Self-protection Mechanism in D-cycloserine-producing *Streptomyces lavendulae*: GENE CLONING, CHARACTERIZATION, AND KINETICS OF ITS ALANINE RACEMASE AND D-ALANYL-D-ALANINE LIGASE, WHICH ARE TARGET ENZYMES OF D-CYCLOSERINE

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1The abbreviations used are aa, amino acid(s); D-Ala, D-alanine; ALR, alanine racemase; alrS, a gene encoding ALR from DCS-producing *Streptomyces lavendulae*; c.c., correlation coefficient; CD, circular dichroism; DCS, D-cycloserine; DDL, D-alanyl-D-alanine ligase, ddlS, a gene encoding DDL from DCS-producing *Streptomyces lavendulae*; ddlA, a gene encoding DDL from *E. coli* or *Salmonella typhymurium*; ddlB, a gene encoding DDL from *E. coli*; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; K12alr, a gene encoding ALR from *E. coli* K-12 W3110; D-Lac, D-lactate; LCS, L-cycloserine; M., *Mycobacterium*; MES, 2-morpholinoethanesulfonic acid; orf, open reading frame; PG, peptidoglycan; PLP, pyridoxal 5’-phosphate; TLC, thin-layer chromatography.

Running title: Alanine Racemase and D-Ala-D-Ala Ligase from D-Cycloserine-Producing *S. lavendulae*
SUMMARY

An antibiotic, \( \text{d-cycloserine (DCS)} \), inhibits the catalytic activities of alanine racemase (ALR) and \( \text{d-alanyl-d-alanine ligase (DDL)} \), which are necessary for the biosynthesis of the bacterial cell wall. In this study, we cloned both genes encoding ALR and DDL, designated \( \text{alrS and ddsS} \), respectively, from DCS-producing \( \text{Streptomyces lavendulae ATCC25233} \). Each gene product was purified to homogeneity and characterized. \( \text{E. coli} \), transformed with a pET vector carrying \( \text{alrS or ddsS} \), displays higher resistance to DCS than the same host carrying the \( \text{E. coli ALR- or DDL-encoded gene inserted into the pET vector} \). Although the \( \text{S. lavendulae DDL} \) was competitively inhibited by DCS, the \( K_i \) value (920 \( \mu \text{M} \)) was obviously higher (40~100-fold) than those for the \( \text{E. coli DdlA (9 \( \mu \text{M} \)) or DdlB (27 \( \mu \text{M} \))} \). The high \( K_i \) value of the \( \text{S. lavendulae DDL} \) suggests that the enzyme may be a self-resistance determinant in the DCS-producing microorganism. Kinetic studies for the \( \text{S. lavendulae ALR} \) suggest that the time-dependent inactivation rate of the enzyme by DCS is absolutely slower than that of the \( \text{E. coli ALR} \). We conclude that ALR from DCS-producing \( \text{S. lavendulae} \) is also one of the self-resistance determinants.

INTRODUCTION

Since the discovery of streptomycin, tuberculosis, a disease caused by infection of \( \text{Mycobacterium (M.) tuberculosis} \), has decreased annually; however, currently, it is once again on the rise. The increase in morbidity is likely due to the decline in immunity caused by changes in the environment and diet (1). In addition, the advent of multi-drug-resistant \( \text{M. tuberculosis} \) is also a cause of the return of tuberculosis (2).

\( \text{d-Cycloserine (d-4-amino-3-isoxazolidone: DCS)} \), which is a cyclic structural analogue of \( \text{d-alanine (d-Ala)} \) and is produced by \( \text{Streptomyces (S.) garyphalus and S. lavendulae} \), is a clinical medicine for the treatment of tuberculosis. The antibiotic is an effective anti-mycobacterial agent, but it is rarely prescribed and is used only in combined therapies due to its serious side effects (3). The side effects are caused by the binding of DCS to \( N\)-methyl-\( \text{d-aspartate receptors as an agonist} \). However, application of these adverse
effects to treatments for neural disease (4), such as Alzheimer's (5) and Parkinsonism (6), have been dedicatedly researched.

The peptidoglycan (PG) layer, which is contained in a bacterial cell wall, is the main component that enables bacteria to be resistant to osmotic pressure. The formation of UDP-\(N\)-acetyl muramyl pentapeptide, which is a precursor of PG, is followed by a cross-link reaction of the precursors. In the cross-linking process, \(\text{D-Ala}\) plays an important role as a bridge molecule (7). Since \(\text{D-amino acids}\), including \(\text{D-Ala}\), are not primarily found in natural resources, bacteria generate \(\text{D-Ala}\) from \(\text{L-Ala}\) by the catalysis of Ala racemase (ALR). This enzyme needs a pyridoxal 5'-phosphate (PLP) as a cofactor and catalyzes the racemization of both Ala enantiomers. \(\text{E. coli}\) and \(\text{Salmonella typhimurium}\) possess two kinds of closely related ALR-encoded genes (\(\text{alr}\) and \(\text{dadX}\) in \(\text{E. coli}\), and \(\text{dal}\) and \(\text{dadB}\) in \(\text{Salmonella typhimurium}\)) (8-10). For example, the racemase encoded by \(\text{dal}\) of \(\text{Salmonella typhimurium}\) is necessary for peptidoglycan synthesis and displays a 40% identity to a catabolic racemase encoded by \(\text{dadB}\) (11).

\(\text{D-Ala}\), generated by ALR, is a substrate to form \(\text{D-alanyl-D-alanine} (\text{D-Ala-D-Ala}) (12). The dipeptide is formed by the action of an ATP-dependent enzyme, \(\text{D-Ala-D-Ala ligase (DDL)}\), and incorporated into the PG precursor by the catalytic activity of the \(\text{D-Ala-D-Ala-adding enzyme (7). E. coli produces two kinds of DDL, designated DdlA and DdlB, which are encoded by \text{ddlA and ddlB}, respectively. Salmonella typhimurium expresses DDL, which has high similarity to the E. coli DdlA (13).}

DCS interferes with the activities of both ALR and DDL, which are necessary for the synthesis of PG contained in the cell wall of bacteria. Since these enzymes are unique to bacteria, they may become potential targets for the screening of selective anti-bacterial agents (14). ALR and DDL have been considered to be competitively inhibited because DCS is structurally similar to \(\text{D-Ala}\) (13, 15). However, it was recently reported that DCS inhibits the catalytic activity of ALR via a time-dependent inactivation manner (13). In addition, the antibiotic and its enantiomer, \(\text{L-cycloserine (LCS)}\), inhibit several kinds of PLP-dependent enzymes in the same manner (16-18).

Antibiotic-producing microorganisms must be protected from the lethal effect of their own product. We recently cloned a 3.5-kb DNA fragment carrying a gene that confers resistance to DCS from DCS-producing \(\text{S. garyphalus}\) by a shot-gun cloning technique (19). The hydrophy plot analysis of a protein deduced from the nucleotide sequence of the gene encoding DCS resistance revealed that the protein may carry membrane-integral
domains spanning the membrane 10 times, suggesting that the DCS-resistance gene product may be a factor associated with DCS transport. Interestingly, an incomplete gene was found to be located upstream of the transmembrane protein gene from *S. garyphalus*. The incomplete gene consists of 246 bp, and the putative protein has a 52.6% identity with a D-Ala-D-Ala ligase from *Pseudomonas aeruginosa* (20). On the other hand, although the cloned fragment has a few open reading frames (orfs), it has no orf, which makes it similar to a gene encoding ALR. Since DDL and ALR are target enzymes of DCS, it is of great interest to know whether these enzymes from the DCS-producing microorganism show resistance to DCS.

In the present study, an effort was made to clone ALR- and DDL-encoding genes from DCS-producing *S. lavendulae* ATCC25233. Both of the *S. lavendulae* ALR and DDL, which were overproduced in an *E. coli*-host vector system, were purified and characterized biochemically and kinetically. The present study suggests that the *Streptomyces* ALR and DDL function as self-resistance determinants.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains and Plasmids* – *E. coli* strains TG1, JM109, and DH5α and plasmids pUC18 and pUC19 were used for the cloning experiments. *E. coli* XL1-Blue MRA (P2) was used for the construction of phage libraries. *E. coli* BL21 (DE3)-pLysS and a plasmid pET-21a(+) (Novagen, Germany) were used for protein expression. *E. coli* was grown in an LB medium (21) at 37°C or 28°C. If necessary, ampicillin (100 μg/ml) and/or chloramphenicol (34 μg/ml) were supplemented to the LB medium. For the cultivation of *E. coli* XL1-Blue MRA (P2), 0.2% maltose and 10 mM MgSO₄ were supplemented to the LB medium. *S. coelicolor* A3(2) (strain no. M145), which has been used as the typical strain of the International *Streptomyces* Genome Project (22), and DCS-producing *S. lavendulae* ATCC25233 were grown at 28°C in a GMP medium (23) or a YEME medium (24).

*DNA Manipulations* – Plasmid DNA from *E. coli* was isolated by the standard method described previously (21). The chromosomal DNA from *S. coelicolor* A3(2) and *S. lavendulae* was isolated from 100 ml of a culture grown at 28°C for 72 h according to a
method described earlier (24). Phage DNA from plaque was isolated by the standard method described elsewhere (21).

Analysis of Genes Encoding the DCS-Resistance Determinant from S. lavendulae

The chromosomal DNA (500 \( \mu \)g) from S. lavendulae was partially digested with BamHI, purified by the phenol/chloroform extraction method, and precipitated by ethanol. The DNA fragments, cleaved within 10 to 20-kb, were separated by sucrose gradient (10-40%) centrifugation and precipitated by ethanol (21). After the 5’-dephosphorylation of DNA with bacterial alkaline phosphatase, the resulting DNA fragments were ligated to a BamHI-digested Lambda DASH II vector (Stratagene, USA). In vitro packaging was performed using a Gigapack III Gold Packaging Extract (Stratagene, USA) according to the supplier’s instructions. The resulting phages were infected to E. coli XL1-Blue MRA (P2) and plated onto an NZYM medium (21) containing 1.2% agarose to generate plaques.

The plaques generated on the NZYM agarose plate were transferred to a nylon membrane (Hybond-N+, Amersham Biosciences, Sweden), and the phage DNA was fixed to the membrane by the alkaline treatment (21). Hybridization was performed at 65°C by using a 1.2-kb DNA fragment from pCSPC9, which contains the DCS-resistance gene of S. garyphalus (19), as a probe DNA. The probe labeling, hybridization, and detection were performed with an AlkPhos Direct Labeling and Detection Kit (Amersham Biosciences, Sweden) according to the supplier’s instructions. One positive clone was obtained by plaque hybridization. The phage DNA, isolated from the positive plaque, had a 14-kb DNA insert from S. lavendulae.

The phage DNA containing a 14-kb DNA from S. lavendulae was digested with BamHI. The resulting DNA fragments (about 1.2, 2.0, 2.9, and 8.0 kb) were subcloned into pUC18 or pUC19. Using the resulting chimeric plasmids, the DNA sequence was determined with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) and ABI PRIZM 310 Genetic Analyzer (Applied Biosystems, USA). Of the 14-kb DNA fragment, in the present study, we determined the nucleotide sequence of a 2,820-bp DNA fragment including the S. lavendulae DDL gene. Genetic analysis was performed by using the GENETYX-Mac software (Software Development, Japan) and the Frame Analysis program (25). The homology search was done with the FASTA program on a Web site. The DNA sequence determined in this study has been submitted to the DNA Data Bank of Japan (DDBJ accession number: AB176675).
Cloning and Analysis of an ALR Gene from *S. lavendulae* – The chromosomal DNA from *S. lavendulae* ATCC25233, which was digested with *Bam*H1, was fractionated on 0.8% agarose gel electrophoresis and transferred to a Hybond-N+ membrane using the standard protocol (21). Southern hybridization analysis was done using a putative ALR gene (1,176-bp) from *S. coelicolor* (22) as a probe. To obtain the probe DNA, PCR amplification was done using the *S. coelicolor* genomic DNA as a template together with a sense primer (5’-ATGAGCGAGACAATCGTCGACGACGGACCG-3’) and an anti-sense primer (5’-TCATTGTTGACGTAGACGCGGACGGGACCCGG-3’). PCR was done under the following conditions: an initial 5 min at 96°C and 3 min at 70°C; then, 24 cycles of 1 min at 96°C and 3 min at 70°C; and, finally, a 3-min extension period at 72°C. Probe labeling, hybridization, and detection were performed using an AlkPhos Direct Labeling and Detection Kit according to the manufacturer’s instructions.

The probe DNA was hybridized to *Bam*H1-digested genomic DNA that had a size of 3.0 kb. Therefore, *Bam*H1 digests of 2.5–3.5 kb were extracted from the agarose gel, purified, ligated to *Bam*H1-digested pUC19, and then introduced into *E. coli* TG1. The resulting genomic libraries were screened using the colony hybridization technique (21). From approximately 8,000 clones, 52 candidates carrying the putative ALR gene were obtained using the putative *alr* from *S. coelicolor* as a probe DNA. One of the chimeric plasmids, isolated from these candidate colonies, was hybridized to the probe. Results from the International *Streptomyces* Genome Project confirmed that a protein deduced from the nucleotide sequence of the *S. lavendulae* gene, which was inserted into the candidate plasmid, displayed a high similarity with the *S. coelicolor* putative ALR.

DNA sequencing was performed with the ABI PRIZM 310 Genetic Analyzer using the BigDye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer’s protocols. By combination of subcloning and chromosome-walking techniques, the entire nucleotide sequence of the cloned DNA fragment containing the *S. lavendulae* ALR gene, designated *alrS*, was determined and analyzed for the existence of orf with a GENETYX-Mac software. The orfs were predicted using a frame analysis program (25). The similarity among proteins was searched using the FASTA program on the website. The sequence data obtained in this study has been submitted to the DDBJ (accession number AB176676).
Overexpression and Purification of S. lavendulae DDL – A gene encoding DDL from S. lavendulae was amplified by PCR using a sense primer, 5’-CACCATATGCGAATCGTGATCTTGTGTGGAGAAGC-3’ (NdeI site underlined), and an antisense primer, 5’-CACCTCGAGTCAGCGGGTGCGGAGGGACAC-3’ (XhoI site underlined). PCR was done under the following conditions: 1 cycle of 5 min at 96ºC, 1 min at 55ºC, and 2 min at 72ºC followed by 29 cycles of 1 min at 96ºC, 1 min at 55ºC, and 2 min at 72ºC. The amplified DNA was digested with NdeI and XhoI and subcloned into the same sites of pET-21a(+) to generate pET-ddlS. E. coli BL21 (DE3)-pLysS harboring pET-ddlS was grown at 28ºC in 6 liters of an LB medium to an O.D. 600 nm = 0.5, whereupon isopropyl-β-d-thiogalactopyranoside (IPTG) was added to the culture at the final concentration of 1 mM to induce the expression of ddlS. The E. coli cells were grown for 8 h at 28ºC. The purification of the S. lavendulae DDL was carried out at 4ºC: the E. coli cells were suspended in Buffer I [50 mM sodium phosphate (pH 7.5), 10 mM MgCl₂, 2 mM dithiothreitol (DTT), and 1 mM EDTA·Na₂] and disrupted by sonication. The cell debris was removed by centrifugation at 17,000 g for 30 min. Solid ammonium sulfate was gradually added to the supernatant to 20% saturation and centrifuged to obtain the supernatant fluid. Solid ammonium sulfate was added to the supernatant fluid to 50% saturation, and the resulting precipitate was collected by centrifugation. The precipitate was dissolved in a small volume of Buffer I and dialyzed against the same buffer. After the dialysate was applied on a DEAE-Sepharose column (2.5 x 10 cm, Amersham Biosciences, Sweden) previously equilibrated with Buffer I, the column was washed with the same buffer. Elution was done with a 0-500 mM KCl linear gradient concentration in Buffer I. The fractions containing the S. lavendulae DDL were pooled and dialyzed against Buffer II [20 mM Tris-HCl (pH 7.5), 2.5 M KCl, 1 mM ATP, 10 mM MgCl₂, and 1 mM 2-mercaptoethanol] and then subjected to an Octyl-Sepharose column (1.5 x 15 cm, Amersham Biosciences, Sweden) previously equilibrated with Buffer II. Since no S. lavendulae DDL was bound to the column, the solution that passed through the column was collected. Finally, the solution carrying the DDL activity was dialyzed against Buffer III [50 mM sodium phosphate (pH 7.5), 10 mM MgCl₂, 2 mM DTT, 1 mM EDTA·Na₂, and 0.2 mM ATP] and applied on a DEAE-Sepharose column (1 x 15 cm) equilibrated with Buffer III. The S. lavendulae DDL did not bind to the column, possibly because of the presence of ATP. Therefore, the solution that passed through the column was collected. Through these steps, the S. lavendulae DDL was purified to homogeneity.
Overexpression and Purification of S. lavendulae ALR – An ALR gene of S. lavendulae was amplified by PCR using the sense primer 5'-CACCATATGAAACGAGACACCGACGCGCGTG-3' (the underline indicates the NdeI cleavage site) and the anti-sense primer 5'-TATCTCGAGGCCGCCGAGGTAGA CCCGGG-3' (the underline indicates the XhoI cleavage site). The amplified DNA was digested with NdeI and XhoI and then subcloned into the same sites of pET-21a(+) to yield pET-alrS. The pET-alrS plasmid expresses ALR having His6-tag at the C-terminus. E. coli BL21 (DE3)-pLysS harboring pET-alrS was grown in 3 liters of an LB medium at 28°C. At the exponential phase of growth (O.D.600 nm = 0.6), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at the concentration of 0.1 mM to express the ALR gene. After an additional incubation for 4.5 h, the cells were harvested by centrifugation and washed with a binding buffer [20 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 5 mM imidazole]. The washed cells (20 g wet weight) were suspended into 200 ml of the same buffer and disrupted by sonication at 4°C for 60 min. The cell debris was removed by centrifugation at 24,000 g twice for 20 min. The resulting cell-free extract was brought to 65% saturation with solid ammonium sulfate. The resulting precipitate was collected by centrifugation at 24,000 g for 20 min, dissolved in a binding buffer, and dialyzed against the same buffer. The dialysate was applied to a Ni(II)-chelated His-Bind Resin (Novagen, Germany) column (1.0 x 30 cm) according to the manufacturer's protocol. The column was washed with a wash buffer [20 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 60 mM imidazole] and eluted linear-gradienly with 60–350 mM imidazole. The fractions having ALR activity were pooled and concentrated by ultrafiltration with the Amicon Ultra (Millipore, USA). The concentrate was loaded onto a Sephadex G-100 super fine (Amersham Biosciences, Sweden) column (1.5 x 120 cm) equilibrated with a 200 mM Tris-HCl buffer (pH 8.5) supplemented with 50 mM NaCl and eluted with the same buffer. The purified enzyme fractions were collected, concentrated by ultrafiltration, and stored at 4°C until use.

Overexpression and Purification of E. coli ALR – A gene encoding ALR from E. coli K12 W3110 was amplified by PCR using the primers 5’-CACCATATGCAAGCGGCAACTGTTGTGATT-3’ (the underline indicates the NdeI cleavage site) and 5’-TATCTCGAGATCCACGTATTTTCATCGCGAC-3’ (the underline indicates the XhoI
cleavage site) according to the genome information (26). The amplified DNA was inserted into pET-21a(+) to generate pET-K12alr. The cell-free extract of *E. coli* BL21 (DE3)-pLysS harboring pET-K12alr was applied to a Ni(II)-chelated His-Bind Resin column (1.0 x 30 cm) and washed with the binding buffer. Elution was done with the same buffer containing 500 mM imidazole. The enzyme fraction was concentrated by 65% ammonium sulfate precipitation, and the resulting precipitate was dissolved in a 50 mM ammonium phosphate buffer (pH 8.2) containing 50 mM NaCl followed by dialysis against the same buffer. The dialyzed solution was subjected to a DEAE-Sepharose column (1.0 x 30 cm) equilibrated with the same buffer. The fraction that passed through the column containing ALR from *E. coli* K12 was concentrated by ultrafiltration.

**Enzyme Assay of DDL** – The DDL activity was monitored by the continuous ADP release-coupled assay method as described previously (27). This assay monitors the absorbance at 340 nm. The DDL activity at different pH values was compared with buffers prepared as follows: a solution containing 100 mM Tris, 100 mM glycine, and 100 mM MES was adjusted to pH 6.0 and pH 10.5. By mixing the two solutions, buffers with pH values of 6.0, 7.0, 8.0, 9.0, 9.5, and 10.0 were prepared. All reactions were carried out at 37°C. The protein concentrations were determined by using a Bio-Rad Protein Assay (Bio-Rad, USA), which is based on the method of Bradford (28).

**Enzyme Kinetic Study of DDL** – Kinetic assays for the purified *S. lavendulae* DDL were carried out by the continuous ADP release-coupled assay method (27) at 37°C. By application of the steady-state approximation to the proposed reaction sequence, which is shown as Eq. 1, a rate equation (Eq. 2) can be obtained, which gives parabolic Lineweaver-Burk plots (Eq. 3). The *V*<sub>max</sub> value can be obtained from the y intercept of Eq. 3. Subsequently, a plot of [S](1/1/<sub>V</sub> – 1/<sub>V</sub><sub>max</sub>) against 1/[S] gives a straight line (Eq. 4), whose y intercept (1/<sub>V</sub><sub>max</sub>) and slope (1/<sub>V</sub><sub>max</sub>/<sub>K</sub><sub>m</sub>) provide the two <sub>K</sub><sub>m</sub> values.
Because the $K_1$ value in the above equations is very small (13), the value can be ignored when the concentration of the substrate ([S]) is high; therefore, Eqs. 2 and 3 can be represented as Eqs. 5 and 6, respectively. We determined the $K_2$ value alone using these equations (Eqs. 5 and 6).

$$V = \frac{V_{\text{max}} [S]^2}{K_1 K_2 + K_2 [S] + [S]^2} \quad \text{(Eq. 2)}$$

$$\frac{1}{V} = \frac{1}{V_{\text{max}}} + \frac{K_2}{V_{\text{max}} [S]} + \frac{K_1 K_2}{V_{\text{max}} [S]^2} \quad \text{(Eq. 3)}$$

$$[S] \left( \frac{1}{V} - \frac{1}{V_{\text{max}}} \right) = \frac{K_2}{V_{\text{max}}} + \frac{K_1 K_2}{V_{\text{max}} [S]} \quad \text{(Eq. 4)}$$

Enzyme Assay and Kinetic Study of ALR –The enzyme assay and kinetic study of ALR using circular dichroism (CD) spectrometry were performed by a novel method (29). The CD signals of samples were measured using a spectropolarimeter (JU-720 type, JASCO, Japan).

To understand the inhibition mode of DCS to the S. lavendulae ALR, the assay was repeated using a reaction mixture incubated with DCS at various concentrations (0.05–0.5 mM) for more than 10 min before the reaction. [D-Ala], [L-Ala], and $v$ were also calculated as described (29), and numerical analysis was performed to maximally fit to Eqs. 7 and 8, which are equations applied for competitive and non-competitive inhibition, respectively.
In Eqs. 7 and 8, $K_{i1}$ and $K_{i3}$ are inhibition constants for the D- to L-direction and $K_{i2}$ and $K_{i4}$, for the L- to D-direction, and $[I]$ means the concentration of the inhibitor (DCS).

**Time-Dependent Inactivation Assay using CD Spectrometry of ALR**—The remaining activity after inactivation of ALR by the enantiomers of cycloserine was determined as follows: the enzyme (12.5 μg/ml) was incubated with the given concentrations of DCS (0.4-3.0 mM) or LCS (5-20 mM) at 25°C. At specific intervals, 20 μl of the reaction mixture was added to a solution (3 ml) consisting of a 30 mM ammonium phosphate buffer (pH 8.2) and 4 mM D-Ala, and the CD signals (at 205 nm) were then recorded as a function of time at 25°C.

**Constructions of pET-alrS-ddlS, pET-ddlA, pET-ddlB, pET-K12alr-ddlA, and pET-K12alr-ddlB**—Each gene from *E. coli* K12 W3110, designated ddlA and ddlB, was amplified by PCR using the primers 5’-TATCATATGGAAAAACTCGGCTAGGAA-TC-3’ (the underline indicates the NdeI cleavage site) and 5’-CCCCAGCTTTTACATTGTT-GGTTTTCAATGC-3’ (the underline indicates the HindIII cleavage site) for *ddlA* and 5’-CACCATATGACTGATAAAATCGGGTCCCTTG-3’ (the underline indicates the NdeI cleavage site) and 5’-CACAAGCTTTTATGTTAATCCGCAGTCCAGAAT-3’ (the underline indicates the HindIII cleavage site) for *ddlB*, and the amplified DNA was inserted into pET-21a(+) to generate pET-ddlA and pET-ddlB, respectively. The *ddlA* and *ddlB* fragments including the T7 promoter and terminator region were then amplified by PCR using the primers 5’-CACGCATGCAGAAATTAATACGACTCAC-3’ and 5’-TACTGCAGAAAAACCCCTCAAGAC-3’ (the underline indicates the SphI cleavage site), and the amplified DNA was inserted into the SphI-digested pET-K12alr to generate

\[
v = \frac{V_{\text{max}1}[\text{D-Ala}]}{K_{m1}(1+[I]/K_{i1})} - \frac{V_{\text{max}2}[\text{L-Ala}]}{K_{m2}(1+[I]/K_{i2})} \frac{[\text{D-Ala}]}{1 + \frac{[\text{L-Ala}]}{K_{m1}} + \frac{[\text{L-Ala}]}{K_{m2}}}
\]

(Eq. 7)

\[
v = \frac{V_{\text{max}1}[\text{D-Ala}]}{K_{m1}(1+[I]/K_{i3})} - \frac{V_{\text{max}2}[\text{L-Ala}]}{K_{m2}(1+[I]/K_{i4})} \frac{1}{1 + \frac{[\text{D-Ala}]}{K_{m1}} + \frac{[\text{L-Ala}]}{K_{m2}}}
\]

(Eq. 8)
pET-K12alr-ddlA and pET-K12alr-ddlB, respectively. On the other hand, after pET-ddlS was double-digested with XhoI and BglII, a 1.1-kb DNA fragment carrying ddlS was blunted and inserted into pET-alrS, which was digested with SphI and blunted to generate pET-alrS-ddlS.

**DCS-Resistance of E. coli Transformed with the Plasmid Carrying the ALR- and/or DDL encoded genes from S. lavendulae and E. coli** —The DCS resistance of E. coli BL21 (DE3)-pLysS carrying the plasmids pET-alrS, pET-ddlS, pET-alrS-ddlS, pET-K12alr, pET-ddlA, pET-ddlB, pET-K12alr-ddlA, and pET-K12alr-ddlB was tested by measuring O.D. at 600 nm of cultures grown in an M9 agar medium (1.0% w/v) supplemented with 100 mg ampicillin /ml, 34 mg chloramphenicol /ml, 0.1 mM IPTG, and 0-200 mg DCS /ml. E. coli cultures were grown at 37°C overnight. As a control, E. coli BL21(DE3)-pLysS harboring pET-21a(+) was used.

**RESULTS AND DISCUSSION**

*Cloning of Genes Encoding the DCS-Resistance Determinant from DCS-Producing S. lavendulae* —We recently cloned a 3.5-kb DNA fragment from DCS-producing S. garyphalus, which includes a DCS-resistance gene, designated orfB (19). We suggest that the orfB gene product, which may carry membrane-integral domains spanning the membrane 10 times, may be a transporter for the efflux of DCS to the outside cells. To determine whether orfB is conserved in another DCS-producing microorganism, we examined whether a gene homologous to orfB is located on the chromosome from DCS-producing S. lavendulae ATCC25233.

A genomic library of S. lavendulae, prepared in a λ phage-derived vector, was screened with the S. garyphalus orfB as a probe DNA. A 2.8-kb DNA portion of the 14-kb DNA fragment inserted in the phage vector, which was hybridized with orfB, was analyzed for the nucleotide sequence. Frame analysis (25) suggested that three orfs, designated orf I, II, and III, are present in the 2.8-kb region (Fig. 1). The central gene, designated orf II, consists of 903 bp and encodes a protein with a molecular weight of 30,930. The protein exhibits a 98.7% identity (300 aa overlap) to the protein encoded by orfB from S. garyphalus (19). That is, orf II shown in Fig. 1 should be called orfB, which
suggests that the gene is conserved in both DCS-producing *Streptomyces* strains. A gene located downstream of *orf II*, designated *orf III* (456 bp), encodes a protein consisting of 151 aa with a molecular weight of 15,882. The *orf III*-encoded protein displays the highest similarity to an unknown protein from *S. coelicolor* (42.4% identity, 151 aa overlap) (22). The *orf III* gene product has an 89% identity to a protein encoded by a gene designated *orfC*, which is located in the 3′-adjacent region of the DCS-resistance gene in *S. garyphalus* (19). Interestingly, a protein encoded by a gene, designated *orf I* (1,038 bp), exhibits a significant similarity (42.0% identity) to a DDL from *Pseudomonas aeruginosa* (20). In a previous study (19), an incomplete gene from *S. garyphalus*, which is predicted to be the 3′-portion of the gene, was found to be present just upstream of *orfB*. The incomplete gene product from *S. garyphalus* is completely identical to a protein encoded by *orf I* from *S. lavendulae*. In addition, the order and transcriptional direction between *orf I* and *orfB* in *S. lavendulae* are the same as those in *S. garyphalus*. The predicted molecular weight and pI of the *orf I*-encoded protein (345 aa) are 35,987 and 4.81, respectively. As described below, the protein was confirmed to exhibit DDL activity using the gene product, which was purified to homogeneity. Therefore, *orf I* and the gene product are referred to as *ddlS* and DDL, respectively, from here on.

Figure 2 shows a comparison of the aa sequence of DDL from *S. lavendulae* with those from various bacteria. The amino acids which interact with ATP and d-Ala (30) are conserved, except for Leu^{320}, which corresponds to Leu^{282} of the *E. coli* DdlB. In some cases, the Leu residue is replaced by Met (Fig. 2). Although the consensus sequence of the ω-loop in these DDLs is Ser(or Ala or Thr)-Lys-Tyr-Ile(or Met or Ser) (31), the loop in the *S. lavendulae* DDL is Ala-Lys-Tyr-Gln. The Gln residue, present in the ω-loop, is characteristic of d-Ala-d-Ser ligases, which belong to VanC, found in vancomycin-resistant bacteria (31, 32).

**Cloning and Sequence Analysis of a Gene Encoding ALR from *S. lavendulae***–We found that an *orf* that is contained in the 2.8-kb DNA fragment cloned from *S. lavendulae* is homologous to the putative ALR from *S. coelicolor* A3(2) M145 but is not complete. Therefore, we newly cloned an additional 500-bp DNA fragment, which is adjacent to the 2.8-kb DNA fragment, by conducting a chromosomal-walking experiment. The nucleotide sequence analysis of the 3,296-bp DNA fragment suggests that it contains a gene encoding a complete ALR protein from *S. lavendulae*. As shown in Fig. 3, frame
analysis (25) of the 3,296-bp DNA fragment suggests the presence of three complete orfs, designated *orf1*, *orf2*, and *orf3*. An *orf*, *orf1*, consists of 1,134 bp, and a protein deduced from the nucleotide sequence has 378 aa, with a molecular mass of 39.9 kDa. The protein shows a 74.9% identity to a putative ALR from *S. coelicolor* (22). This *orf* is referred to as *alrS* from here on. The nucleotide sequence of *alrS* was deposited in the DDBJ (accession no. AB176676). The aa sequence of the putative *S. lavendulae* ALR also shows a significant homology to ALRs from mycobacteria (33, 34). A Lys residue in the *S. lavendulae* ALR, which is the putative binding site for PLP (35, 36), is present (Lys38), similarly to other ALRs expressed by a few microbial sources. A Tyr residue, which plays an essential role in the racemization of alanine (37), is also conserved in the *Streptomyces* ALR (Tyr270).

*E. coli* and *Salmonella typhimurium* have been known to possess two kinds of closely related ALR-encoded genes (*alr* and *dadX* in *E. coli*, and *dal* and *dadb* in *Salmonella typhimurium*) (8-10). A genomic Southern analysis performed using *alrS* as a probe indicated that the *alrS*-related gene, which is hybridized to the probe, was the only one in the *S. lavendulae* chromosome (data not shown).

A protein encoded by *orf2* (1,176-bp) consists of 392 aa and exhibits the highest identity (71.8%) to a putative lipase from *S. coelicolor* (22). A protein encoded by *orf3* (462-bp) consists of 154 aa and displays the highest identity (79.0%) to a putative ATP/GTP-binding protein from *S. coelicolor* (22). The organization of these orfs in *S. lavendulae* ATCC 25233 is identical to that in *S. coelicolor* A3(2).

Overproduction and Purification of the *S. lavendulae* DDL –Since DDL is a target enzyme of DCS, it would be significant to determine whether the DDL of DCS-producing *S. lavendulae* exhibits resistance to DCS. We overproduced the *S. lavendulae* DDL using an *E. coli* host-vector system and purified it to homogeneity (Fig. 4). The DDL shows a molecular mass of about 38 kDa on SDS-PAGE, which is almost the same as that calculated from the deduced aa sequence. The molecular mass, measured by gel filtration chromatography performed on a Sephacryl S-300 HR column (1.5 x 120 cm, Amersham Biosciences, Sweden), is about 67 kDa, suggesting that the *S. lavendulae* DDL is a dimeric protein, like DDL from *E. coli*, designated DdlB (13).

Purification of ALRs from *S. lavendulae* and *E. coli* –Each ALR from *S. lavendulae*
and *E. coli* K12 W3110 was overproduced as a protein with the C-terminal His6-tag in *E. coli* and purified to homogeneity (Fig. 5). As shown in Fig. 5, the molecular masses of the purified *S. lavendulae* and *E. coli* ALRs, as estimated by SDS-PAGE, are 42 and 40 kDa, respectively. The bacterial ALRs have been reported to be classified into two types of subunit structures, that is, a monomer or a homodimer structure (38). Gel filtration chromatography with Sephacryl S-200HR (1.5 x 120 cm, Amersham Biosciences, Sweden) revealed that the *S. lavendulae* ALR has a molecular mass of about 80 kDa, suggesting that it may have a homodimer structure.

**Enzyme Properties of the *S. lavendulae* DDL** – DdlB from *E. coli* was observed to display a higher DDL activity at pH 9.2 than at pH 6.0-7.5 (39). Therefore, the catalytic activity of the *S. lavendulae* DDL was measured by varying the pH in the reaction mixture. We observed that the *Streptomyces* DDL activity is 15 μM·min⁻¹ at pH 7.0 and 57 μM·min⁻¹ at pH 10.0, respectively, suggesting that the enzyme exhibits higher activity as the pH values increase. The α-loop in the *S. lavendulae* DDL has an Ala-Lys-Tyr-Gln sequence, raising the question of whether the enzyme displays D-Ala-D-Ser ligase activity. A TLC assay (40) confirmed that the *S. lavendulae* DDL did not display D-Ala-D-Ser ligase or D-Ala-D-Lac activities (data not shown). The latter observation is consistent with the fact that D-Ala-D-Lac ligases, such as VanA (41, 42) and VanB (43, 44) from vancomycin-resistant bacteria, possess the consensus α-loop sequence of Pro-Glu-Lys-Gly (31). The α-loop consensus in D-Ala-D-Lac ligases from lactic acid bacteria, including *Lactobacillus confusus*, *L. salivarius*, and *L. plantarum*, has the Asn-(Lys/Met)-Phe-Val sequence (31).

The kinetic parameters of the *S. lavendulae* DDL were measured using an ADP release-coupled assay method (27) and compared with those for the *Salmonella typhimurium* DdlA, the *E. coli* DdlA and DdlB, and the *Enterococcus faecium* VanA (13, 42). The turnover number (*kₕₐₜ*) of the *S. lavendulae* DDL was 4-10 fold lower than those of the *E. coli* DdlA and DdlB and the *Salmonella* DdlA (TABLE I). In addition, the *Kₘ* value of the *Streptomyces* DDL for the second D-Ala substrate (*K₂*) was 4- and 2-fold higher than those of DdlA and DdlB, respectively. The *kₕₐₜ* value of the *S. lavendulae* DDL was much closer to that of VanA ligase. The *Kₘ* value of the *S. lavendulae* DDL for ATP was 3-fold higher than those of DdlA and DdlB. The value was almost the same as that of VanA. The dipeptide, D-Ala-D-Ala, is known to act as a reversible inhibitor of the
forward reaction (i.e., the formation of d-Ala-d-Ala from d-Ala). The $K_i$ value of d-Ala-d-Ala for the *S. lavendulae* DDL was 60 μM, which is close to those for the *Salmonella typhimurium* DdlA (61 μM) and the *E. coli* DdlA (49 μM) and DdB (70 μM). However, the *S. lavendulae* DDL was competitively inhibited by d-Ala-d-Ala, just like the *Streptococcus faecalis* DDL (45), whereas the *E. coli* DdlA and DdB and the *Salmonella* DdlA were non-competitively inhibited (13). The reason that there is a difference between the inhibition modes in these enzymes is currently unclear.

DCS inhibits DdlAs and DdB competitively, with $K_i$ values in the range of 9-27 μM (TABLE I). Although the *S. lavendulae* DDL was competitively inhibited by DCS, the $K_i$ value of DCS for the protein (920 μM) was obviously higher (40-100 fold) than those for DdlAs and DdB. This value was close to that for the VanA ligase. The high $K_i$ value suggests that the *S. lavendulae* DDL may be involved in the self-resistance mechanism in DCS-producing *S. lavendulae*. The kinetic properties of the *S. lavendulae* DDL were similar to those of DdlAs and DdB ($K_2$ for the second d-Ala and $K_1$ for d-Ala-d-Ala) and, in part, to those of VanA ($k_{cat}$, $K_m$ for ATP, and $K_i$ for DCS), suggesting that the structure of the substrate-binding sites of the *S. lavendulae* DDL might be different from those of the enzymes. Therefore, the crystallization of the *S. lavendulae* DDL is in progress to determine its three-dimensional structure.

Kinetic Studies of Both ALRs –The kinetic parameters of the *S. lavendulae* ALR and the *E. coli* K12 W3110 ALR were determined using a CD assay developed by us (29). The $K_m$ values of both ALRs were not significantly different from each other, whereas the $k_{cat}$ value of the *S. lavendulae* ALR was twice as large as that of the *E. coli* ALR.

The resultant parameters of *S. lavendulae* ALR computed as a competitive or non-competitive inhibition model are shown in TABLE II. The equilibrium constants ($K_{eq}$) (46) in each analysis are 1.27 (for competitive) and 1.12 (for non-competitive), which are almost the same as the theoretical value (1.0). However, the value of c.c. in each case is equal (0.977 and 0.978), and the $K_m$ values are largely different from the results without DCS (29). Therefore, neither competitive nor non-competitive inhibition is applied to the inhibition mode of DCS to the *S. lavendulae* ALR.

Time-Dependent Inactivation by DCS of ALRs –Since it is difficult to apply the inhibition mode of DCS to each mechanism based on steady-state equilibrium (Eqs. 7 and
an attempt was made to apply the inhibition mode of DCS based on the time-dependent inactivation manner (16). This manner originates from the fact that DCS reacts with PLP bound to the enzyme (E) and forms a complex of a PLP-unbound enzyme (E’) and a 3-hydroxyisoxazole pyridoxamine 5’-phosphate derivative (X) (16, 17) (Scheme 1).

\[
E + I \rightleftharpoons E \cdot I \rightleftharpoons E' \cdot X \quad \text{(Scheme 1)}
\]

To investigate the effect of PLP degeneration on the remaining activity of ALR, the ALR activities after incubation with DCS at given times were analyzed by observing the CD signal at 205 nm (Fig. 6A and B). The slope of the regression line was defined as the ALR activity (v) at each incubation interval, and the decrease of the remaining activity was evaluated from Eq. 9.

\[
v_t/v_o = \exp(-k_{app}t) \quad \text{(Eq. 9)}
\]

The value of \( k_{app} \) is an apparent rate constant. At the beginning of the reaction, \([E’\cdot X]\) is regarded as zero, and the reverse reaction \( k_2 \) is ignored in Scheme 1. The inhibition constant is defined as

\[
K_I = \frac{[E] \cdot [I]}{[E \cdot I]} = \frac{([E]_0 - [E' \cdot X]) \cdot [I]}{[E \cdot I]}, \quad \text{where } [E]_0 \text{ means the total amount of enzyme; thus, the rate of DCS conversion is given as}
\]

\[
d[E'\cdot X]/dt = k_2 \cdot [E \cdot I]
\]

\[
= \frac{k_2 [I]}{K_I + [I]} ([E]_0 - [E’\cdot X]) \quad \text{(Eq. 10)}
\]

This equation means that \( k_{app} \) can be regarded as \( k_2 \cdot [I]/(K_I + [I]) \) at the initial phase of the reaction. Using these equations, \( K_I \) and \( k_2 \) are determined from double-reciprocal plots (Fig. 6C, Table III).

As shown in Table III, the \( K_I \) values of DCS for both ALRs are similar, but the \( k_2 \) value of \( S. \ lavendulae \) ALR is smaller than that of \( E. \ coli \) ALR. This kinetic experiment for the \( S. \ lavendulae \) ALR suggests that the time-dependent inactivation rate of the enzyme by DCS is absolutely slower than that of the \( E. \ coli \) ALR. It may be concluded that ALR from DCS-producing \( S. \ lavendulae \) is also one of the self-resistance determinants.
Comparison of the Inhibitory Effect of DCS with That of LCS on the S. lavendulae ALR Activity

Proteins which carry PLP as a cofactor, such as aminotransferases, are inhibited by LCS, like DCS. In fact, since the catalytic activity of the Bacillus stearothermophilus ALR is inhibited by LCS (16), we also examined the inhibitory effects of LCS on the S. lavendulae and E. coli ALR activities. Table III lists the kinetic parameters of both enzymes, which were determined by a CD spectrometric assay. The $k_2/K_I$ value ($4.5 \times 10^{-3} \text{s}^{-1}$) of DCS for the S. lavendulae ALR is smaller than that ($9.2 \times 10^{-3} \text{s}^{-1}$) for the E. coli ALR, suggesting that the former enzyme displays resistance to DCS when compared with the latter one. However, the $k_2/K_I$ value ($0.48 \times 10^{-3} \text{s}^{-1}$) of LCS for the E. coli ALR is lower than that of DCS. The time-dependent inactivation of the S. lavendulae ALR activity by LCS was not observed, suggesting that the enzyme exhibits more resistance to LCS than DCS. Structural evidence that ALR from S. lavendulae exhibits resistance to enantiomers of cycloserine will be provided in our next paper, which will be submitted simultaneously with this one.

The S. lavendulae ALR and DDL Function as DCS-resistance Determinants

Kinetic studies of the S. lavendulae ALR and DDL suggest that these enzymes may play an important role in the self-resistance of DCS-producing microorganisms. To verify this hypothesis, we examined whether E. coli carrying alrS or ddlS exhibits resistance to DCS in vivo. Therefore, we constructed several chimeric plasmids, designated pET-alrS, pET-ddlS, pET-K12alr, pET-ddlA, and pET-ddlB, which are generated by the insertion of the ALR or DDL gene from S. lavendulae and E. coli K12 W3110, into pET-21a(+). After E. coli transformed with each plasmid was grown in an M9 medium (4 ml) for 10 h, a 400-μl portion of the culture was mixed with an agar-melted M9 medium (4 ml) containing 1% (w/v) agar. The 180-μl portion was immediately transferred into a 96-well plate which contained a DCS solution (20 μl) at the given concentration (0, 3, 6, 12.5, 25, 50, and 100 μg/ml) and incubated for 14 h. The growth of the transformed cells, cultured in the M9 agar medium, was monitored by measuring the absorbance at 600 nm. Figure 7A shows that E. coli harboring pET-K12alr displays resistance to DCS as a result of the overexpression of ALR (33, 47). However, E. coli, transformed with pET-alrS, could grow under the condition of a higher concentration of DCS than the same host harboring pET-K12alr.

It has been reported that the overexpression of d-Ala-d-Ala ligase increases resistance
to DCS (47). In this study, we observed that *E. coli* harboring pET-ddlS is more resistant to DCS than the same host harboring pET-ddlA or pET-ddlB (Fig. 7B). This result suggests that the *Streptomyces* DDL, which is produced by *E. coli* harboring pET-ddlS, has lower affinity to DCS than the *E. coli* DdlA and DdlB.

*E. coli* transformed with pET-alrS-ddlS, which carries both ALR- and DDL-encoded genes from *S. lavendulae*, displayed higher resistance to DCS than the same cell transformed with pET-alrS or pET-ddlS (Fig. 7C). To know the resistance level to DCS by the co-expression of ALR and DDL from *E. coli* K-12 W3110, we constructed pET-K12alr-ddlA and pET-K12alr-ddlB by the insertion of ddlA or ddlB into pET-K12alr, respectively. Figure 7C shows that the co-expression of the *E. coli* alr and ddlA (or ddlB) confers absolutely higher resistance to DCS than the single expression of each gene. However, the increase in DCS resistance is clearly lower than the co-expression of alrS and ddlS from *S. lavendulae*. These results may indicate that, although the co-expression of ALR with DDL from DCS-producing microorganisms synergistically enhances the resistance to DCS, the DCS-resistance ability may be intrinsic to these enzymes expressed by the organism. In fact, *E. coli* transformed with pET-alrS-ddlS can grow vigorously, even in an LB medium supplemented with 1,600 μg DCS/ml (data not shown).

Figure 8 shows the expression level of ALR and DDL in the cell-free extract from *E. coli* transformed with each plasmid, which carries each enzyme-encoded gene(s) from *S. lavendulae* or *E. coli*: *E. coli* harboring pET-K12alr, pET-ddlA, or pET-ddlB overexpressed the *E. coli* ALR, DdlA, or DdlB, respectively. *E. coli* harboring pET-K12alr-ddlA or pET-K12alr-ddlB produced significant amounts of the *E. coli* ALR and DdlA or the *E. coli* ALR and DdlB, respectively. However, *E. coli* carrying alrS, ddlS, or alrS-ddlS expressed lower amounts of ALR, DDL, or ALR-DDL from *S. lavendulae*, respectively. These results suggest that the *Streptomyces* ALR and DDL contributes to resistance to DCS, even at lesser amounts. In other words, the ALR and DDL of DCS-producing microorganisms may function as resistance determinants to DCS.

Structural evidence by the X-ray crystallographic analysis that the *S. lavendulae* ALR confers resistance to DCS will be provided in another manuscript to be submitted simultaneously with this one (48).
ACKNOWLEDGEMENT

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REFERENCES

2. Pablos-Mendez, A., Raviglione, M.C., Laszlo, A., Binkin, N., Rieder, H.L., Bustreo, F.,
   190-194
   23, 5182-5187
    3255-3260
    5775-5783
    Am. Chem. Soc.* 120, 2268-2274


Figure Legends

FIG. 1.  Frame analysis of the DNA sequence from DCS-producing <i>S. lavendulae</i>. Of the cloned 14-bp DNA fragment from <i>S. lavendulae</i>, the 2,820-bp DNA sequence was determined in this study. The three complete genes were designated <i>orf I</i>, <i>orf II</i>, and <i>orf III</i>. The <i>orf I</i> encodes D-Ala-D-Ala ligase.

FIG. 2.  Alignment of the amino acid sequence of DDL from <i>S. lavendulae</i> with those from various bacteria. Alignment was carried out with the CLUSTAL W program on the Web site. DDL sequences of <i>Escherichia coli</i> (Eco DdlA and Eco DdlB), <i>Salmonella typhimurium</i> (Sty DdlA), <i>Haemophilus influenzae</i> (Hin DdlB), <i>Enterococcus faecalis</i> (Efa), <i>E. gallinarum</i> (Ega), and <i>E. hirae</i> (Ehi) were obtained from the DNA or protein database. The <i>S. lavendulae</i> DDL is represented as Sla DDL. The amino acids that putatively interact with ATP, the first D-Ala (D-Ala1), and the second D-Ala (D-Ala2) (20) are represented as bold letters. The aa residues, conserved in all sequences, are marked with an asterisk (*) below the sequences. The position of the ω-loop sequence is indicated by a bold line.

FIG. 3.  Frame analysis of the 3.3-kb DNA fragment from DCS-producing <i>S. lavendulae</i>. Three complete open reading frames were designated <i>orf1</i>, <i>orf2</i>, and <i>orf3</i>. The <i>orf1</i> encodes Ala racemase.

FIG. 4.  SDS-PAGE profiles of the purified <i>S. lavendulae</i> DDL. Lane 1, molecular weight standards; Lane 2, purified <i>S. lavendulae</i> DDL. The molecular sizes are indicated on the left-hand side of the panel.

FIG. 5.  The SDS-PAGE profiles of ALRs. Lane 1, molecular size markers; Lane 2 and Lane 3 show ALRs purified from <i>S. lavendulae</i> and <i>E. coli</i>, respectively.

FIG. 6.  Effect of PLP degeneration on the remaining activity of ALR after incubation with DCS. After ALR from <i>S. lavendulae</i> or <i>E. coli</i> was incubated with 2.0 mM DCS for the given times (0, 2, and 8 min), it was added to a solution (3 ml) consisting of a 30 mM sodium phosphate buffer (pH 8.2) and 4 mM D-Ala. The CD signal at 205 nm in the
reaction mixture is recorded as dots (A and B). The double reciprocal plots for each enzyme are shown as (C). The ALRs from *S. lavendulae* and *E. coli* are shown as closed circles and squares, respectively.

**FIG. 7. Resistance to DCS in *E. coli* carrying ALR and/or DDL.** The survival (%) by a given concentration of DCS was expressed as a ratio of *E. coli* harboring each plasmid grown in the presence of DCS to the same cells grown in the absence of DCS. The cell growth was monitored as OD at 600 nm. (A) Plasmids carrying *alrS* and K12*alr* are shown as open and closed triangles, respectively. (B) Plasmids carrying *ddlS*, *ddlA*, and *ddlB* are shown as open squares, closed squares, and closed diamonds, respectively. (C) Plasmids carrying *alrS-ddlS*, K12*alr-ddlA*, and K12*alr-ddlB* are shown as open diamonds, closed squares, and closed diamonds, respectively. In Fig. 7A, 7B, and 7C, *E. coli* harboring pET-21a(+) was used as a control strain (closed circles).

**FIG. 8. Expression level of ALR and DDL contained in the cell-free extract from *E. coli* harboring each plasmid.** Cell-free extract of *E. coli* harboring each plasmid. Lane 1, molecular size markers; Lane 2, pET vector without the inserted DNA as a control; Lane 3, pET-*alrS*; Lane 4, pET-*ddlS*; Lane 5, pET-*alrS-ddlS*; Lane 6, pET-K12*alr*; Lane 7, pET-*ddlA*; Lane 8, pET-*ddlB*; Lane 9, pET-K12*alr-ddlA*; Lane 10, pET-K12*alr-ddlB*.
### TABLE I

**Kinetic parameters for the S. typhimurium DdlA, the E. coli DdlA and DdlB, the E. faecium VanA, and the S. lavendulae DDL**

<table>
<thead>
<tr>
<th>Substrate/inhibitor</th>
<th>S. typh. DdlA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>E. coli DdlA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>E. coli DdlB&lt;sup&gt;b&lt;/sup&gt;</th>
<th>VanA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>DDL&lt;sup&gt;d&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>D-alanine</td>
<td>k&lt;sub&gt;cat&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>644</td>
<td>444</td>
<td>1018</td>
<td>295</td>
</tr>
<tr>
<td></td>
<td>K&lt;sub&gt;1&lt;/sub&gt; (µM)</td>
<td>1.9</td>
<td>5.7</td>
<td>3.3</td>
<td>3400</td>
</tr>
<tr>
<td></td>
<td>K&lt;sub&gt;2&lt;/sub&gt; (mM)</td>
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<td>0.55</td>
<td>1.2</td>
<td>38</td>
</tr>
<tr>
<td>ATP</td>
<td>K&lt;sub&gt;m&lt;/sub&gt; (µM)</td>
<td>38</td>
<td>38</td>
<td>40</td>
<td>116</td>
</tr>
<tr>
<td>D-Ala-D-Ala</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; (µM)</td>
<td>61 (NC)</td>
<td>49 (NC)</td>
<td>70 (NC)</td>
<td>2300 (NC)</td>
</tr>
<tr>
<td></td>
<td>K&lt;sub&gt;i&lt;/sub&gt; (µM)</td>
<td>14 (C)</td>
<td>8.9 (C)</td>
<td>27 (C)</td>
<td>730 (C)</td>
</tr>
<tr>
<td>D-cycloserine</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; (µM)</td>
<td>0.09 (± 0.02)</td>
<td>0.14 (± 0.03)</td>
<td>0.24 (± 0.03)</td>
<td>0.6 (± 0.1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Measured by the continuous ADP release coupled assay (27).
<sup>b</sup> Data are from the previous literature (13).
<sup>c</sup> Data are from (42).
<sup>d</sup> D-Ala-D-Ala ligase from DCS-producing S. lavendulae

NC = noncompetitive; C = competitive; ND = not determined.

### TABLE II

**Kinetic parameters of ALR computed as a competitive or non-competitive inhibition model**

<table>
<thead>
<tr>
<th></th>
<th>Competitive model (c.c. = 0.977)</th>
<th>Non-competitive model (c.c. = 0.978)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D→L direction</td>
<td>L→D direction</td>
</tr>
<tr>
<td>Km (mM)</td>
<td>0.7 (± 0.2)</td>
<td>0.7 (± 0.1)</td>
</tr>
<tr>
<td>k&lt;sub&gt;cat&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>4.2 (± 0.4) × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>3.3 (± 0.2) × 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt; (mM)</td>
<td>0.09 (± 0.02)</td>
<td>0.14 (± 0.03)</td>
</tr>
</tbody>
</table>

### TABLE III

**Kinetic parameters for the inhibition of ALR by DCS and LCS determined from the CD spectrometric assay**

<table>
<thead>
<tr>
<th></th>
<th>DCS</th>
<th>LCS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K&lt;sub&gt;i&lt;/sub&gt; (mM)</td>
<td>k&lt;sub&gt;2&lt;/sub&gt; × 10&lt;sup&gt;-3&lt;/sup&gt; (mM·s&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>S. lavendulae</td>
<td>0.87 (± 0.08)</td>
<td>3.9 (± 0.5)</td>
</tr>
<tr>
<td>E. coli</td>
<td>1.2 (± 0.1)</td>
<td>11 (± 2)</td>
</tr>
</tbody>
</table>

<sup>a</sup>ND means "not determined” due to that the decrease of ALR activity was not observed in the given time range.
FIG. 1. Noda et al.

orf I (= ddlS)
orf II (= orf B)
orf III

Base numbers

G + C (%)
FIG. 3. Noda et al.
FIG. 4. Noda et al.
FIG. 5. Noda et al.
FIG. 6. Noda et al.

A

wave length (nm)

0 100 200 300 400

control

2 min

8 min

S. lavendulae ALR

B

wave length (nm)

0 100 200 300 400

control

3 min

6 min

E. coli ALR

C

1/I

0 0.5 1 1.5 2 2.5 3

0 5 10 15

U_1/2
FIG. 7. Noda et al.

A

Survival (%) vs. DCS (μg/ml)

B

Survival (%) vs. DCS (μg/ml)

C

Survival (%) vs. DCS (μg/ml)
FIG. 8. Noda *et al.*
Self-protection mechanism in D-cycloserine-producing streptomyces lavendulae: Gene cloning, characterization, and kinetics of its alanine racemase and D-alanyl-D-alanine ligase, which are target enzymes of D-cycloserine

Masafumi Noda, Yumi Kawahara, Azusa Ichikawa, Yasuyuki Matoba, Hiroaki Matsuo, Don-Geun Lee, Takanori Kumagai and Masanori Sugiyama

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