NT-I.

Keywords: *Stutzerimonas stutzeri* NT-I, Dimethyl diselenide, selenium, biovolatilization, Methyltransferase
Statements about COI: The authors declare no conflict of interest associated with this manuscript.

*** Correspondence:**
Osamu Otsuka
KFC Ltd.
1-19 Ookuwa, Kazo-city, Saitama 347-0010, Japan
Tel : +81-480-76-0095
Fax : +81-480-65-8608
E-mail : ootsuka.osamu@kfc-net.co.jp

Received: September 30, 2024
Accepted: January 07, 2025
Released online: January 16, 2025

Introduction

Selenium (Se) is produced as a by-product of copper in nonferrous metal smelters [1]. Selenium is an industrially important metal resource utilized in photocopiers, glass dyeing, and semiconductor materials, and in pharmaceutical and supplement applications [2]. Moreover, Se circulates in the global environment in various valences and forms. Soluble selenooxyanions, such as selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), present in the soil are converted to volatile dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe), vaporized, and oxidized in the atmosphere to SeO_4^{2-} and SeO_3^{2-}, which are then

 DOI https://doi.org/10.11299/metallomicsresearch.MR202405

Table 1 | Volatile selenium–producing microorganisms and associated synthetic substances

Bacterial strain	Substrate		Substances	Reference
	Concentration	Species		
*Enterobacter cloacae SLD*1a-1	0.01-1.0 mM	SeO_3^{2-}	**DMSe**	[4]
Methylococcus capsulatus (Bath) (NCIBM11132)	20-40 mg·L^{-1}	SeO_3^{2-}	DMSe, DMDSe, DMSeS, Methylselenol, Methylselenoacetate	[5]
	20-40 mg·L^{-1}	Bio-Se^0	DMDSe, DMSeS, DMSe	
	20-40 mg·L^{-1}	Chem-Se^0	DMDSe, DMSeS, DMSe	
Methylosinus trichosporium OB3b (NCIMB 11131)	10-20 mg·L^{-1}	SeO_3^{2-}	DMDSe, DMSeS	[5]
	10-20 mg·L^{-1}	Bio-Se^0	DMDSe, DMSe	
	10-20 mg·L^{-1}	Chem-Se^0	**DMSe**	
Rhodocyclus tenuis DSM 109	1 mM	SeO_4^{2-}	DMDSe, DMSe	[6]
	1 mM	SeO_3^{2-}	**DMSe**	
Rhodobacter sphaeroides DSM 158	114 mg·L^{-1}	SeO_4^{2-}	**DMSe**	[7]
	0.80 mg·L^{-1}	SeO_4^{2-}	—	
	104 mg·L^{-1}	SeO_3^{2-}	DMDSe, DMSeS, **DMSe**	
	0.86 mg·L^{-1}	SeO_3^{2-}	—	
Stenotrophomonas bentonitica	100 mM	SeO_4^{2-}	DMDSe, DMSeS, **DMSe**	[8]
	2 mM	SeO_4^{2-}	—	
	0.1-2 mM	SeO_3^{2-}	**DMDSe**, DMSeS	[9]
Stenotrophomonas maltophilia	0.5 mM	SeO_4^{2-}	**DMDSe**, DMSeS, DMSe	[10]
	0.5 mM	SeO_3^{2-}	**DMDSe**, DMSeS, DMSe	
Stutzerimonas stutzeri NT-I	0.5-5 mM	SeO_4^{2-}	**DMDSe**, DMSeS, DMSe	[11,12]
	0.5-5 mM	SeO_3^{2-}	**DMDSe**, DMSeS, DMSe	
	0.5-5 mM	Bio-Se^0	**DMDSe**, DMSeS, DMSe	
Escherichia coli DH5α pGEM	0.5 mM	SeO_3^{2-}	—	This study
	0.5 mM	Bio-Se^0	—	
Escherichia coli DH5α pGEM-mdsN	0.5 mM	SeO_3^{2-}	**DMDSe**	This study
	0.5 mM	Bio-Se^0	**DMDSe**	

Note: Primary synthesized substances are shown in bold letters. — indicates no substances synthesized.

carried by rainfall [3]. Microorganisms capable of converting SeO_4^{2-} or SeO_3^{2-} into volatile Se compounds have been reported[4-12], suggesting that microbial Se metabolism is involved in global Se cycle.

Among the reports of microorganisms synthesizing volatile Se compounds, there are many reports that multiple methylated selenocompounds such as DMSe and dimethyl selenosulfide (DMSeS) were detected simultaneously (**Table 1**). Some organisms, such as *Enterobacter cloacae* SLD1a-1, synthesize only DMSe [4]. However, to date, no microorganisms that synthesize only DMDSe have been reported. In addition, some microorganisms have different volatile selenium compounds depending on the type of substrate [5,6]. Volatile Se compounds synthesized by *Methylosinus trichosporium* OB3b (NCIMB 11131) include DMDSe and DMSe derived from SeO_3^{2-}, DMDSe and DMSe from bioselenium (Bio-Se^0), and DMSe from elemental selenium(Se^0) [5]. In addition, some microorganisms synthesize volatile Se compounds depending on the substrate concentration [7-9]. However, some microorganisms can synthesize DMDSe in different substrates [10,11]. *Stutzerimonas stutzeri* NT-I reduces SeO_4^{2-}, SeO_3^{2-}, and Se^0 to form DMDSe and DMSeS [11,12]. We previously developed a method to reduce selenooxyanions in solution to volatile

DMDSe and then recover volatile DMDSe with a concentrated nitric acid by utilizing the high SeO_4^{2-} metabolism ability of *S. stutzeri* NT-I [12]. Subsequently, selenium recovered from wastewater was purified to high purity and successfully recycled [12]. It is also used to remove Se from soil by synthesis volatile Se compounds from soluble Se in soil and wastewater [13].

Although some microorganisms synthesize DMDSe and volatile Se compounds, and volatile Se compounds is used for resource recovery and soil remediation, the synthesis pathway of DMDSe has not yet been identified. The ability of *S. stutzeri* NT-I to synthesize DMDSe has been characterized [12]; thus, the DMDSe synthesis pathway can be estimated by analyzing this process. We searched for candidate genes related to DMDSe synthesis from genomic analysis of *S. stutzeri* NT-I and introduced the genes into *Escherichia coli* DH5α to express DMDSe synthesis in recombinant *E. coli* DH5α.

Materials and methods

Growth media and conditions

Stutzerimonas stutzeri NT-I was cultured in Bacto trypticase soy broth (TSB; Becton-Dickinson) or on TSB plates containing 1.5% agar. TSB medium supplemented with ampicillin to a final concentration of 30 $\mu g \cdot mL^{-1}$ or TSB plate medium with 1.5% agar and 2% Xgal 50 µL per plate was used to cultivate recombinant *E. coli* DH5α. Bacterial growth in the culture medium was calculated from the optical density (OD_{600}) at 600 nm using a spectrophotometer (V-600, JASCO Corporation).

DNA cloning and sequencing

The genomic DNA of *S. stutzeri* NT-I was extracted using ISOPLANT (Nippon Gene Co., Ltd.). Using the extracted DNA as a template, the Se methylation candidate gene *mdsN* from *S. stutzeri* NT-I was amplified via polymerase chain reaction (PCR) using the forward primer 5 '-GCGAGAGATTCTCGAC -3', reverse primer 5'-CTCTCCTGTTCTGAATCAGT -3', and TaKaRa La Taq® polymerase (Takara Bio Inc., Japan). The amplification products were TA-cloned (plasmid pGEM-mdsN) using the pGEM-T Easy Vector System (Promega Corporation). White colonies were selected by blue–white selection. Plasmid pGEM-mdsN was extracted from *E. coli* DH5α, and the sequence of the insert was determined using the forward primer 5 '-GTTTTCCCAGTCACGAC -3' and reverse primer 5 '-CAGGAAACAGCTATGAC -3' on a 3730xl DNA Analyzer (Applied Biosystems, Inc.). The *E. coli* DH5α transformed with the pGEM vector was referred to as the *E. coli* DH5α pGEM-control. The obtained sequences were subjected to a homology search using the BLASTN and BLASTX software, supplied by the National Center for Biotechnology Information.

Bio-selenium preparation

Tryptic soy broth (TSB) medium (Becton Dickinson; 30 $g \cdot L^{-1}$) was used for cultivating the *S. stutzeri* NT-I. A loopful of a colony of *S. stutzeri* NT-I was inoculated into 50 mL TSB in a 100 mL Erlenmeyer flask and cultivated at 30 °C for 24 h on a rotary shaker at 120 rpm. A total of 0.5 mL of the culture was then transferred into 50 mL TSB in a 100 mL Erlenmeyer flask and cultivated for 12 h under the same conditions. Bacterial cells were harvested by centrifugation at 1,500 × g, 4 °C for 20 min. The harvested bacterial cells were suspended in adding a sterilized saline solution to $OD_{600} = 1.0$, and then used as the pre-culture solution. The TSB cultivation medium (3 L) was placed inside a jar fermenter (Bioneer C500N type 5L (S) supplied by B.E. Marubishi), which was then autoclaved for 15 min at 101.33 kPa and 121 °C. After autoclaved, sodium selenate was added in the TSB medium to the final Se concentration 5 $mmol \cdot L^{-1}$, which was used as the simulated wastewater. A total of 30 mL of the pre-culture solution was added to this simulated wastewater, cultivation performed under controlled: cultivation temperature, 38 °C; pH, 9.0; agitation speed, 250 rpm; and air flow rate, 1 $L \cdot min^{-1}$. Selenate in the simulated wastewater is almost completely reduced to SeO_3^{2-} by *S. stutzeri* NT-I in 12 h, while after 48 h approximately 90% of SeO_3^{2-} is reduced to Se^0. After 48 h, air flow was stopped and the cultivation was continued for another 24 h.

The culture was centrifuged at 6,000 × g, 4 °C for 20 min to harvest the mixture of the cells and Se^0. The precipitates were washed with 600 mL of distilled water, and then centrifuged for at 6,000 × g, 4 °C for 20 min. The

supernatant was discarded. Next, 600 mL of 70% ethanol was added to the precipitations, which were recovered by centrifugation at 20,000 × g, 4 °C for 20 min. This precipitates were referred to Bio-Se^0. Bio-selenium was then dried by using an automatic oven at 40 °C for 24 h. The color of the Bio-Se^0 was red.

Selenite reduction test

One colony was scraped from a plate of *S. stutzeri* NT-I and recombinant *E. coli* DH5α pGEM-mdsN, *E. coli* DH5α pGEM-control, inoculated into 50 mL of TSB medium (pH 7.0) in a 100 mL volume flask, and cultivated at 30 °C for 24 h on a rotary shaker at 120 rpm. A total of 0.5 mL of the culture was then transferred into 50 mL TSB in a 100 mL Erlenmeyer flask and cultivated for 12 h under the same conditions. Subsequently, bacterial cells were collected by centrifugation (15,000 × g, 20 °C for 5 min), and the collected bacterial cells were suspended in sterile saline and adjusted to an OD_{600}=1.0.

Then, 30 mL of the suspension was added to 3 L of TSB medium containing 0.5 mM SeO_3^{2-} and 30 μg·mL^{-1} ampicillin in a 5L-volume jar fermenter (Bioneer-C500 N Model 5 L (S), B.E.MARUBISHI CO., LTD.). Culturing was carried out at 38 °C, pH 7.0, aeration at 1 L·min^{-1}, and agitation speed at 120 rpm. The pH was adjusted using 30% sodium hydroxide solution and 2 N hydrochloric acid solution. Dissolved oxygen (DO) and pH were measured using a DO electrode OX-2500 and a pH combination electrode MPS-220 (B.E. MARUBISHI Co., Ltd.), respectively. The recovery method of volatilized Se compounds was referred to by Winkel *et al.* [14]. The exhaust from the jar fermenter was passed through 150 mL of concentrated nitric acid solution (*i.e.*, a gas trap) dispensed into a 250 mL capacity reagent bottle using a Teflon tube (inner diameter, 5 mm; outer diameter, 6 mm). The cultures and concentrated nitric acid were collected each 24h.

Qualitative methylated selenium

S. stutzeri NT-I, *E. coli* DH5α pGEM-control, and *E. coli* DH5α pGEM-mdsN were each plated with one colony, inoculated into 50 mL of TSB medium in a 100 mL volume flask, and incubated aerobically at 37 °C for 24 h. The culture medium was inoculated into 1 mL TSB medium, incubated under the same conditions for 12 h, and used as the preculture medium. Bacteria were obtained by centrifugation (2,300 × g, 4 °C for 20 min) from the preculture solution. Bacterial cells were washed twice with sterile saline and resuspended in saline to an OD_{600} = 1.0. Then, 10 mL of TSB medium was added to a 100 mL volume vial, and Bio-Se^0 or SeO_3^{2-} was added as a substrate to achieve a final Se concentration of 0.5 mM. Then, 100 μL of the resuspension solution was added to the flask, which was then stoppered with butyl rubber and incubated at 37 °C for 12 h with shaking. After 12 h of incubation, 250 μL of the gas-phase sample was collected using a micro-syringe, and the gas-phase components were qualitatively analyzed by GC-MS (FocusGC DSQII, Thermo Fisher Scientific K.K).

Qualitative and quantitative analysis of elements

Before measuring the Se concentration, the samples were centrifuged (15,000 × g, 20 °C for 5 min) to separate the supernatant from the precipitate. The supernatants were filtered with a membrane filter (0.2 μm pore size, Steradisc 13, Kurabo, Osaka, Japan), and the resultant filtrates were used to measure Se concentration. Selenate and SeO_3^{2-} concentrations were determined using an ion chromatography (IC) system (ICS-1100, Thermo Fisher Scientific K.K.) equipped with a DS6 heated conductivity cell detector and an ASRS300 suppressor. An IonPac AS12A separation column (Thermo Fisher Scientific K.K) equipped with an AG12A guard column (Thermo Fisher Scientific K.K) was used. The mobile phase comprised 3 mM sodium carbonate solution prepared with ultrapure water (Barnstead NANO Pure, Thermo Fisher Scientific K.K.) at a flow rate of 1.5 mL·min^{-1}. The total soluble Se concentration in the supernatant was determined using inductively coupled plasma atomic emission spectrometry (ICP-AES) (iCAP 6300 Duo, Thermo Fisher Scientific K.K.). The soluble Se concentrations other than SeO_4^{2-} and SeO_3^{2-} were calculated by subtracting the SeO_4^{2-} and SeO_3^{2-} concentrations from the total soluble Se concentration. The concentrations of the metal elements in the volatile substances collected in gas trap were also measured using ICP-AES. The precipitates were washed with 2 mL of ultrapure water and collected by centrifugation (15,000 × g,

20 °C for 5 min). The washing procedure was repeated twice. Next, the precipitates were digested in a mixed acid solution (1,500 µL of 60% nitric acid solution, and 50 µL of 95% sulfuric acid solution) using a vortex for 10 min. After centrifugation (15,000 × g, 20 °C for 5 min), the supernatants were collected in a volumetric flask. The acid-digestion supernatants were mixed in a volumetric measuring flask with ultrapure water to a total volume of 10 mL. The total Se concentration in the digested samples was determined by ICP-AES. All samples were measured in triplicate, and the average values were used for the analysis.

Analysis of selenium species in the gas phase

The exhausted gaseous samples were analyzed using GC-MS. A fused-silica capillary column (30 m × 0.250 mm (inner diameter)) (DB-624, Agilent Technologies) was used. Splitless injections were performed, and the split valve was opened 1 min after injection. Helium was used as the carrier gas at 1.0 mL·min^{-1}. The column temperature was programmed from 40 to 240 °C at a rate of 10 °C·min^{-1} after being maintained at 40 °C for 5 min and then kept at 240 °C for 1.5 min. Both the injector and the interface (between GC and MS) temperatures were kept at 200 °C. Ionization was performed in the positive ion mode at an ion source temperature of 220 °C. Gaseous samples were injected at 250 µL by 250 µL of gas tight syringe. DMSe, DMDSe, and DMDS (Tokyo Chemical Industry Co., Ltd.) were used as standard solutions.

Results

Cloning of selenium methylation-related gene

The genome of *S. stutzeri* NT-I has been characterized previously, yielding 115 contig sequences with a total contig base number of 63,558,482 bp [15]. Comparative genomic analysis of the reference strain, *Stutzerimonas stutzeri* A1501, revealed 43 contigs unique to *S. stutzeri* NT-I (data not shown). *S. stutzeri* NT-I-specific contigs contained SeO_4^{2-} reduction genes such as the SeO_4^{2-} reduction gene *serA* (Access No. ACV70151), which is a characteristic of *S. stutzeri* NT-I. This suggests that the 43 contigs unique to *S. stutzeri* NT-I characterize its high Se metabolism capacity of *S. stutzeri* NT-I. Genes involved in the synthesis of volatile Se compounds in microorganisms and plants other than *S. stutzeri* NT-I have also been reported. Ranjard *et al*. reported that the gene *tpm*, which encodes thiopurine methyltransferase from *Pseudomonas syringae pathovar pisi*, was transformed into *E. coli* DH10B, and SeO_3^{2-} was added as a substrate to synthesize DMSe and DMDSe [16]. Swearingen *et al.* reported that the gene *ubiE* encoding the ubiquinone/menaquinone biosynthesis C-methyltransferase from *Geobacillus stearothemophilus* V was introduced into *E. coli* K12 to express its ability to synthesize DMSe and DMDSe from SeO_4^{2-} and SeO_3^{2-} [17]. Zhou *et al.* reported that the gene *BoCOQ5-2* encoding COQ5 methyltransferase from *Brassica oleracea varitalica* was transformed into *E. coli* to synthesize DMDSe and DMSe from SeO_4^{2-} [18]. All the enzymes that confer the ability to synthesize volatile Se compounds are methyltransferase genes. However, the genes involved in DMDSe synthesis have not yet been reported.

An open reading frame (ORF) search (ORFFinder NCBI) of 43 contigs unique to *S. stutzeri* NT-I revealed that the gene (Accession No.: PSNT00042, 44319-44918 bp) encoding class I SAM-dependent methyltransferase was included in 43 contigs unique to *S. stutzeri* NT-I. Thus, the gene encoding the class I SAM-dependent methyltransferase was named *mdsN* and designated as a potential selenomethylation gene for *S. stutzeri* NT-I.

The candidate gene *mdsN* was amplified by PCR and subjected to the agarose gel electrophoresis (**Fig. 1**). The PCR amplification product was inserted into the pGEM-T easy vector to transform *E. coli* DH5α. A transformant in which the PCR amplification product of the expected size was inserted was obtained by blue–white selection on the LB-Amp plate medium. The pGEM-mdsN plasmid extracted from the transformant was subjected to restriction enzyme treatment with *Eco*RI. The inserted sequence was excised into an approximately 600 bp fragment (**Fig. 2**). These results indicate that PCR-amplified mdsN could be inserted into the pGEM-easy vector. Sequence analysis of the inserted sequence showed 100% identity with a class I SAM-dependent methyltransferase (Accession No.: PSNT00042, 44319-44918bp) from *S. stutzeri* NT-I registered in NCBI.

E. coli DH5α reduces SeO_3^{2-} and synthesizes Se^0 [19]. However, there is no report on the synthesis of DMDSe and DMSe by the reduction of Se^0 in *E. coli* DH5α. Therefore, *E. coli* DH5α pGEM-control was used as a negative control.

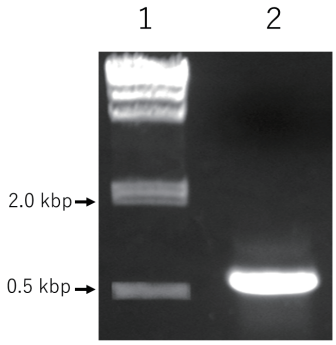

Fig. 1 **Electrophoresis of PCR products**
Lane 1: λ DNA/HindIII markers;
Lane 2: *mdsN* (about 600 bp)

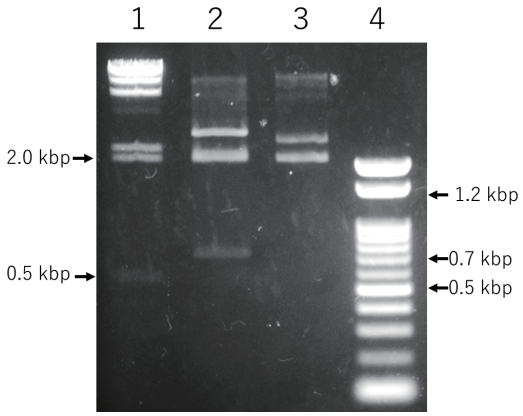

Fig. 2 **Electrophoresis of plasmids**
Lane 1: λ DNA/*Hin*dIII markers,
Lane 2: After *Eco*RI restriction enzyme treatment of pGEM-mdsN;
Lane 3: pGEM-mdsN;
Lane 4: 100 bp DNA ladder marker.

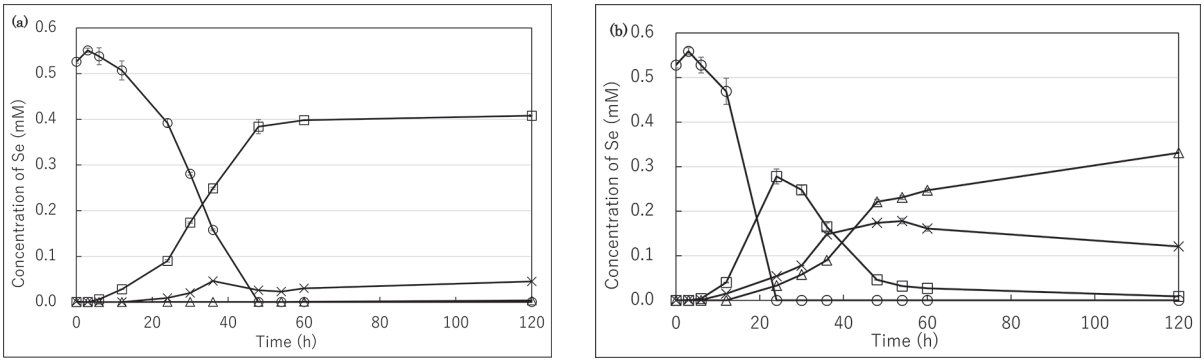

Fig. 3 **Time course of selenium concentrations of selenite reduction test**
(a) *E. coli* DH5α pGEM.
(b) *E. coli* DH5α pGEM-mdsN. Circle: Selenite, Square: Elemental selenium, Cross: Soluble Se without selenate and selenite, Triangle: Volatile selenium (gas trap).

Selenite reduction test of *E. coli* DH5α pGEM-mdsN

E. coli DH5α pGEM-mdsN and negative control *E. coli* DH5α pGEM-control were cultured in TSB medium containing SeO_3^{2-} (**Fig. 3a**). The time course of Se concentrations in the culture medium showed that *E. coli* DH5α pGEM-control reduced SeO_3^{2-} to Se^0 within 48 h after SeO_3^{2-} addition (**Fig. 3a**). Subsequently, Se^0 accounted for 0.41 mM (78% of its initial value) at 120 h. The amount of Se^0 did not decrease between 48 and 120 h. Analysis of metallic elements in the gas trap at 120 h revealed 0.02 mM of sulfur, suggesting that *E. coli* synthesized small amounts of volatilized sulfur compounds (**Table 2**).

Conversely, *E. coli* DH5α pGEM-mdsN reduced SeO_3^{2-} to Se^0 at 24 h after SeO_3^{2-} addition (**Fig. 3b**). Subsequently, elemental selenium decreased from 48 to 120 h to nearly zero. In contrast to the decrease in Se^0 from 48 to 120 h, an increase in Se was detected in the gas trap, suggesting that *E. coli* DH5α pGEM-mdsN synthesized volatilized Se compounds from Se^0 in the culture broth. At 120 h, the Se detected by the gas trap accounted for 62% of the initial value. Analysis of metal elements in the gas trap at 120 h showed that 0.08 mM sulfur and 0.33 mM Se were detected. The amount of Se detected in the gas trap of *E. coli* DH5α pGEM-mdsN was the same as that of *S. stutzeri* NT-I (**Table 2**). Therefore, it was suggested that the transformation of *mdsN* into *E. coli* DH5α had the same high Se methylation ability as that of *S. stutzeri* NT-I. The amount of sulfur produced by *E. coli* DH5α pGEM-mdsN in

Table 2 | Elemental analysis results in the gas trap (mM)

Bacterial strain	S	Se
Escherichia coli DH5α pGEM-control	0.02	<0.01
Escherichia coli DH5α pGEM-mdsN	0.08	0.33
Stutzerimonas stutzeri NT-I	0.51	0.37

the gas trap was 4-fold higher than that by *E. coli* DH5α pGEM-control but only approximately 16% of that in the *S. stutzeri* NT-I, suggesting that sulfur volatilization is not enhanced in *E. coli* DH5α pGEM-mdsN.

These results suggest that *mdsN* is strongly involved in the synthesis of volatile Se compounds because it can synthesize volatilized Se compounds in *E. coli* DH5α transfected with the class I SAM-dependent methyltransferase gene from *S. stutzeri* NT-I.

Quantitative determination of volatile selenium compounds

To qualitatively identify the volatile Se compounds synthesized from *E. coli* DH5α pGEM-mdsN with recombinant methyltransferase from *S. stutzeri* NT-I, the gas phase of the culture vessel was analyzed (**Fig. 4**). *S. stutzeri* NT-I synthesizes DMSe and DMDSe when SeO_3^{2-} is used as the substrate, with DMDSe being mainly synthesized (**Fig. 4a**)[11]. When *E. coli* DH5α pGEM-mdsN was incubated with SeO_3^{2-}, only DMDSe was detected in the gas phase (**Fig. 4b**). Conversely, nothing was detected in the gas phase of the culture vessel containing the control *E. coli* DH5α pGEM-control and the negative control experiment containing only SeO_3^{2-} without bacteria (**Fig. 4c, 4f**).

To determine whether volatile Se compounds can be synthesized from Bio-Se^0, a similar experiment was performed by changing the substrate to Bio-Se^0 synthesized by *S. stutzeri* NT-I (**Fig. 5**). The elemental compositions of Bio-Se^0 were quantitatively analyzed using an ICP-AES. Other than Se, inorganic components of Bio-Se^0 comprise six elements: calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), phosphorus (P), and sulfur (S). Bio-selenium was washed with distilled water during its preparation, which removed the TSB medium-derived impurities. Ca, K, Mg, Na, P, and S were suggested to have been derived from bacterial cells. Concentrations of Se in Bio-Se^0 was 11% (mass %)[20]. In the culture of *S. stutzeri* NT-I with Bio-Se^0 as the substrate, DMDSe and a small amount of DMSe were observed, similar to the SeO_3^{2-} added culture (**Fig. 5a**). In contrast, no Se species were detected in the gas phase when Bio-Se^0 alone was added to the TSB medium without bacteria (**Fig. 5d**). Because no turbidity change was observed in the culture solution containing only Bio-Se^0, it is considered that all the *S. stutzeri* NT-I used in the synthesis may have been killed in Bio-Se^0, and there was no microbial contamination in the Bio-Se^0.

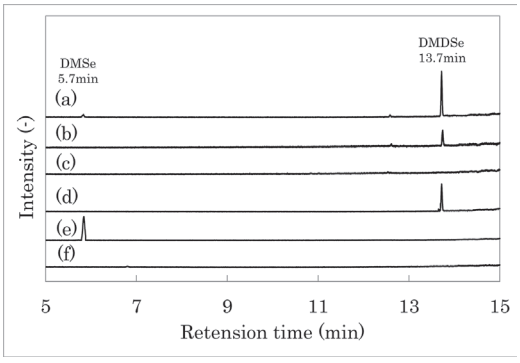

Fig. 4 **GC-MS spectra of gas phase from selenite reduction test**
(a) *S. stutzeri* NT-I,
(b) *E. coli* DH5α pGEM-mdsN,
(c) *E. coli* DH5α pGEM-control,
(d) DMDSe std,
(e) DMSe std, and (f) selenite without bacteria.

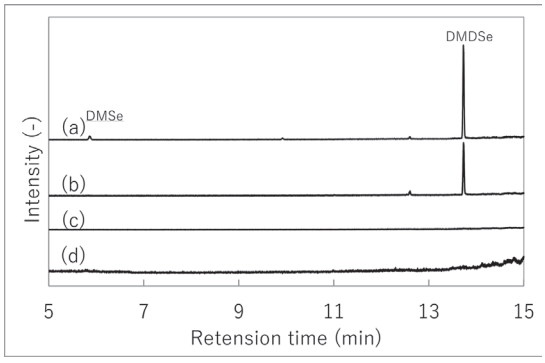

Fig. 5 **GC-MS spectra of gas phase from Bio-Se^0 reduction test**
(a) *S. stutzeri* NT-I,
(b) *E. coli* DH5α pGEM-mdsN,
(c) *E. coli* DH5α pGEM-control, and
(d) Bio-Se^0 without bacteria.

When Bio-Se^0 was used as a substrate, only DMDSe was detected in the gas phase of *E. coli* DH5α pGEM-mdsN (**Fig. 5b**). In the control experiment, nothing was detected in the gas phase of the culture vessel of *E. coli* DH5α pGEM-control (**Fig. 5c**).

These results suggest that *E. coli* DH5α pGEM-mdsN transfected with the gene encoding class I SAM-dependent methyltransferase acquired the ability to synthesize DMDSe.

Homology analysis of the gene involved in DMDSe synthesis

The *E. coli* DH5α pGEM-mdsN strain acquired the ability to synthesize DMDSe, and the nucleotide sequence of the gene encoding the inserted class I SAM-dependent methyltransferase (Accession No.: PSNT00042, 44319-44918bp) was analyzed. A homology search using BLASTN revealed homology of the nucleotide sequence was 98% with *Stenotrophomonas sp.* As-6 chromosome (Accession No. CP127404). The amino acid sequence showed 100% homology with a class I SAM-dependent methyltransferase (Accession No.: WP_205404086) from *Gammaproteobacteria*. However, the independent synthesis of DMDSe by the class I SAM-dependent methyltransferases of *Gammaproteobacteria* has not yet been reported.

In addition, considering that there might be another enzyme involved in DMDSe synthesis near the class I SAM-dependent methyltransferase, we analyzed the sequence of 5,000 bp around *mdsN* (Accession No.: PSNT00042, 39319-49918 bp) encoding the class I SAM-dependent methyltransferase in the *S. stutzeri* NT-I genome but found no genes involved in Se metabolism (data not shown).

Discussion

We hypothesized that a methyltransferase in the *S. stutzeri* NT-I-specific contig is involved in the synthesis of methylated Se in *S. stutzeri* NT-I and cloned the gene encoding the enzyme. We carried out qualitative analysis of synthesized volatile Se compounds using the transgenic *E. coli* DH5α pGEM-mdsN. A novel gene involved in DMDSe synthesis, that was identified and named *mdsN*. The gene encodes a class I SAM-dependent methyltransferase. When *mdsN* was introduced into *E. coli* DH5α, the recombinant *E. coli* DH5α pGEM-mdsN acquired the ability to synthesize Se to DMDSe, which corresponds to 62% of the initial Se concentration.

The enzymes *tpm*, *ubiE*, and *BoCOQ5-2* involved in the synthesis of both DMSe and DMDSe have been reported [16-18]. However, the genes involved in the synthesis of DMDSe, such as *mdsN*, have not been reported.

The results of qualitative experiments on volatilized Se compounds and SeO_3^{2-} reduction tests in *E. coli* DH5α pGEM-mdsN indicate that the concentration of SeO_3^{2-} decreases and Se^0 increases, followed by a decrease in Se^0. This suggests that *E. coli* DH5α pGEM-mdsN reduces SeO_3^{2-} to Se^0 and then synthesizes DMDSe using Se^0 as a substrate.

These results suggest that *E. coli* DH5α pGEM-mdsN has a pathway to synthesize DMDSe using Se^0 reduced from SeO_3^{2-} as a substrate. The results of qualitative analysis of volatilized Se compounds using Bio-Se^0 (**Figs. 4 and 5**) showed that *S. stutzeri* NT-I synthesized both DMSe and DMDSe, while strain *E. coli* DH5α pGEM-mdsN synthesized only DMDSe. This suggests that *S. stutzeri* NT-I contains genes encoding enzymes involved in DMSe synthesis in addition to class I SAM-dependent methyltransferases. Therefore, further studies on the reaction of *S. stutzeri* NT-I to synthesize methylated Se will lead to the elucidation of DMDSe and DMSe synthetic pathways that have not yet been elucidated.

In this paper, we report a novel finding that *E. coli* DH5α pGEM-mdsN, in which *mdsN* from *S. stutzeri* NT-I was transformed into *E. coli* DH5α, synthesized DMDSe from SeO_3^{2-} and Bio-Se^0 from *S. stutzeri* NT-I. The identification of a novel gene involved in DMDSe synthesis is expected to advance our understanding of the DMDSe synthesis pathway.

References

[1] Wang C. Selenium minerals and the recovery of selenium from copper refinery anode slimes. *J. South. Afr. Inst. Min. Metall.* 2016; 116(6): 593–600. doi: 10.17159/2411-9717/2016/v116n6a16

[2] Srivastava P, Kowshik M. Anti-neoplastic selenium nanoparticles from Idiomarina sp. PR58-8. *Enzyme and Microbial Technology.* 2016; 95: 192–200. doi: 10.1016/j.enzmictec.2016.08.002

[3] Winkel LHE. *et al.* Environmental Selenium Research: From Microscopic Processes to Global Understanding. *Environ. Sci. Technol.* 2012; 46(2): 571–579. doi: 10.1021/es203434d

[4] Dungan RS, Frankenberger WT Jr. Factors affecting the volatilization of dimethylselenide by Enterobacter cloacae SLD1a-1. *Soil Biology and Biochemistry.* 2000; 32(10): 1353-1358. doi: 10.1016/S0038-0717(00)00044-4

[5] Eswayah AS, Smith TJ, Scheinost AC, Hondow N, Gardiner PHE. Microbial transformations of selenite by methane-oxidizing bacteria. *Appl Microbiol Biotechnol.* 2017; 101(17): 6713–6724. doi: 10.1007/s00253-017-8380-8

[6] McCarthy S, Chasteen T, Marshall M, Fall R, Bachofen R. Phototrophic bacteria produce volatile, methylated sulfur and selenium compounds. *FEMS Microbiol Lett.* 1993; 112(1): 93–97. doi: 10.1111/j.1574-6968.1993.tb06429.x

[7] Van Fleet-Stalder V, Chasteen TG, Pickering IJ, George GN, Prince RC. Fate of Selenate and Selenite Metabolized by Rhodobacter sphaeroides. *Appl Environ Microbiol.* 2000; 66(11). doi: 10.1128/aem.66.11.4849-4853.2000

[8] Ruiz-Fresneda MA, *et al.* Combined bioreduction and volatilization of SeVI by Stenotrophomonas bentonitica: Formation of trigonal selenium nanorods and methylated species. *Science of The Total Environment.* 2023; 858(2): 160030. doi: 10.1016/j.scitotenv.2022.160030

[9] Ruiz-Fresneda MA, Eswayah AS, Romero M, Gardiner PHE, Solari PL, Merroun ML. Chemical and structural characterization of SeIV biotransformations by Stenotrophomonas bentonitica into Se0 nanostructures and volatiles Se species. *Environmental Science Nano.* 2020; 2020(7): 2140. doi: 10.1039/D0EN00507J

[10] Dungan RS, Yates SR, Frankenberger WT Jr. Transformations of selenate and selenite by Stenotrophomonas maltophilia isolated from a seleniferous agricultural drainage pond sediment. *Environmental Microbiology.* 2003; 5(4): 287–295. doi: 10.1046/j.1462-2920.2003.00410.x

[11] Kagami T, *et al.* Effective selenium volatilization under aerobic conditions and recovery from the aqueous phase by Pseudomonas stutzeri NT-I. *Water Research.* 2013; 47(3): 1361–1368. doi: 10.1016/j.watres.2012.12.001

[12] Otsuka O, Yamashita M. Selenium recovery from wastewater using the selenate-reducing bacterium Pseudomonas stutzeri NT-I. *Hydrometallurgy.* 2020; 197: 105470. doi: 10.1016/j.hydromet.2020.105470

[13] Otsuka O, Yamashita M. Methods for recovering and recycling selenium from wastewater and soil. *Metallomics Research.* 2022; 2(3): rev28–39. doi: 10.11299/metallomicsresearch.MR202208

[14] Winkel L, Feldmann J, Meharg AA. Quantitative and Qualitative Trapping of Volatile Methylated Selenium Species Entrained through Nitric Acid. *Environ. Sci. Technol.* 2010; 44(1): 382-387. doi: 10.1021/es902345m

[15] Kuroda M, Sei K, Yamashita M, Ike M. Draft Genome Sequence of Stutzerimonas stutzeri NT-I, Which Reduces Selenium Oxyanions into Elemental Selenium and Volatile Selenium Species. *Microbiol Resour Announc.* 2022; 11(12): e01016–22. doi: 10.1128/mra.01016-22

[16] Ranjard L, Nazaret S, Cournoyer B. Freshwater Bacteria Can Methylate Selenium through the Thiopurine Methyltransferase Pathway. *Applied and Environmental Microbiology.* 2003; 69(7): 3784–3790. doi: 10.1128/aem.69.7.3784-3790.2003

[17] Swearingen JW Jr, *et al.* Expression of the ubiE Gene of Geobacillus stearothermophilus V in Escherichia coli K-12 Mediates the Evolution of Selenium Compounds into the Headspace of Selenite- and Selenate-Amended Cultures. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY.* 2006; 72(1): 963-967. doi: 10.1128/AEM.72.1.963-967.2006

[18] Zhou X, *et al.* Involvement of a broccoli COQ5 methyltransferase in the production of volatile selenium compounds. *Plant Physiology.* 2009; 151(2): 528–540. doi: 10.1104/pp.109.142521

[19] Kuroda M, *et al.* Molecular Cloning and Characterization of the srdBCA Operon, Encoding the Respiratory Selenate Reductase Complex, from the Selenate-Reducing Bacterium Bacillus selenatarsenatis SF-1. *J. BACTERIOL.* 2011; 193. doi: 10.1128/JB.01197-10

[20] Otsuka O, Yanaba Y, Yoshikawa T, Yamashita M. Fundamental Studies on Oxidizing Roasting of the 'Bioselenium'. *Mater. Trans.* 2016; 57(7): 1183–1191. doi: 10.2320/matertrans.M2016060