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Molecular Cloning of a D-Cycloserine Resistance Gene from D-Cycloserine-producing *Streptomyces garyphalus*

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A 3.5-kb DNA fragment that confers resistance to D-cycloserine (DCS) was cloned from the chromosomal DNA of a DCS-producing *Streptomyces garyphalus* into *Streptomyces lividans* by a shot-gun cloning technique. Nucleotide sequence analysis revealed the existence of four open reading frames (ORFs B, C, D, and E), together with two incomplete ORFs, A and F. By introduction of the cloned fragment into *Escherichia coli*, the host obtained resistance to DCS. We showed that ORF B, which consists of 903 bp, is a DCS resistance gene. The hydropathy plot analysis of a protein deduced from ORF B revealed that the protein carries membrane-integral domains spanning the membrane 10 times, which suggests that the DCS-resistance determinant may be a factor associated with DCS transport.

The peptidoglycan (PD), a main component of a bacterial cell wall that is essential to protect the cell from osmotic lysis, contains D-alanine (D-Ala). The D-amino acid is generated from L-Ala by Ala racemase and used as a substrate to make D-Ala-D-Ala by D-Ala-D-Ala ligase[1]. The resulting dipeptide is incorporated into the PD precursor by the D-Ala-D-Ala-adding enzyme[2]. Because the three enzymes are present only in bacterial cells, they may become potential targets to screen antibiotics selective to bacteria[3].

D-Cycloserine (DCS), produced by *Streptomyces* (*S.*) *garyphalus* and *S. lavendulae*, is clinically used to treat tuberculosis, which is mainly caused by *Mycobacterium tuberculosis*. Because DCS is structurally related to D-Ala, the drug competitively prevents the action of both Ala racemase and D-Ala-D-Ala ligase. Thus, DCS functions as an inhibitor of bacterial cell-wall biosynthesis[4,5].

DCS-producing microorganisms must be protected from the lethal effect of their own products. A research group has suggested that the Ala racemase of DCS-producing *S. garyphalus* may be correlated with DCS resistance[6].

In the present study, we independently cloned a DCS-resistance gene from DCS-producing *S. garyphalus* using a shot-gun cloning technique. The amino acid sequence of a DCS-resistance determinant, which is deduced from the nucleotide sequence, was dissimilar to that of Ala racemase from various bacteria. We show here that the DCS-resistance determinant carries membrane-integral domains, which suggests that it may function as a DCS-transporter.

Materials and Methods

Bacterial Strains, Plasmids, and Media

A DCS-producing strain *S. garyphalus* (CSH) 5-12, which is stocked in Meiji Seika Kaisha, Ltd., was grown in a YEME medium [0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1% glucose, 34% sucrose, and 5 mM $MgCl_2$, pH 7.0]. *S. lividans* 66, which is sensitive to 500 μg DCS/ml, was used as a host for the shot-gun cloning experiment. *Escherichia coli* JM109 was used as a host for subcloning experiments and the preparation of deletion mutants. Plasmids, pIJ702[7] for the shot-gun cloning, pUC118 for sequence analysis, and pUC18 and pUC19[8]

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for subcloning, were used in this study. An LB medium[9] was used for the cultivation of *E. coli* cells. R2YE[10] and NB [0.3% beef extract and 0.8% peptone, pH 6.8] media were used for the *Streptomyces* cell growth.

Shot-gun Cloning Experiment

Chromosomal DNA from *S. garyphalus*, grown in a YEME medium, was isolated according to the method described previously[10]. The chromosomal DNA was digested with *Bam*HI and ligated to a vector, pIJ702, digested with *Bgl*II. *S. lividans* 66 protoplasts were transformed with the library and regenerated on an R2YE medium containing 100 μg thiostrepton/ml. The resulting transformants were transferred to an NB medium containing DCS and then grown at 31°C for 96 hours.

Sequence Analysis

The deletion mutants required to determine the nucleotide sequence of the cloned DNA fragment from *S. garyphalus* were created by using a Kilo-Sequence Deletion Kit (Takara, Japan). With the aid of helper phage M13KO7, a single strand DNA of the deletion plasmids was prepared[9] and then sequenced by the dideoxy chain termination method[11] using an AutoRead Sequencing Kit and the A.L.F. DNA sequencer (Pharmacia, Sweden). Based on the sequence data, ORFs were analyzed using the Frame analysis program[12]. The homology search at an amino acid level was done using the FASTA program.

PCR

PCR was performed using *ExTaq* polymerase (Takara, Japan) and Program Temp Control System PC-700 (Astec, Japan). To amplify the DNA fragments containing a complete DCS-resistance gene, the following sets of the primers were used: for pCSPC6, 5′-GACGGAATTCCC-GAGGAGTTCGCCT-3′ (6F) and 5′-TCCCGGATCCTCA-TGACGGCCGCCG-3′; for pCSPC9, 6F and 5′-GCGTG-AGCTCTCACGACGACCGCGC-3′; for pCSPC12, 5′-GCCCGAATTCCTACTCGCGCGCCGACTTCC-3′ and 5′-GCGTGCATGCTCACGACGACCGCGC-3′. The 6F and 5′-ACGAGGATCCTCACCGGCGCGGCCGGCGTG-3′ primers were used to amplify the DNA fragment containing a truncated DCS-resistance gene (pCSPC10). All PCR conditions were as follows: 1 cycle of 3 minutes at 95°C, 45 seconds at 65°C, and 90 seconds at 72°C, followed by 29 cycles of 2 minutes at 95°C, 45 seconds at 65°C, and 90 seconds at 72°C. The amplified DNAs were gel-purified and cloned into pUC18.

Determination of the Minimum Inhibitory Concentration (MIC)

MICs of various antibiotics to *E. coli* were determined by an agar-dilution method as described previously[13], except that an LB medium was used instead of the Mueller Hinton medium.

Hydropathy Analysis

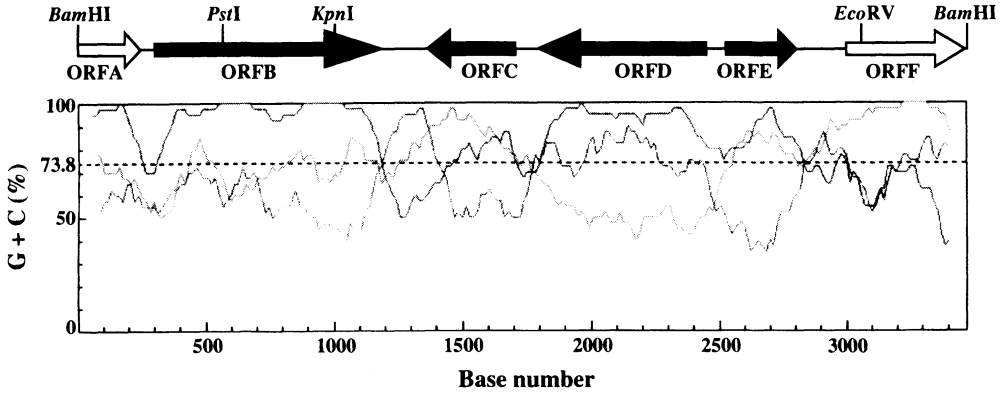
Hydropathy plot analysis, which is based on the method of KYTE and DOOLITTLE[14], was performed using GENETYX software (Software Development, Japan).

Results and Discussion

Cloning and Sequence Analysis of a DNA Fragment that Confers Resistance to DCS

The growth of *S. lividans* 66 was completely inhibited by 500 μg DCS/ml. We tried to clone a DCS-resistant gene from the chromosomal DNA of DCS-producing *S. garyphalus* into pIJ702. As a result of shot-gun cloning using *S. lividans* 66 as a host, we obtained only one transformant resistant to 1,500 μg DCS/ml. A plasmid, designated pKM3, isolated from the transformant, had a 3.5-kb DNA fragment from *S. garyphalus* in pIJ702. We analyzed the entire nucleotide sequence, showing that the fragment consists of 3,472 bp. Frame analysis[12], which is frequently used to predict the ORF of *Streptomyces* genes, suggested that four complete (ORFs B, C, D, and E) and two incomplete (ORFs A and F) ORFs are present in the 3.5-kb DNA fragment (Fig. 1). An incomplete ORF A consists of 246 bp, which is predicted to be the 3′-portion of a gene. The putative ORF A protein has a 52.6% identity with a D-Ala-D-Ala ligase from *Pseudomonas aeruginosa*[15]. ORF B, which consists of 903 bp, is located 33 bp downstream of ORF A at the same orientation. The protein encoded by ORF B exhibits a 59.2% identity with a putative integral membrane protein of *S. coelicolor*[16]. ORF C and ORF D, located sequentially downstream of ORF B, have the opposite direction to ORF A and ORF B. A protein encoded by ORF C consisting of 357 bp shows high similarity to a hypothetical protein of *S. coelicolor* (46.6% identity)[16]. GGAGG is located 11 bp upstream of the start codon (GTG) of ORF C, which may be a ribosome-binding site. ORF D, which consists of 606 bp, is located 122 bp upstream of ORF C. The ORF D-encoding protein exhibits the highest similarity (45.0% identity) to a putative methyltransferase from *S. coelicolor*[16]. ORF E, which consists of 267 bp and is located upstream of ORF D, has the same orientation as ORF A. The ORF E-encoding

Fig. 1. Frame analysis of the 3,472-bp DNA sequence from *S. garyphalus*.



Open and closed arrows indicate incomplete and complete ORFs, respectively.

protein shows dissimilarities to the proteins present in the protein database. Another incomplete ORF, ORF F, which consists of 490 bp, is deduced to be the 5′-portion of a gene, and the amino acid sequence of the protein encoded by ORF F has 50% identity to a putative lipoprotein of *S. coelicolor*[16]. The nucleotide sequence data, obtained in this study, has been deposited in the DDBJ (accession number: AB112918).

Determination of the DCS-resistance Gene

By subcloning experiments using *E. coli* JM109 as a host strain, we determined an ORF that confers resistance to DCS. A 3.8-kb *Sph*I and *Sac*I DNA fragment consisting of a *S. garyphalus* DNA (3.5 kb) and a part (0.3 kb) of pIJ702 was blunted and inserted into the *Sma*I site of pUC18 to generate p18CSR. To construct p19CSR, which has the *lac* promoter on the opposite side of the cloned DNA fragment, a 3.8-kb *Eco*RI and *Hin*dIII DNA fragment from p18CSR was inserted into the same site of pUC19.

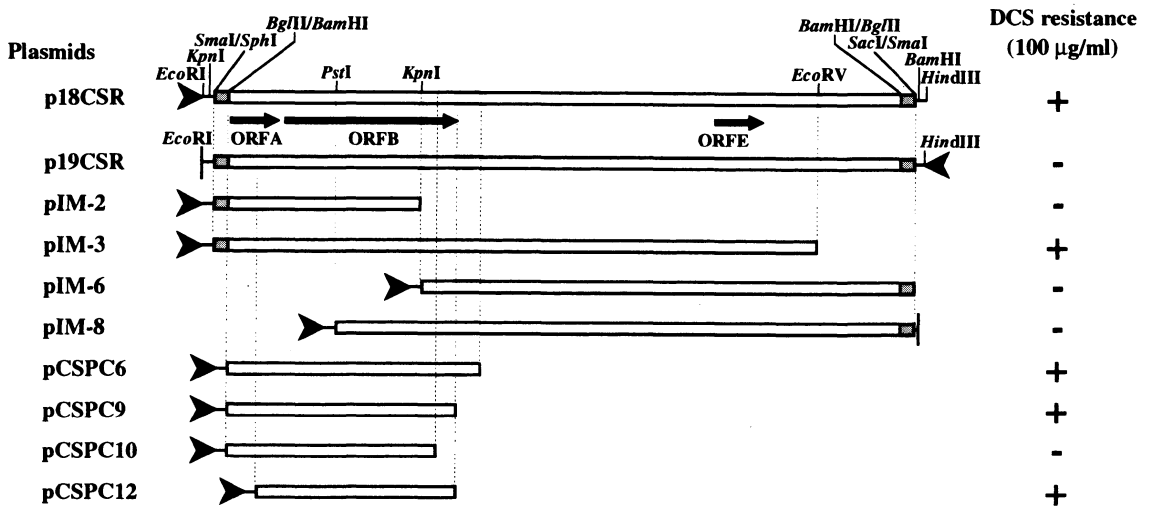
E. coli JM109 and the same host harboring p19CSR were susceptible to 12.5 μg DCS/ml, whereas *E. coli* JM109 harboring p18CSR was resistant to over 100 μg DCS/ml. The cloned 3.5-kb fragment derived from *S. garyphalus* is in the opposite orientations in p19CSR and p18CSR (Fig. 2). These results suggest that the *Streptomyces* gene, which confers resistance to DCS, is expressed in *E. coli* under the control of the *lac* promoter in p18CSR. Judging from the orientation of the ORFs in the 3.5-kb fragment from *S. garyphalus*, the DCS-resistance gene was deduced to be ORF B or ORF E.

The *Kpn*I-digested and *Pst*I/*Bam*HI-digested fragments of p18CSR, which contain a part of the cloned 3.5-kb *S. garyphalus* DNA, were subcloned into the *Kpn*I-digested and *Pst*I/*Bam*HI-digested pUC19 to generate pIM-2 and pIM-8, respectively. Similarly, *Eco*RI/*Eco*RV-digested fragments of p18CSR were subcloned into the *Eco*RI/*Sma*I-digested pUC18 to generate pIM-3. A 1.2-kb DNA fragment between two *Kpn*I sites was removed from p18CSR to generate pIM-6. By these subcloning experiments, we revealed that *E. coli* harboring pIM-2, which has a truncated ORF B, is susceptible to 100 μg DCS/ml and that the same host harboring pIM-6 or pIM-8, which contains ORF E but not ORF B, is also susceptible (Fig. 2). However, *E. coli* harboring pIM-3, which contains the complete ORF B, exhibited resistance to 100 μg DCS/ml (Fig. 2). These results suggest that DCS resistance is conferred by ORF B. Moreover, *E. coli* harboring pCSPC6, pCSPC9, or pCSPC12, which contains a PCR-amplified DNA having a complete ORF B, showed resistance to DCS. *E. coli* harboring pCSPC10, which has a truncated ORF B, did not exhibit resistance to DCS (Fig. 2). The MIC of the DCS to *E. coli* harboring pCSPC12 was 200 μg/ml, which demonstrates that ORF B is involved in DCS resistance.

Putative Function of the ORF B Gene Product

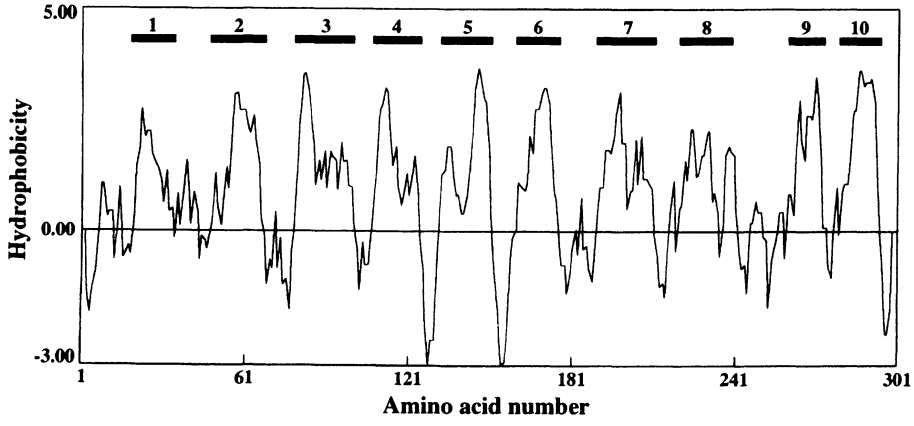
Since the protein that is encoded by the DCS-resistance gene ORF B is homologous to typical membrane proteins, we performed a hydrophathy plot analysis for the ORF B-encoding protein. The analysis revealed that the membrane-

Fig. 2. Subcloning analysis of the 3.5-kb DNA fragment from *S. garyphalus*.



An open box indicates the DNA fragment from *S. garyphalus*. A shaded box indicates the DNA derived from a *Streptomyces* vector pIJ702. The *lac* promoter is indicated by an arrowhead.

Fig. 3. Hydrophathy plot analysis of the protein encoded by ORF B.



The thick bars indicate the putative membrane-integral domains.

integral domains spanning the membrane 10 times are present in the ORF B-encoding protein (Fig. 3), which suggests that the ORF B gene product may be a membrane protein necessary for the excretion of DCS from the cell. To clarify whether the DCS-resistance determinant is specific for DCS, the susceptibility of *E. coli* JM 109 harboring pCSPC12 to clinically used antibiotics such as kanamycin, arbekacin, chloramphenicol, tetracycline, bleomycin, vancomycin, erythromycin, ofloxacin, and fosfomycin was tested. The MICs for these antibiotics to *E. coli* JM109 harboring pCSPC12 were the same as those to the host cell (data not shown), which suggests that the ORF B-encoding protein functions specifically as a DCS-resistance determinant.

A previous study by another research group has reported that an Ala racemase, which is not inhibited by DCS, is present in a DCS-producing *S. garyphalus*[6]. However, we found a DCS-resistance determinant that is different from

Ala racemase. Some antibiotic-producing microorganisms possess a membrane protein that responsible for the efflux of their own products. Such membrane proteins include two types with respect to the efflux of antibiotics: one is an antiporter-type[17,18], and the other is an ATP-binding cassette (ABC)-type[19,20]. Antiporter-type proteins, such as tetracycline-resistance[21,22] and chloramphenicol-resistance[23] gene products, have membrane-integral domains, which span the membrane 12 times, and contain the consensus Ser-Asp-Arg-X-Gly-Arg-Arg sequence. The DCS-resistance determinant from *S. garyphalus* found in this study has no sequences that are conserved in ABC-type membrane proteins. The hydropathy analysis showed the presence of 10 membrane-spanning domains in the DCS-resistance determinant from *S. garyphalus*, indicating that it may be an antiporter-type membrane protein. However the Ser-Asp-Arg-X-Gly-Arg-Arg sequence, conserved in the antiporter-type membrane proteins, is absent in the protein. Therefore, the DCS-resistance determinant from *S. garyphalus* may be an another type of transporter.

E. coli expressing the DCS-resistance gene (ORF B) from *S. garyphalus* showed no resistance to the tested antibiotics except DCS, suggesting that the ORF B-encoding protein confers resistance to DCS. Interestingly, a homology search with a protein database revealed that *S. coelicolor* possesses a putative membrane protein homologous to the DCS-resistance determinant from *S. garyphalus*. However, *S. coelicolor* was susceptible to 500 μg DCS/ml, like *S. lividans*. Therefore, although we can't rule out a possibility that the ORF B-encoding protein may have another function, it is presumed that the protein functions as a DCS-resistant determinant in DCS-producing *S. garyphalus*.

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