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‡, Dong-Geun Lee, Takanori Kumagai, and Masanori Sugiyama§

From the Department of Molecular Microbiology and Biotechnology, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-Ku, Hiroshima 734-8551, Japan

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An antibiotic, d-cycloserine (DCS), inhibits the catalytic activities of alanine racemase (ALR) and 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 alrS and ddlS, respectively, from DCS-producing Streptomyces lavendulae ATCC25223. Each gene product was purified to homogeneity and characterized. Escherichia coli, transformed with a PET vector carrying alrS or ddlS, displays higher resistance to DCS than the same host carrying the E. coli ALR- or DDL-encoded gene inserted into the PET vector. Although the S. lavendulae DDL was competitively inhibited by DCS, the Ki value (920 μM) was obviously higher (40–100-fold) than those for E. coli DdIA (9 μM) or DdIB (27 μM). The high Ki value of the S. lavendulae DDL suggests that the enzyme may be a self-resistance determinant in the DCS-producing microorganism. Kinetic studies for the S. lavendulae ALR suggest that the time-dependent inactivation rate of the enzyme by DCS is absolutely slower than that of the E. coli ALR. We conclude that ALR from DCS-producing S. lavendulae is also one of the self-resistance determinants.

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

D-Cycloserine (0-4-amino-3-isoxazolidone (DCS)) is a cyclic structural analogue of d-alanine (d-Ala) and is produced by Streptomyces garyphalus and Streptomyces 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 because of its serious side effects (3). The side effects are caused by the binding of DCS to N-methyl-d-aspartate receptors as an agonist. However, application of these adverse effects to treatments for neural diseases (4) such as Alzheimer’s (5) and Parkinsonism (6) have been dedicatedly researched.

The peptidoglycan 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 peptidoglycan, is followed by a cross-link reaction of the precursors. In the cross-linking process, D-Ala plays an important role as a bridge molecule (7). Because D-amino acids, including D-Ala, are not primarily found in natural resources, bacteria generate D-Ala from L-Ala by the catalysis of ALR racemase (ALR). This enzyme needs a pyridoxal 5′-phosphate (PLP) as a cofactor and catalyzes the racemization of both Ala enantiomers. Escherichia coli and Salmonella typhimurium possess two kinds of closely related ALR-encoded genes (alr and dalX in E. coli, and dal and dadB in S. typhimurium) (8–10). For example, the race-

DSL interferes with the activities of both ALR and DDL, which are necessary for the synthesis of peptidoglycan contained in the cell wall of bacteria. Because 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 competitively inhibited because DCS is structurally similar to d-Ala (13, 15). However, it was recently reported that DCS inhibits the catalytic activity of ALR in a time-dependent inactivation manner (16). In addition, the an-

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‡ Present address: Dept. of Dermatology, Shimane Medical University, 88-1 Enya-cho, Izumo, Shimane 693-8501, Japan.
§ To whom correspondence should be addressed. Tel.: 81-82-257-5280; Fax: 81-82-257-5284; E-mail: sug@hiroshima-u.ac.jp.

The abbreviations used are: DCS, d-cycloserine; aa, amino acid(s); ALR, alanine racemase; alrS, a gene encoding ALR from DCS-producing S. lavendulae; DDL, d-alanyl-d-alanine ligase; ddlS, a gene encoding DDL from DCS-producing S. lavendulae; dda, a gene encoding DDL from E. coli or S. typhimurium; ddb, a gene encoding DDL from E. coli; K12dtr, a gene encoding ALR from E. coli K-12 W3110; LCS,
Alanine Racemase and D-Ala-D-Ala Ligase from D-Cycloserine Producer

from ATCC25233 were grown at 28 °C in a GMP medium (23) or a YEME. The plasmid DNA from S. lavendulae was extracted from the agarose gel, purified, ligated to BamHI-digested Lambda DASH II vector, and packaged was performed using a Gigapack III Gold Packaging Extract (Stratagene) according to the supplier's instructions. One positive clone was obtained by plaque hybridization. The plaque DNA, isolated from the positive plaque, had a 14-kb DNA insert from S. lavendulae.

The plasmid 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) and ABI PRIZM 310 genetic analyzer (Applied Biosystems). Of the 14-kb DNA fragment, in the present study, we determined the nucleotide sequence of a 2820-bp DNA fragment including the S. lavendulae ddl gene. Genetic analysis was performed by using GENETYX-Mac software (Software Development, Tokyo, Japan) and the Frame Analysis program (25). The homology search was done with the FASTA program. 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 BamHI, 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 (1176 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′-ATGAGCGAGACAACT-3′) and an antisense primer (5′-TCTATTGGTT-GACGTAGCAGCGGCGGACCCCGG-3′). PCR was done using 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 BamHI-digested genomic DNA that had a size of 3.0 kb. Therefore, BamHI digests of 2.5–3.5 kb were extracted from the agarose gel, purified, ligated to BamHI-digested pUC19, and then introduced into E. coli TG1. The resulting genomic libraries were screened using the colony hybridization technique (21). From ~8000 clones, 52 candidates carrying the putative ALR gene were identified by the positive hybridization 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. Using a 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 open reading frames (ORFs). The ORFs were analyzed 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′-CACCATGGAATACTGGTGCAT-3′ (Ndel site underlined), and an antisense primer, 5′-CACCTGGA- GTAGCGCGGTGTCAGCGGAGAGG-3′ (Xhol site underlined). PCR was done under the following conditions: 1 cycle of 5 min at 96 °C, 1 min at 55 °C, and 2 min at 72 °C followed by 29 cycles of 1 min at 96 °C, 1 min at 55 °C, and 2 min at 72 °C. The amplified DNA was digested with Ndel and Xhol and ligated into the Ndel- and Xhol-digested pET-dllS (number AB176676). The resulting plasmid was transformed into E. coli BL21(DE3)-pLysS harboring pET-dllS was grown at 28 °C in 6 liters of LB medium to an OD600 of 0.5. Then, 0.5 ml of 3 M Na2S and 1 ml of 0.1 M D Thigalactopyranoside were added to the culture at the optimal concentration for DDL induction. 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.
buffer. After the dialysate was applied on a DEAE-Sepharose column (2.5 × 10 cm, Amersham Biosciences) 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 \textit{S. lavendulae} DDL were pooled and dialyzed against Buffer II (20 mM Tris-HCl (pH 7.5), 2.5 mM KCl, 1 mM ATP, 10 mM MgCl₂, and 1 mM 2-mercaptoethanol) and then subjected to an Octyl-Sepharose column (1.5 × 15 cm, Amersham Biosciences) previously equilibrated with Buffer II. Since no \textit{S. lavendulae} DDL was bound to the column, the solution that passed through the column was collected. Through these steps, the \textit{S. lavendulae} DDL was purified to homogeneity.

\textit{Overexpression and Purification of S. lavendulae ALR}—An ALR gene of \textit{S. lavendulae} was amplified by PCR using the sense primer, 5′-CA-CCATATGACGACGACGACGGCGCGCTG-3′ (the underline indicates the NdeI cleavage site), and the antisense primer, 5′-TATCTCGAGGGCCGCCGAGTGACGCGC-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-ALR. The pET-ALR plasmid expresses ALR having His₆ tag at the C terminus. \textit{E. coli} BL21(DE3)-plysS harboring pET-ALR was grown in 3 liters of LB medium at 28 °C. At the exponential phase of growth (OD₆₀₀₄₅ ≈ 0.6), isopropyl-β-D-thiogalactopyranoside was added to the culture at a 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 for 20 min, dissolved in a binding buffer, and dialyzed against the same buffer. The dialysate 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) column (1 × 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 50 mM imidazole) and eluted with a linear gradient of 60–350 mM Na₂, and 0.2 mM ATP) and applied on a DEAE-Sepharose column (1.5 × 15 cm) equilibrated with Buffer III. The \textit{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 \textit{S. lavendulae} DDL was purified to homogeneity.

\textit{Enzyme Assay and Kinetic Study of DDL}—Kinetic assays for the purified \textit{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 of the proposed reaction sequence, which is shown as Equation 1, a rate equation (Equation 2) can be obtained, which gives parabolic Lineweaver-Burk plots (Equation 3). The \( V_{\text{max}} \) value can be obtained from the y intercept of Equation 3. Subsequently, a plot of \([S]/(V_{\text{max}} - V)\) against 1/[S] gives a straight line (Equation 4), in which the y intercept \((K_{\text{m}}V_{\text{max}})/V_{\text{max}} \) and slope \((K_{\text{m}}V_{\text{max}})/V_{\text{max}} \) provide the two \( K_{\text{m}} \) values.

\[
K_v = \frac{V_{\text{max}}[S]}{K_v + [S] + [S]} + [S] \quad \text{(Eq. 1)}
\]

\[
1 = \frac{V_{\text{max}}[S]}{K_v + [S] + [S]} = \frac{K_v}{V_{\text{max}}[S]} + \frac{1}{V_{\text{max}}[S]} \quad \text{(Eq. 2)}
\]

\[
[S] \quad \frac{1}{1 + \frac{1}{V_{\text{max}}[S]} - \frac{K_v}{V_{\text{max}}[S]} - \frac{1}{V_{\text{max}}[S]} \quad \text{(Eq. 3)}
\]

\[
K_v V_{\text{max}}[S] = K_v + [S] \quad \text{(Eq. 4)}
\]

Because the \( K_v \) value in the above equations is very small (13), the value can be ignored when the concentration of the substrate ([S]) is high; therefore, Equations 2 and 3 can be represented as Equations 5 and 6, respectively. We determined the \( K_v \) value alone using these equations (Equations 5 and 6).

\[
K_v + [S] = \frac{V_{\text{max}}[S]}{K_v} \quad \text{(Eq. 5)}
\]

\[
1 = \frac{V_{\text{max}}[S]}{K_v + [S]} = \frac{K_v}{V_{\text{max}}[S]} + \frac{1}{V_{\text{max}}[S]} \quad \text{(Eq. 6)}
\]

\textit{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.² The CD signals of samples were measured using a spectropolarimeter (JU-720 type, JASCO, Japan). To understand the inhibition mode of DCS to the 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 [D-Ala] were also calculated as described,³ and numerical analysis was performed to maximally fit to Equations 7 and 8, which are equations applied for competitive and noncompetitive inhibition, respectively.

\[
v = \frac{V_{\text{max}}[\text{D-Ala}]}{K_{\text{m1}}(1 + [I]/K_{i1})} - \frac{V_{\text{max}}[\text{L-Ala}]}{K_{\text{m2}}(1 + [I]/K_{i2})} \quad \text{(Eq. 7)}
\]

\[
v = \frac{V_{\text{max}}[\text{L-Ala}]}{K_{\text{m1}}(1 + [I]/K_{i1})} \quad \frac{V_{\text{max}}[\text{D-Ala}]}{K_{\text{m2}}(1 + [I]/K_{i2})} \quad \text{(Eq. 8)}
\]

² M. Noda, Y. Matoba, T. Kumagai, and M. Sugiyama, submitted for publication.
In Equations 7 and 8, $K_{i1}$ and $K_{i2}$ are inhibition constants for the D- to L-direction and $K_{i3}$ 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 L CS (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'-T-3'.
ATCATATTGGAAAAACTCCGGTGAGGATC-3′ (the underline indicates the NdeI cleavage site) and 5′-CCCAAGACTTACAGTG-GGT- TTTCAATGC-3′ (the underline indicates the HindIII cleavage site) for ddlA and 5′-CACCATATGACTGATAAAATCGCGTGCTG-3′ (the underline indicates the NdeI cleavage site) and 5′-CACAGGCTTTAGT- CCGCAAGTTTCCAGAATC-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′-CACGCATGCGAAATTA- GGT-3′ (the underline indicates the SphI cleavage site) and 5′-TTTCAATGC-3′ (the underline indicates the HindIII cleavage site) for ddlA, and the amplified DNA was inserted into the SphI-digested pET-K12arl to generate pET-K12arl-ddlA and pET-K12arl-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-ddlS, which was digested with SphI and blunted to generate pET-aldS-ddlS.

**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 orf I (19). We suggest that the orf I gene product, which may carry membrane-integral domains spanning the membrane 10 times, may determine whether the orf I gene product has an 89% identity to a DCS-resistant protein from *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 *P. 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 orfI. 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 orfF 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 hereafter as ddlS and DDL, respectively.

Fig. 2 shows a comparison of the aa sequence of DDL from *S. lavendulae* with those from various bacteria. The amino acids that interact with ATP and d-Ala (30) are conserved except for Leu320, which corresponds to Leu282 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 3′-adjacent region of the DCS resistance gene in *S. garyphalus* (19). That is, orf I was cloned from *S. garyphalus*. We recently cloned a 3.5-kb DNA fragment from DCS-producing *S. garyphalus*, which includes a DCS resistance gene, designated orf I (19). We suggest that the orf I gene product, which may carry membrane-integral domains spanning the membrane 10 times, may determine whether the orf I gene product has an 89% identity to a DCS-resistant protein from *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 *P. 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 orfI. 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 orfF 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 hereafter as ddlS and DDL, respectively.

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 fragment.
DNA fragment, which is adjacent to the 2.8-kb DNA fragment, by conducting a chromosome-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 *orf*1, *orf*2, and *orf*3. One of these, *orf*1, 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 nucleotide sequence of *orf*1 was deposited in the DDBJ (accession no. AB176676). The aa sequence of the putative ALR from *S. coelicolor* (22). This *orf*1 is referred to as *a1rS* hereafter. The nucleotide sequence of *a1rS* 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 also shows a significant homology to ALRs from mycobacteria (33, 34). A Lys residue in the ALR, which is identical to that of *S. coelicolor* A3 (22).

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

<table>
<thead>
<tr>
<th>Substrate/inhibitor</th>
<th><em>S. typhimurium</em> DdlA</th>
<th><em>E. coli</em> DdlA</th>
<th><em>E. coli</em> DdlB</th>
<th>VanA</th>
<th>DDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Alanine</td>
<td><em>k_\text{cat} (\text{min}^{-1})</em></td>
<td>644</td>
<td>444</td>
<td>1018</td>
<td>295</td>
</tr>
<tr>
<td></td>
<td><em>K_m (\mu M)</em></td>
<td>1.9</td>
<td>5.7</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td><em>K_0.5 (\mu M)</em></td>
<td>0.54</td>
<td>0.55</td>
<td>1.2</td>
<td>38</td>
</tr>
<tr>
<td>D-Ala-D-Ala</td>
<td><em>K_m (\mu M)</em></td>
<td>38</td>
<td>116</td>
<td>40</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td><em>K_0.5 (\mu M)</em></td>
<td>61 (NC)</td>
<td>49 (NC)</td>
<td>70 (NC)</td>
<td>2,300 (NC)</td>
</tr>
<tr>
<td>D-Cycloserine</td>
<td><em>K_m (\mu M)</em></td>
<td>14 (C)</td>
<td>8.9 (C)</td>
<td>27 (C)</td>
<td>730 (C)</td>
</tr>
</tbody>
</table>

aData are from the previous literature (13).

**Table II** Kinetic parameters of ALR computed as a competitive or noncompetitive inhibition model

<table>
<thead>
<tr>
<th>Competitive model ((C^*_e = 0.977))</th>
<th>Noncompetitive model ((C^*_e = 0.978))</th>
<th>(D \rightarrow L) Direction</th>
<th>(L \rightarrow D) Direction</th>
<th>(D \rightarrow \alpha) Direction</th>
<th>(L \rightarrow \alpha) Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_m (\mu M))</td>
<td>0.7 (\pm 0.2)</td>
<td>0.7 (\pm 0.1)</td>
<td>1.1 (\pm 0.2)</td>
<td>0.9 (\pm 0.1)</td>
<td></td>
</tr>
<tr>
<td>(k_\text{cat} (\text{min}^{-1}))</td>
<td>4.2 (\pm 0.4) \times 10^3</td>
<td>3.3 (\pm 0.2) \times 10^3</td>
<td>5.2 (\pm 0.2) \times 10^4</td>
<td>3.8 (\pm 0.2) \times 10^5</td>
<td></td>
</tr>
<tr>
<td>(K_i (\mu M))</td>
<td>0.09 (\pm 0.02)</td>
<td>0.14 (\pm 0.03)</td>
<td>0.24 (\pm 0.03)</td>
<td>0.6 (\pm 0.1)</td>
<td></td>
</tr>
</tbody>
</table>

**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 \(\mu\)mol min\(^{-1}\) at pH 7.0 and 57 \(\mu\)mol min\(^{-1}\) at pH 10.0, respectively, suggesting that the enzyme exhibits higher activity as the pH values increase. The \(\omega\)-loop in the *S. lavendulae* DDL has an Ala-Lys-Tyr-Gln sequence, raising the question of whether the enzyme displays \(\alpha\)-Ala-\(\alpha\)-Ser ligase activity. A TLC assay (40) confirmed that the *S. lavendulae* DDL did not display \(\alpha\)-Ala-\(\alpha\)-Ser ligase or \(\alpha\)-Ala-\(\alpha\)-Lac activity (data not shown). The latter observation is consistent with the fact that \(\alpha\)-Ala-\(\alpha\)-Lac ligases, such as VanA (41, 42) and VanB (43, 44) from vancomycin-resistant bacteria, possess the consensus \(\omega\)-loop sequence of Pro-Glu-Lys-Gly (31). The \(\omega\)-loop consensus in \(\alpha\)-Ala-\(\alpha\)-Lac ligases from lactic acid bacteria, including *Lactobacillus confusus*, *Lactobacillus salivarius*, and *Lactobacillus plantarum*, has the Asn-(Lys/Met)-Phe-Val sequence (31).

**Overproduction and Purification of the *S. lavendulae* DDL—**Because 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 \(\times\) 120 cm, Amersham Biosciences), 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 His\(_6\) 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 classified into two types of subunit structures, a monomer and a homodimer structure (38). Gel filtration chromatography with Sephacryl S-200HR (1.5 \(\times\) 120 cm, Amersham Biosciences) revealed that the *S. lavendulae* ALR has a molecular mass of about 80 kDa, suggesting that it may have a homodimeric structure.
that of VanA ligase. The $K_m$ value of the *S. lavendulae* DDL for ATP was 3-fold higher than those of DdlA and DdbB. This 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 *S. typhimurium* DdlA (61 μM) and the *E. coli* DdlA (49 μM) and DdbB (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 DdbB and the *Salmonella* DdlA were noncompetitively inhibited (13). The reason that there is a difference between the inhibition modes in these enzymes is currently unclear.

DCS inhibits DdlAs and DdbB 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 DdbB. This value was close to that of 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 DdbB ($K_i$ for the second D-Ala and $K_i$ for D-Ala-D-Ala) and, in part, to those of VanA ($k_{cat}$, $K_i$ 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, crystallization experiments are in progress to determine the three-dimensional structure of the *S. lavendulae* DDL.

**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 that we developed. 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 noncompetitive 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 noncompetitive), which are almost the same as the theoretical value (1.0). However, the value of the correlation coefficient ($C_c$) in each case is equal (0.977 and 0.978), and the $K_m$ values are largely different from the results without DCS. Therefore, neither competitive nor noncompetitive inhibition is applied to the inhibition mode of DCS to the *S. lavendulae* DDL.

**Time-dependent Inactivation by DCS of ALRs**—Because it is difficult to apply the inhibition mode of DCS to each mechanism based on steady-state equilibrium (Equations 7 and 8), 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 protein (Equation 7) and forms a complex of a PLP-unbound enzyme (Equation 8) (Scheme 1).

\[
E + \text{DCS} \rightarrow E-\text{DCS} \rightarrow E^*-\text{DCS} \rightarrow E^* \rightarrow E + \text{DCS}
\]

**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. 6, A and B). The slope of the regression line was defined as the ALR activity (v) at each incubation interval, and
TABLE III

<table>
<thead>
<tr>
<th></th>
<th>DCS</th>
<th>LCS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$</td>
<td>$k_2 \times 10^{-3}$</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>

* ND, not determined because the decrease of ALR activity was not observed in the given time range.

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 the absorbance (A) at 600 nm.

FIG. 8. Expression level of ALR and DDL contained in the cell-free extract from 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-K12alr; lane 7, pET-ddlA; lane 8, pET-ddlB; lane 9, pET-K12alr-ddlA; lane 10, pET-K12alr-ddlB.

The survival (%) was expressed as a ratio of the number of survivors to the number of cells inoculated.

The survival (%) by a given concentration of DCS was expressed as a ratio of the number of survivors to the number of cells inoculated.

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 $K_i = [E'\cdot I]/[E'\cdot I] = ([E'\cdot I] - [E'\cdot X] + [E'\cdot I])/[E\cdot I]$, where $[E'\cdot I]$ means the total amount of enzyme; thus, the rate of DCS conversion is given as

$$
dE'[\cdot X]/dt = k_{2}/K_i [E'\cdot I]$$

This equation means that $k_{app}$ can be regarded as $k_{2}/[I]/K_i + [I]$ at the initial phase of the reaction. Using these equations, $K_i$ and $k_2$ are determined from double reciprocal plots (Fig. 6C and 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 as they are by DCS. In fact, because 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}$ s$^{-1}$) of DCS for the S. lavendulae ALR is smaller than that of the E. coli ALR, suggesting that the former enzyme displays resistance to DCS when compared with the latter. However, the $k_2/K_i$ value (0.48$ \times 10^{-3}$ 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 is provided in an accompanying paper (29).

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 (1%) 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. Fig. 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 higher expression of DCS than the 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 K12 W3110, we constructed pET-K12alr-ddlA and pET-K12alr-ddlB by the insertion of ddlA or ddlB into pET-K12alr, respectively. Fig. 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 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 a LB medium supplemented with 1600 μg of DCS/ml (data not shown).

Fig. 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 are provided in an accompanying paper (29).

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Alanine Racemase and D-Ala-D-Ala Ligase from D-Cycloserine Producer

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, Dong-Geun Lee, Takanori Kumagai and Masanori Sugiyama

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