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Metagenome Resource for D-Serine Utilization in a DsdA-Disrupted *Escherichia coli*

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To find alternative genetic resources for D-serine dehydratase (E.C. 4.3.1.18, *dsdA*) mediating the deamination of D-serine into pyruvate, metagenomic libraries were screened. The chromosomal *dsdA* gene of a wild-type *Escherichia coli* W3110 strain was disrupted by inserting the tetracycline resistance gene (*tet*), using double-crossover, for use as a screening host. The W3110 *dsdA::tet* strain was not able to grow in a medium containing D-serine as a sole carbon source, whereas wild-type W3110 and the complement W3110 *dsdA::tet* strain containing a *dsdA*-expression plasmid were able to grow. After introducing metagenome libraries into the screening host, a strain containing a 40-kb DNA fragment obtained from the metagenomic souce derived from a compost was selected based on its capability to grow on the agar plate containing D-serine as a sole carbon source. For identification of the genetic resource responsible for the D-serine degrading capability, transposon-μ was randomly inserted into the 40-kb metagenome. Two strains that had lost their D-serine degrading ability were negatively selected, and the two 6-kb contigs responsible for the D-serine degrading capability were sequenced and deposited (GenBank code: HQ829474.1 and HQ829475.1). Therefore, new alternative genetic resources for D-serine dehydratase was found from the metagenomic resource, and the corresponding ORFs are discussed.

Keywords: D-serine utilization, metagenome, *dsdA* disruption, transposon-μ, homologous recombination

D-Form amino acids are not generally found in nature, although a trace amount is found in some bacteria, worms, and other invertebrates [2], circulation organs cells of mammals, and human brain cells [1, 17]. The *N*-methyl-D-aspartate (NMDA) receptor requires co-activation by glycine and glutamate. D-Serine, similar to glycine, is also able to bind at the NMDA receptor [8]. D-Serine and its derivatives are of interest because it has been suggested as a starting material or an intermediate for synthesis of pharmaceuticals [5, 10] such as D-cycloserine, a tuberculosis-treating drug, as well as medical treatment for neuropsychiatric disorders such as Schizophrenia, Alzheimer's Disease, autism, depression, benign forgetfulness, childhood learning disorders, closed head injury, and attention deficit disorder [16]. The chemical synthesis of D,L-serine followed by racemation or separation using chiral chromatography have been proposed as a manufacturing method, but no biological process has been attempted. Biological synthesis of L-serine, however, has been studied using metabolically engineered bacterial strains [4, 6, 11, 15].

D-Serine dehydratase (E.C. 4.3.1.18, *dsdA*) mediates the deamination of D-serine into pyruvate, and is a critical enzyme for D-serine degradation although it cannot spontaneously mediate the reverse reaction. To develop a metabolically engineered strain for biological D-serine synthesis, a novel genetic resource for the reversible D-serine-related reaction (*i.e.*, racemase) is required. A metagenome refers to all genetic material present in a certain environmental sample consisting of the genomes of many nonculturable individual organisms, and therefore a metagenome would provide the opportunity to find novel genetic resources.

In this study, we report the construction of a *dsdA* knockout mutant of *E. coli* and screening of a novel genetic resource for D-serine degradation from a metagenomic library, which might have potential for D-serine biosynthesis leading to the biological production of D-serine.

Materials and Methods

Plasmid Construction

Routine DNA manipulation techniques were used as previously described [13]. The plasmids and DNA used in this study are listed

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Table 1. Plasmids and DNA used in this study.

	Description	Source
pLex	Constitutive expression vector harboring Amp^R	Invitrogen Co.
pLex-*dsdA*	pLEX harboring *dsdA* with *Bam*HI and *Xho*I restriction cut	This study
pBR325	Cloning vector harboring Amp^R, Cm^R, Tet^R	Reference [14]
pLex-*dsdA*::*tet*	pLEX-*dsdA* harboring *tet* gene at *Nco*I site in *dsdA*	This study
pCVD442	Suicide vector harboring *sacB*, Amp^R	Reference [12]
pCVD442-*dsdA*::*tet*	pCVD442 harboring *dsdA*::*tet* (2.5 kb) with *Sac*I, *Xba*I restriction cut	This study
pCC1Fos	40 kb metagenome library harboring fosmid, Chl^R	Epicentre Co.
Transposon-μ	Randomly inserting sequences, HyperMu KAN-1	Epicentre Co.
G208402-7	Screened 40-kb DNA fragment possessing the capacity concerning D-serine metabolism in pCC1Fos	This study
DNA fragment #22 and #28	6-kb DNA sequences in G208402-7, lost the D-serine metabolism capacity by transposon-μ insertion	This study

in Table 1. The *dsdA* gene was amplified by PCR, based on the genome from *E. coli* W3110, using oligonucleotides 5'-AGCTGT GGATCCTATGGAAAACGCTAAAATG-3' (*Bam*HI site underlined) and 5'-CAGTCTCGAGTTAACGGCCTTTTGCCAG-3' (*Xho*I site underlined). After T-vector ligation (TA cloning vector; RBC, Taipei, Taiwan) and digestion by *Bam*H1–*Xho*I, the PCR product of *dsdA* (1.3 kb) was purified and further ligated into an expression vector, pLex (Invitrogen Co., Carlsbad, CA, USA), which yielded pLex-*dsdA*. The oligonucleotides of 5'-CCATGGATGAAATCTAACAAT GCGCTC-3' (*Nco*I site underlined) and 5'-CCATGGTCAGGTCGAG GTGGCCCGGCT-3' (*Nco*I site underlined) were used for PCR amplification of the tetracycline resistance gene (*tet*) based on pBR325 [14]. The PCR product of *tet* (1.2 kb) was purified after T-vector ligation and *Nco*I digestion, and further ligated with the *Nco*I-treated pLex-*dsdA*, resulting in pLex-*dsdA*::*tet*. The *Xba*I–*Sac*I digestion fragment of pLex-*dsdA*::*tet* (2.5 kb) and the same digestion of pCVD442, a 6.3-kb suicide vector, were ligated, yielding pCVD442-*dsdA*::*tet*. The pCVD442 and *E. coli* S17-1*λpir* strain, a strain that enables the suicide vector to replicate, were kindly donated by Dr. Ho-Keun Lee, Kyunghee University, Seoul, Korea [12]. The constructed vectors were transformed by electroporation (MicroPulser; Bio-Rad, Hercules, CA, USA) into the S17-1*λpir* strain. Each PCR fragment was verified by DNA sequencing (DNA sequencing facility, Bionex, Seoul, Korea). The oligonucleotides were synthesized by the DNA sequencing facility of Bioneer Co. (Daejeon, Korea).

Screening Host Construction

E. coli DH5α was used for normal DNA manipulation, S17-1*λpir* for pCVD442-derived plasmid manipulation, and W3110 for homologous recombination. The pCVD442-*dsdA*::*tet* was transformed into *E. coli* W3110 and spread on LB agar containing 30 μg/ml of ampicillin (medium A). The single colony was transferred into medium A broth (4 ml) and cultured for 16 h at 37°C. The cells were diluted to 10^{-4} with LB broth and subcultured for 7 h at 37°C, which enriched the homologous recombination. Then 300 μl of enriched cells were spread on an agar medium B (10 g tryptone, 5 g yeast extract, and 50 g sucrose, per liter) and incubated at 30°C for 16 h. The colonies were transferred to both the agar medium A and agar medium B. The colonies that did not grow on ampicillin-containing medium A but grew on sucrose-based medium B were selected [2, 7, 9].

Media

LB medium containing 30 μg/ml of ampicillin (medium A) was used for plasmids manipulations. Medium B harboring 10 g tryptone, 5 g yeast extract, 50 g sucrose, per liter, was used for homologous recombination. Medium C was a minimal medium containing D-serine as a sole carbon source and composed of 2 g D-serine, 6.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 1.0 g NH_4Cl, 0.5 g NaCl, 1 mg thiamine·HCl, 1 mmol $MgSO_4$, and 0.13 mmol $CaCl_2$, per liter. Cell growth was measured by a spectrophotometer at 600 nm. The growth rate was estimated by measuring cell growth in the medium C (50 ml) at 37°C, 230 rpm.

Metagenome Library Screening

Metagenome libraries (19 kinds including compost, soil, and beach samples, 4.4×10^4 clones) were parceled out from Microbank (KRIBB, Daejeon, Korea). Each library contained about 40-kb metagenome derived from various sources in pCC1Fos fosmid (Epicentre, Madison, WI, USA). The culture harboring metagenome libraries was incubated in a LB medium supplemented with 15 μg/ml of chloramphenicol and 0.02% arabinose for amplification and then harvested using a plasmid miniprep kit. The extracted library was mixed with λ packing extract (MaxPlax Lambda Packaging Extracts, Epicentre) and incubated at 30°C for 3 h to allow formation of phage λ particles. The libraries in phage λ particles were mixed with the actively growing *E. coli* W3110 *dsdA::tet* dispersed in LB medium containing 10 mM $MgSO_4$ at room temperature for 25 min to deliver the metagenome into the screening host. The metagenome-carrying screening host was spread on the medium C agar plate and incubated for 10 days at 37°C.

D-Serine Degradation Activity

The D-serine degradation activity of screened cells was confirmed by comparison of the amino acids profiles derived from cell extracts reacted with D-serine. Actively growing cells were harvested by centrifugation of 1 ml of culture broth and disrupted using a sonicator (Vibracell Sonics & Materials Inc., Danbury, CT, USA.) set at 30 W for 1 min at 1-s intervals on ice. After removal of the cell debris by centrifugation, the supernatant was used for analysis of protein content and D-serine degradation activity. One hundred μl of cell extract was reacted with 100 mM D-serine for 30 min at 37°C in the presence of 10 μM pyridoxal phosphate.

Amino acids in the reaction mixture were converted into fluorescent isoindole derivatives using the *o*-phthaldialdehyde (OPA) and *N*-isobutyl-L-cysteine (IBC) method, followed by HPLC analysis [3]. Amino acids in the reaction mixture were mixed with the same volume of derivatization reagent [900 ml of 0.2 M sodium borate buffer (pH 10), 100 ml of methanol, 1 g of OPA, and 2 g of IBC] at 37°C for 20 min. Amino acids were separated on a COSMOSIL C_{18} column (4.6×150 mm; Nacalai Tesque, Kyoto, Japan) and analyzed with a FID-detector at 260 nm and 455 nm (FID 474; Waters, Milford, MA, USA). Eluent A was composed of 15% (v/v) methanol and 0.04 M sodium phosphate buffer [pH 6.2, 85% (v/v)]. Eluent B was a mixture of methanol (555 ml), tetrahydrofuran (30 ml), and 0.04 M sodium phosphate (pH 6.2, 670 ml). The column was equilibrated with 85% eluent A for 15 min, followed by gradient flow of eluent B until 100% for 15−50 min, and finally 85% eluent A for 10 min at a flow rate of 1 ml/min.

Construction of Transposon Library

To find the locus of interest, transposon-μ was randomly inserted in the selected metagenome by using a insertion mutation kit according to the manufacturer's recommendation (HyperMu KAN-1, Epicentre). After delivery of the transposon library, colonies unable to grow in the medium C were negatively selected. The negatively selected fosmid was extracted and its DNA was sequenced bi-directionally based on the inserted transposon sequence at the DNA sequencing facility of Solgent Co. (Daejeon, Korea).

RESULTS

Construction of *dsdA* Mutated Strain and Complementation Test

Suicide plasmid pCVD442 containing *dsdA*::*tet* was constructed for development of the W3110 *dsdA*::*tet* strain using homologous recombination (Fig. 1A). The *dsdA* gene from the W3110 genome was PCR-subcloned into an expression vector, yielding pLex-*dsdA*. To destruct the functional expression of *dsdA*, the *tet* gene from pBR325 was PCR-amplified and inserted into the *Nco*I site in the *dsdA* gene of pLex-*dsdA*. The *tet*-inserted *dsdA* gene (*dsdA*::*tet*) in plasmid pLex was named pLex-*dsdA*::*tet*. The *dsdA*::*tet* cassette (2.5 kb) was transferred to a suicide vector, pCVD442. The pCVD442 vector was replicable only in a strain harboring the *pir* gene, such as *E. coli* S17-1*λpir*, and was not replicable in DH5α and W3110 strains. The constructed pCVD442-*dsdA*::*tet* was transformed into W3110. The colonies grown on ampicillin-containing medium A, which were supposed to harbor the *bla* gene derived from single homologous recombination of pCVD442 in the chromosome, were transferred to the medium B broth for 16 h to allow double homologous recombination. The culture broth was diluted and spread on the medium B agar plate. After cultivation at 30°C for 16 h, 110 colonies were tooth-picked onto medium A and medium B agar plates. Twenty colonies grown on medium B agar, which were not found on medium A agar, were selected based on the double homologous recombination resulting in *bla* gene deletion. The chromosomal *dsdA* of selected colonies was tested by colony PCR and tetracycline resistance. The 20 selected colonies were dispersed in 20 μl of DW and boiled for 5 min to release genomic DNA, and then the supernatant was used for PCR template using oligonucleotides for the *dsdA* gene. The colonies showing the PCR fragment size of the *dsdA*::*tet* cassette (2.5 kb) instead of the original *dsdA* size (1.3 kb) were selected. They also were able to grow on tetracycline (2 μg/ml)-containing medium B agar plate. The 2.5-kb PCR fragments from the selected strain genomes were concluded to be *dsdA*::*tet*, based on the sequencing results, which was replaced from the original *dsdA* (Fig. 1B).

To verify the phenotype of *dsdA* disruption, a complementation test was performed (Fig. 2). The constructed W3110 *dsdA*::*tet* strain was streaked on an agar plate containing D-serine as the sole carbon source (medium C)

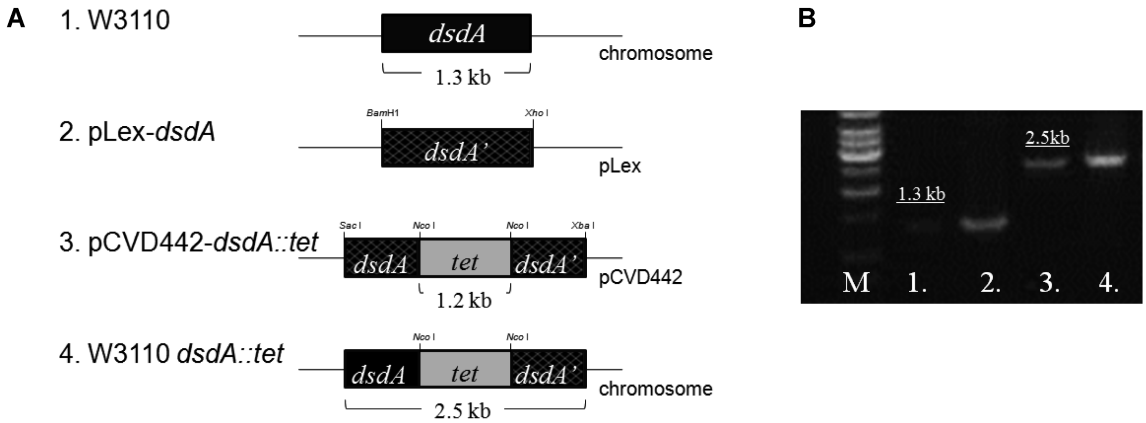

Fig. 1. Schematic diagram of development of *dsdA*::*tet* mutant *Escherichia coli*.
A. Diagrams of the chromosome of wild-type *E. coli* (1); *dsdA*-expression vector (2); *dsdA*-disrupted suicide vector (3); *dsdA*-disrupted chromosome of *E. coli* (4). **B**. Agarose gel electrophoresis of marker (M); PCR-product amplifying *dsdA* from the wild-type chromosome (lane 1); from the *dsdA*-expression vector (lane 2); from the *dsdA*-disrupted suicide vector (lane 3); from the *dsdA*-disrupted mutant chromosome (lane 4).

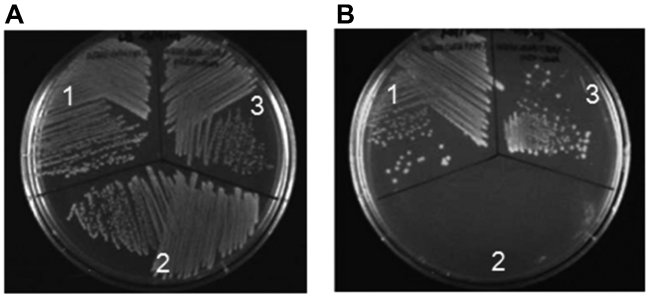

Fig. 2. Growth of W3110, W3110 *dsdA*::*tet*, and W3110 *dsdA*::*tet*/ pLex-*dsdA* on complex medium and on D-serine sole carbon source minimal medium.
A. LB medium. **B**. Minimal medium containing D-serine as a sole carbon source. Strains: (1) W3110, (2) W3110 *dsdA::tet*, (3) W3110 *dsdA::tet* / pLex-*dsdA*.

with wild type W3110 and complement W3110 *dsdA*::*tet*/ pLex-*dsdA*. *E. coli* W3110 formed colonies on the plate in three days, whereas the *dsdA* knocked-out W3110 was unable to grow. The complement W3110 *dsdA*::*tet* / pLex-*dsdA* was able to grow in the same way as strain W3110. The growth rates of W3110 and W3110 *dsdA*::*tet*/pLex-*dsdA* in the liquid medium C during the logarithmic phase were 0.27 and 0.10 h^{-1}, respectively.

Metagenomic Library Screening

Metagenome libraries in the fosmid pCC1fos were delivered into W3110 *dsdA*::*tet* after λ packing and infection, and the screening hosts harboring metagenome libraries were spread on an agar medium containing D-serine as a sole carbon source with supplement of 15 μg/ml chloramphenicol. Among the 4.4×10^4 clones of the libraries, a metagenome derived from compost enabled the W3110 *dsdA*::*tet* to form a colony on the selection medium in 10 days, and the harbored 40-kb metagenome DNA was named DNA G208402-7.

To verify that G208402-7 possesses the capacity of D-serine metabolism, a cell extract of the screened strain was reacted with D-serine solution *in vitro* and the reaction mixture was analyzed by HPLC (Fig. 3). Peaks of several amino acids were detected along with the D-serine degradation peak. No change was found in the reaction of cell extract of W3110 *dsdA*::*tet* with D-serine (data not shown). This result indicated that W3110 *dsdA*::*tet* had acquired the D-serine metabolism capability from the 40-kb DNA fragment of G208402-7.

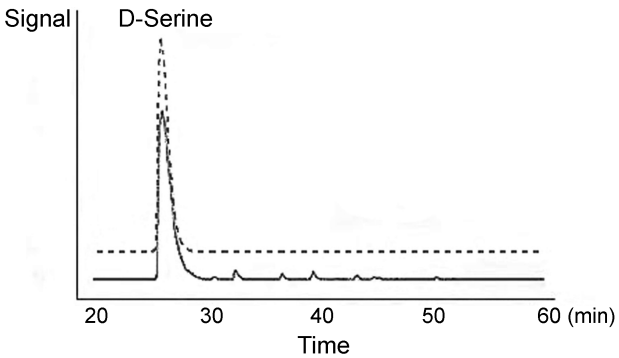

Fig. 3. HPLC chromatograms of *in vitro* reactions including D-serine and a cell extract harboring metagenome G208402-7.
The solid line indicates the chromatogram from the G208402-7-harboring cell extract, and the dashed line the chromatogram from the control.

DNA Sequence Responsible for D-Serine Metabolism

To determine the locus corresponding to the D-serine metabolism, transposon-μ was randomly inserted into the 40-kb DNA of G208402-7, and colonies losing capability to grow on the D-serine sole carbon source medium were negatively selected. Two colonies were selected from the negative selection and the transposon-inserted DNA G208402-7 were named as #22 and #28. The locus of interest of #22 and #28 were bi-directionally sequenced based on the known DNA sequence of the transposon-μ at the first try, and the bi-directional sequencing processes were iterated 3~5 times until the contigs of #22 and #28 reached 6 kb, respectively. The newly sequenced DNA fragments were submitted to GenBank (access codes: HQ829474.1 and HQ829475.1), and the nucleotide sequences were analyzed to find the open reading frames (Supplemental Material).

Discussion

The two 6-kb DNA fragments (HQ829474.1 and HQ829475.1) responsible for the D-serine degrading capability were sequenced and analyzed (Supplemental Material). The BLASTn search of the 6 kb contigs from HQ829474.1 and HQ829475.1 indicated that the sequences have not yet been found in nature (SM 1, 2). The closest BLAST results were the 0.9 kb from *Paracoccus denitrificans* PD1222 chromosome (GenBank CP000490.1) and the 0.7 kb from *Starkera novella* DSM 506 chromosome (GenBank CP002026.1), respectively, which only covers 10~16% of the deposited sequences with maximum identities of 80~82%. The BLASTx for the HQ829474.1 and HQ829475.1 also did not provide any known-function proteins (data not shown).

It is not clear which ORFs are exactly responsible for the D-serine degrading capability from the sequence analysis (SM 3). Although the disrupted ORFs by transposon-μ insertion at each 6-kb DNA fragment could be assigned in the sequence analysis, it is also possible that the insertion might deactivate the regulatory region to not express the accompanying ORF, resulting in the loss of D-serine degrading capability. Only the confirmation by experiments would give a clear result to acquire the corresponding DNA sequences. Nevertheless, it is clear that two or more ORFs are required for the D-serine degrading capability (one from HQ829474.1 and the other from HQ829475.1), because either the transposon-inserted mutation on the DNA

fragments disabled the D-serine degrading capability. The most plausible theory is that the corresponding protein might be a multiplex enzyme, and each peptide is required for the active form.

In conclusion, we have found a metagenome resource for D-serine degradation, based on the phenotype selection of the screening host (W3110 *dsdA*::*tet*). Further study is necessary to understand which DNA regions are responsible, and what the characteristics of the corresponding protein(s) for the D-serine degradation are. Once the protein is characterized (*i.e.*, D-serine racemase), the alternative genetic resources in this study might enable us to apply them for D-serine biosynthesis. The succeeding study is under way.

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