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

Received for publication, April 26, 2004, and in revised form, August 3, 2004
Published, JBC Papers in Press, August 9, 2004, DOI 10.1074/jbc.M404603200

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

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 (d-4-amino-3-isoxazolidone (DCS)), which 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 dedicately 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 (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 dadX in E. coli, and dal and dadB in S. typhimurium) (8–10). For example, the racemase encoded by dal of S. typhimurium is necessary for peptidoglycan synthesis and displays a 40% identity to a catabolic racemase encoded by dadB (11).

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

DCS 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-

* This work was supported by the National Project on Protein Structural and Functional Analysis, Japan. The costs of publication of this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB176675 and AB176676.
‡ Present address: Dept. of Dermatology, Shimane Medical University, 89-1 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: sugi@hiroshima-u.ac.jp.
* The abbreviations used are: DCS, d-cycloserine; aa, amino acids; 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; ddlA, a gene encoding DDL from E. coli or S. typhimurium; ddlB, a gene encoding DDL from E. coli; K12dr, a gene encoding ALR from E. coli K-12 W3110; LCS, L-cycloserine; MES, 2-(N-morpholino)ethanesulfonic acid; ORF, open reading frame; PLP, pyridoxal 5'-phosphate.

This paper is available on line at http://www.jbc.org

46143
tibiotic and its enantiomer, 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 products. We recently cloned a 3.5-kb DNA fragment carrying a gene that confers resistance to DCS from DCS-producing S. garyphalus by a “shotgun” cloning technique (19). The hydropathy 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 gene, which makes it similar to a gene encoding ALR. Because 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 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)-pLyS and plasmid pET-21a (+) (Novagen) were used for protein expression. E. coli was grown in LB medium (21) at 37 or 28 °C. If necessary, ampicillin (100 μg/ml) and/or chloramphenicol (34 μg/ml) were added to the LB medium. For the cultivation of E. coli XL1-Blue MRA (P2), 0.2% maltose and 10 mM MgSO4 were added to the LB medium. Streptomyces coelicolor A3 (2) (strain M145), 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 μg) from S. lavendulae was partially digested with BamHI, purified by the phenol/chloroform extraction method, and precipitated by ethanol. The DNA fragments, cleaved within 10–20 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). In vitro packaging was performed using a Gigapack III Gold Packaging Extract (Stratagene) 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), 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 according to the manufacturer’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, 0.6, 2.8, 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 and sense primer (5′-GAGCTCGTACCCGCGCGACGCGGCGC-3′) and an antisense primer (5′-TCATTGCTT-GACGTAGACGGCGCGACGCGGAG-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 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 colonies, 52 candidates carrying the putative ALR gene were enriched by the putative ALR gene 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 gene 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 identified 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′-CACCATGCTGAAATTCGTGTTTGTTGGTTGAGGAGGAG-G3′ (Ndel site underlined), and an antisense primer, 5′-CACCTGCA-GTCACGCGGTGGGAGGACGAC-3′ (XhoI 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 XhoI and subcloned into Ndel- and XhoI-digested pET-dllS (EMBL plasmid) to generate pET-dllS. E. coli BL21(DE3)-pLyS harboring pET-dllS was grown at 28 °C in 6 l of LB medium to an A600 of 0.5, whereupon isopropyl-β-D-thiogalactopyranoside was added to the culture at final concentration of 1 mM to induce the expression of dllS. The E. coli cells were grown for 8 h at 28 °C. The purification of the S. lavendulae DDL was carried out at 4 °C: the E. coli cells were suspended in Buffer 1 (100 mM potassium phosphate (pH 7.5), 10 mM NaCl, 10 mM MgCl2, and 1 mM EDTA), NaCl was 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 1 and dialyzed against the same
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 *S. lavendulae* DDL were pooled and dialyzed against Buffer II (20 mM Tris-HCl (pH 7.9), 100 mM NaCl, and 0.2 mM ATP) and applied on an Octyl-Sepharose column (1.5 × 15 cm, Amersham Biosciences) 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 dithiothreitol, 1 mM EDTA, and 0.1 mM dithranol) and then subjected to a Octyl-Sepharose column (1.5 × 15 cm, Amersham Biosciences) previously equilibrated with Buffer II. The solution carrying the DDL activity was dialyzed against Buffer III (50 mM sodium phosphate (pH 7.5), 10 mM MgCl₂, 2 mM dithiothreitol, 1 mM EDTA, and 0.2 mM ATP) and applied on a DEAE-Sepharose column (1.5 × 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′-CA-CCATATGACGACAGCCGCGCGTG-3′ (the underline indicates the NdeI cleavage site), and the antisense primer, 5′-TATCTCCG-AGGCGCGGCGAGGTACCGGCCG-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-DL1 (1) to yield pET-ALR. The pET-ALR plasmid expresses ALR His tag at the C terminus. *E. coli* BL21 (DE3)-pLysS harboring pET-DL1 was 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. Through these steps, 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.

**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 Equation 1, a rate equation (Equation 2) can be obtained, which gives parabolic Lineweaver-Burk plots (Equation 3). The *V*ₘₐₓ value can be obtained from the y intercept of Equation 3. Subsequently, a plot of [(S)]/[V*ₘₐₓ*][1/V*ₘₐₓ*] against [S]/[S] gives a straight line (Equation 4), in which the y intercept (Kᵢ/Vₘₐₓ) and slope (Kᵢ/Kᵥₘₐₓ) provide the two Kᵢ values.

Because the Kᵢ 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 Kᵢ value alone using these equations (Equations 5 and 6).
In Equations 7 and 8, $K_i^1$ and $K_i^2$ are inhibition constants for the $D$-to-$L$ direction and $K_i^3$ and $K_i^4$ 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 g/ml) was incubated with the given concentrations of DCS (0.4–3.0 mM) or L-Cys (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-
pET-K12, and pET-K12 alr-ddlA S. lavendulae designated fragment carrying the or DDL-encoded Genes from S. lavendulae and E. coli—was digested with SphI and blunted to generate pET-alrS, pET-ddlS, pET-ddlA.

The orfB gene product has an 89% identity to a DDL from S. coelicolor, which suggests that the gene is conserved in both S. lavendulae—strains. A gene located downstream of orfB, orf II 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 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

**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 2.5-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 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 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 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 Leu230, which corresponds to Leu262 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*. One of these, *orf1*, consists of 1,154 bp, and a protein deduced from the nucleotide sequence has 378 aa, with a molecular mass of 39.9 kDa. The aa sequence of the putative ALR shows a significant homology to ALRs from mycobacteria (33, 34). The aa sequence has 378 aa, with a molecular mass of 39.9 kDa. The nucleotide sequence has 1,134 bp, and a protein deduced from the nucleotide sequence has 378 aa, with a molecular mass of 39.9 kDa. The complete ORFs, designated *orf1*, *orf2*, and *orf3*, suggest the presence of three complete ORFs, designated *orf1*, *orf2*, and *orf3*. One of these, *orf1*, consists of 1,154 bp, and a protein deduced from the nucleotide sequence has 378 aa, with a molecular mass of 39.9 kDa. The aa sequence of the putative ALR shows a significant homology to ALRs from mycobacteria (33, 34). The aa sequence has 378 aa, with a molecular mass of 39.9 kDa.

**TABLE I**

Kinetic parameters for *S. typhimurium* DdIA, *E. coli* DdIA and DdIB, *E. faecium* VanA, and *S. lavendulae* DDL

Activity was measured by the continuous ADP release-coupled assay (27). NC, noncompetitive; C, competitive; ND, not determined.

| Substrate/inhibitor | *S. typhimurium* DdIA | *E. coli* DdIA | *E. coli* DdIB | VanA | DDL |
|---------------------|-----------------------|----------------|----------------|------|-----|
| d-Alanine+Ala | 644 | 444 | 1018 | 295 | 100 |
| kcat (min⁻¹) | 1.9 | 5.7 | 3.3 | 3,400 | ND |
| Km (μM) | 0.54 | 0.55 | 1.2 | 38 | 2.0 |
| ATP Kd (μM) | 38 | 116 | 406 | 130 | 120 |
| d-Ala+d-Ala \(K_m\) (μM) | 61 (NC) | 49 (NC) | 70 (NC) | 2,300 (NC) | 60 (C) |
| d-Cycloserine \(K_m\) (μM) | 14 (C) | 8.9 (C) | 27 (C) | 730 (C) | 920 (C) |

| a | Data are from the previous literature (13). |
| b | Data are from Bugg et al. (42). |
| c | d-Ala-d-Ala ligase from DCS-producing *S. lavendulae*. |

**TABLE II**

Kinetic parameters of ALRs computed as a competitive or noncompetitive inhibition model

| Competitive model \((C_r = 0.977)\) | Noncompetitive model \((C_r = 0.978)\) |
|-----------------|-----------------|
| \(K_m (μM)\) | \(k_cat (min⁻¹)\) | \(K_m (μM)\) | \(k_cat (min⁻¹)\) |
| \(d \rightarrow L\) Direction | \(L \rightarrow d\) Direction | \(d \rightarrow L\) Direction | \(L \rightarrow d\) Direction |
| 0.7 (±0.2) | 4.2 (±0.4) × 10³ | 0.7 (±0.1) | 3.3 (±0.2) × 10³ |
| 0.09 (±0.02) | 0.14 (±0.03) | 0.24 (±0.03) | 0.6 (±0.1) |

**Enzyme Properties of the *S. lavendulae* DDL**—DDL 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 μmol min⁻¹ at pH 7.0 and 57 μmol 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 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 \(\omega\)-loop sequence of Pro-Glu-Lys-Gly (31). The \(\omega\)-loop consensus in D-Ala-D-Lac ligases from lactic acid bacteria, including *Lactobacillus confusus*, *Lactobacillus salivarius*, and *Lactobacillus 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 *S. typhimurium* DdIA, the *E. coli* DdIA and DdIB, and the *Enterococcus faecium* VanA (13, 42). The turnover number \((k_cat)\) of the *S. lavendulae* DDL was 4–10-fold lower than those of the *E. coli* DdIA and DdIB, and the *Salmonella* DdIA (Table I). In addition, the \(K_m\) value of the *Streptomyces* DDL for the second D-Ala substrate \((K_d)\) was 4- and 2-fold higher than those of DdIA and DdIB, respectively.

The \(k_cat\) value of the *S. lavendulae* DDL was much closer to...
shown as plots for each enzyme. The ALRs from the reaction mixture is shown as \( E. coli \) ALR after incubation with DCS. 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 range of 9–27 \( \mu \)M (Table I). Although the \( S. lavendulae \) DDL was competitively inhibited by DCS, the \( K_i \) value of DCS for the protein (920 \( \mu \)M) was obviously higher (40–100-fold) than those for DdlAs and DdlB. 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 DdlB (\( k_i \) 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 (\( C_r \)) 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 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).

\[
\begin{align*}
E + \text{DCS} & \rightleftharpoons E\text{-DCS} \\
E\text{-DCS} & \rightleftharpoons E'\cdot X \\
E'\cdot X & \rightleftharpoons E' + X
\end{align*}
\]

**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

\[v = \frac{k_2}{K_i + K_i} \cdot \text{DCS} \text{ concentration}\]

that of VanA ligase. The \( K_m \) 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 \( \mu \)M, which is close to those for the \( S. typhimurium \) DdlA (61 \( \mu \)M) and the \( E. coli \) DdlA (49 \( \mu \)M) and DdlB (70 \( \mu \)M). 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 DdlB 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 DdlB competitively, with \( K_i \) values in the range of 9–27 \( \mu \)M (Table I). Although the \( S. lavendulae \) DDL was competitively inhibited by DCS, the \( K_i \) value of DCS for the protein (920 \( \mu \)M) was obviously higher (40–100-fold) than those for DdlAs and DdlB. 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 \( S. lavendulae \).

**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 (\( C_r \)) 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 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).

\[
E + \text{DCS} \rightleftharpoons E\text{-DCS} \\
E\text{-DCS} \rightleftharpoons E'\cdot X \\
E'\cdot X \rightleftharpoons E' + X
\]

**Scheme 1**
monitored as the same cells grown in the absence of DCS. The cell growth was 46150 open as closed diamonds, 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 A_{opt, 480}. A, plasmids carrying alrS and K12alr are shown as open and closed triangles, respectively. B, plasmids carrying ddI, ddI, and ddIB are shown as open squares, closed squares, and closed diamonds, respectively. C, plasmids carrying alrS-ddI, K12alr-ddI, and K12alr-ddIB are shown as open diamonds, closed squares, and closed diamonds, respectively. E. coli harboring pET-21a(+) was used as a control strain (A–C, closed circles).

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-ddI; lane 5, pET-alrS-ddI; lane 6, pET-K12alr; lane 7, pET-ddI; lane 8, pET-ddIB; lane 9, pET-K12alr-ddI; lane 10, pET-K12alr-ddIB.

The decrease of the remaining activity was evaluated from Equation 9.

\[
u_v/
u_0 = \exp(-k_{app}t)\]  
(Eq. 9)

The value of \(k_{app}\) is an apparent rate constant. At the beginning of the reaction, \([E’-X]\) is regarded as zero, and the reverse reaction \(k_2\) is ignored in Scheme 1. The inhibition constant is defined as \(K_1 = [E’][I]/[E]_0 = [E’][I]/[E]_0 + [E’][I]/[E]_0\), where \([E]_0\) means the total amount of enzyme; thus, the rate of DCS conversion is given as

\[
d[E’-X]/dt = k_2[E’-X]\]  
(Eq. 10)

This equation means that \(k_{app}\) can be regarded as \(k_2[I]/(K_1 + [I])\) at the initial phase of the reaction. Using these equations, \(K_1\) and \(k_2\) are determined from double reciprocal plots (Fig. 6C and Table III).

As shown in Table III, the \(K_1\) 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 shown in Table III, the ALR is larger than that of DCS. The time-dependent inactivation of the

### Table III: Kinetic parameters for the inhibition of ALR by DCS and LCS determined from the CD spectrometric assay

|         | \(K_1\) (mM) | \(k_2 \times 10^{-3}\) (s\(^{-1}\)) |
|---------|-------------|----------------------------------|
| **DCS** |             |                                  |
| S. lavendulae | 0.87 (±0.08) | 3.9 (±0.5)                      |
| E. coli  | 1.2 (±0.1)  | 11 (±2)                          |
| **LCS** |             |                                  |
|         |             |                                  |
|         | ND*         |                                  |
|         | ND*         |                                  |

*ND, not determined because the decrease of ALR activity was not observed in the given time range.
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 supplemented with 1600 μg/ml DCS (or LCS) at the l portion of the culture was incubated for 16 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 a higher concentration 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 K-12 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 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 an 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 Dddl, 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 Dddl, 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).
41. Dutka-Malen, S., Molinas, C., Arthur, M., and Courvalin, P. (1990) Mol. Gen. Genet. 224, 364–372
42. Bugg, T. D. H., Dutka-Malen, S., Arthur, M., Courvalin, P., and Walsh, C. T. (1991) Biochemistry 30, 2017–2021
43. Ever, S., Reynolds, P. E., and Courvalin, P. (1994) Gene 140, 97–102
44. Meziane-Cherif, D., Badet-Denisot, M. A., Evers, S., Courvalin, P., and Badet, B. (1994) FEBS Lett. 354, 140–142
45. Nuehaus, F. C., Carpenter, C. V., Miller, J. L., Lee, M. N., Gragg, M., and Stichgold, R. A. (1969) Biochemistry 8, 5119–5124
46. Briggs, G. E., and Haldane, J. B. S. (1925) Biochem. J. 19, 338–339
47. Feng, Z., and Barletta, R. G. (2003) Antimicrob. Agents Chemother. 47, 263–291
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

*J. Biol. Chem.* 2004, 279:46143-46152.
doi: 10.1074/jbc.M404603200 originally published online August 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404603200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 11 of which can be accessed free at http://www.jbc.org/content/279/44/46143.full.html#ref-list-1