Molecular Cloning and Heterologous Expression of a Biosynthetic Gene Cluster for the Antitubercular Agent D-Cycloserine Produced by *Streptomyces lavendulae*[▽]

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In the present study, we successfully cloned a 21-kb DNA fragment containing a D-cycloserine (DCS) biosynthetic gene cluster from a DCS-producing *Streptomyces lavendulae* strain, ATCC 11924. The putative gene cluster consists of 10 open reading frames (ORFs), designated *dcsA* to *dcsJ*. This cluster includes two ORFs encoding D-alanyl-D-alanine ligase (*dcsI*) and a putative membrane protein (*dcsJ*) as the self-resistance determinants of the producer organism, indicated by our previous work. When the 10 ORFs were introduced into DCS-nonproducing *Streptomyces lividans* 66 as a heterologous host cell, the transformant acquired DCS productivity. This reveals that the introduced genes are responsible for the biosynthesis of DCS. As anticipated, the disruption of *dcsG*, seen in the DCS biosynthetic gene cluster, made it possible for the strain ATCC 11924 to lose its DCS production. We here propose the DCS biosynthetic pathway. First, L-serine is O acetylated by a *dcsE*-encoded enzyme homologous to homoserine *O*-acetyltransferase. Second, *O*-acetyl-L-serine accepts hydroxyurea *via* an *O*-acetylserine sulfhydrylase homolog (*dcsD* product) and forms *O*-ureido-L-serine. The hydroxyurea must be supplied by the catalysis of a *dcsB*-encoded arginase homolog using the L-arginine derivative, N^G-hydroxy-L-arginine. The resulting *O*-ureido-L-serine is then racemized to *O*-ureido-D-serine by a homolog of diaminopimelate epimerase. Finally, *O*-ureido-D-serine is cyclized to form DCS with the release of ammonia and carbon dioxide. The cyclization must be done by the *dcsG* or *dcsH* product, which belongs to the ATP-grasp fold family of protein.

The soil-dwelling genus *Streptomyces* undergoes a complex morphological differentiation and produces an enormous variety of bioactive secondary metabolites. Because they include clinically useful antibiotics and immunosuppressants, the genus *Streptomyces* occupies an important position as an industrial microorganism.

D-Cycloserine (DCS), a cyclic structural analogue of D-alanine, is produced by "*Streptomyces garyphalus*" and *Streptomyces lavendulae*. This antibiotic is used as an antitubercular agent (21). Since DCS is similar to D-alanine, it prevents the action of both alanine racemase and D-alanyl-D-alanine ligase, which are necessary for the biosynthesis of a bacterial cell wall. Thus, DCS functions as an inhibitor of bacterial cell wall biosynthesis (16, 19). Although the structure of DCS is very simple, the biosynthetic genes for DCS have never been cloned until now.

In general, antibiotic biosynthetic genes form a cluster and are adjacent to their self-resistance genes. We have previously cloned a gene (*orfB*) that confers resistance to DCS on *Streptomyces lividans* and *Escherichia coli* from DCS-producing *S. garyphalus* (CSH) 5-12 (17). The sequence analysis suggests

that the gene may encode a membrane protein that is necessary for the excretion of the DCS outside the cell. We have found that the same gene is also present in *S. lavendulae* ATCC 25233 (20). We have also previously demonstrated that D-alanyl-D-alanine ligase, which is a target enzyme of DCS, functions as a self-resistance determinant in *S. lavendulae* ATCC 25233 (20). Interestingly, the gene encoding D-alanyl-D-alanine ligase, designated *ddlS*, was located just upstream of the putative membrane protein gene, *orfB* (20). Although the self-resistance genes in the DCS producers have been thoroughly analyzed, no attempt to clone the DCS biosynthetic genes has been carried out yet.

To clone the biosynthetic genes for DCS from another DCS-producing *S. lavendulae* strain, ATCC 11924, we first investigated whether this strain harbors *orfB* and *ddlS*. Since both genes were found to be conserved, the flanking region was cloned from the chromosomal DNA. Here, we hypothesize about the biosynthetic pathway for DCS on the basis of our present data and previous studies by another research group (23, 24). The present study is the first report identifying the biosynthetic gene cluster for DCS.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Streptomyces* strains were grown at 28°C on an FB agar medium (9). To select objective clones, appropriate antibiotics were added to the medium (14). *S. lividans* 66 was grown at 28°C in a yeast extract-malt extract liquid medium containing 0.5% glycine for the preparation of protoplasts (14). To produce DCS, *Streptomyces* strains were cultured at 28°C in liquid medium B (15). Plasmids pSET152 and pKC1132 (2) were used

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for gene integration and gene disruption, respectively. A *Streptomyces* expression vector, pIJ8600 (3), which contains a *tipA* promoter (18), was used for the complementation experiment. *E. coli* DH5α and plasmids pUC19, pTA2 (Toyobo), and pBluescript II SK(+) (Stratagene) were used for DNA cloning and sequencing. *E. coli* XL1-Blue MRA(P2) and the lambda FIX II vector (Stratagene) were used for genomic library construction. *E. coli* ET12567/pUZ8002 was used as a transient host for *E. coli-Streptomyces* conjugation (5). *E. coli* BL21(DE3) and a plasmid, pET-28a(+) (Novagen), were used for protein expression. All *E. coli* strains were cultivated at 37°C or 28°C in Luria broth or on Luria agar supplemented with the appropriate antibiotics when necessary (14, 22).

DNA manipulations. The genomic DNA of *Streptomyces* was isolated following a standard protocol (14). The phage DNA was isolated according to a method described elsewhere (22). The plasmid DNA in *E. coli* was isolated using the Wizard *Plus* Minipreps DNA purification system (Promega).

Hybridizations. Southern and plaque hybridizations were performed using a Hybond-N+ membrane (GE Healthcare). Probe labeling, hybridization, and detection were performed with the Alkphos direct labeling and detection System (GE Healthcare) according to the protocol supplied by the manufacturer.

Construction of genomic library. Genomic DNA of *S. lavendulae* ATCC 11924 was partially digested with Sau3AI following purification by phenol-chloroform extraction and 2-propanol precipitation. After DNA fragments with a size of 10 to 20 kb were gel purified, a partial fill-in reaction was carried out. Subsequently, the DNA fragments were purified by phenol-chloroform extraction and ethanol precipitation, followed by ligation to the lambda FIX II vector (Stratagene). *In vitro* packaging was performed using the Gigapack III Gold packaging extract (Stratagene) to generate the genomic library of *S. lavendulae* ATCC 11924.

Cloning of DNA fragments containing DCS biosynthetic genes. Using the PCR-amplified *ddlS* gene of *S. lavendulae* ATCC 25233 as a probe DNA (20), a genomic library of *S. lavendulae* ATCC 11924 was screened by the plaque hybridization technique. After the phage DNA was isolated from a positive plaque, an approximately 12-kb DNA fragment (called DCS1) derived from *S. lavendulae* ATCC 11924 was removed by digestion with XbaI and subcloned into the same site of pUC19 to generate pUC19/DCS1. Since the DCS biosynthetic gene cluster seemed to be incomplete according to the sequence analysis, a DNA fragment adjacent to DCS1 was cloned from the ATCC 11924 genomic library using chromosomal walking. The cloned fragment (called DCS2), with a size of 10 kb, was subcloned into pUC19 to yield pUC19/DCS2. The entire DCS biosynthetic gene cluster was obtained by the sequence analysis of DCS1 and DCS2.

DNA sequencing and analysis. DNA sequencing was performed using ABI Prism 310 and Avant 3100 sequencers (Applied Biosystems) using the Big Dye terminator cycle sequencing ready reaction kit, ver.1.1. Genetic analysis was performed using GENETYX ver.7 for Windows (Software Development). The open reading frames (ORFs) were predicted using FramePlot ver.2.3.2 (11). Homology searches were conducted with the BLAST algorithm (1).

Heterologous expression of DCS biosynthetic gene cluster in *S. lividans*. The putative DCS biosynthetic gene cluster, which consists of 10 genes (*dcsA* to *dcsJ*), including two self-resistance genes, was amplified by PCR using a 5′-phosphorylated sense primer (5′-TCTAGAATGGCGATCATCTGGGCCTGC-3′; XbaI site underlined) and a 5′-phosphorylated antisense primer (5′-GATATCAAGACGG CGGTGCGTGAGGTG-3′; EcoRV site underlined). PCR was carried out under the following conditions: denaturing for 5 min at 98°C, 30 cycles of 10 s at 98°C and 10 min at 70°C, and finally an extension period at 70°C for 15 min. The amplified fragment was cloned into SmaI-digested pUC19 to generate pUC19/*dcs*. After the fragment was confirmed by DNA sequencing, the DCS biosynthetic gene cluster was cut off from pUC19/*dcs* by digestion with XbaI and EcoRV and inserted into the same sites of pSET152 to yield pSET152/*dcs*. The chimeric plasmid was introduced into *S. lividans* 66 by protoplast transformation (14). For DCS production, *S. lividans* cells transformed with pSET152 or pSET152/*dcs* were cultured in medium B at 28°C for 48 h.

Construction of *dcsG* mutant *via* chromosomal gene disruption. A 5.6-kb DNA fragment was cut off from pUC19/DCS1 by digestion with PvuII and ligated to pUC19, which was previously digested with PstI/XbaI and blunt ended by using a DNA blunting kit (Takara), to yield pUC19/DCS11. The internal region of *dcsG* (636 bp) was removed from pUC19/DCS11 by digestion with SalI, followed by self-ligation to give pUC19/Δ*dcsG*. A 5.0-kb fragment containing the disrupted *dcsG* gene was removed from pUC19/Δ*dcsG* by digestion with EcoRI/HindIII and inserted into the same sites of pKC1132 to generate pKC/Δ*dcsG*. pKC/Δ*dcsG* was introduced into methylation-deficient *E. coli* ET12567/pUZ8002, and the resulting transformant was used for conjugal transfer to *S. lavendulae* ATCC 11924. Conjugal transfer from *E. coli* to *Streptomyces* was carried out according to a method described elsewhere (14). An apramycin-

resistant conjugant, obtained by single-crossover homologous recombination, was further cultured on medium without apramycin for several rounds. An apramycin-sensitive *dcsG* mutant, which contains the in-frame-deleted *dcsG* generated by double-crossover homologous recombination, was obtained and designated the Δ*dcsG* mutant.

Complementation of disrupted mutant with *dcsG*. The structural gene of *dcsG* was amplified by PCR using a sense primer, 5′-CATATGGGCATCCTCGCCT TGGTCACCG-3′ (NdeI site underlined), and an antisense primer, 5′-TCTAG ACTAGGGCTTGAGCCGTTCGGCCAG-3′ (XbaI site underlined). The amplified gene was cloned into pTA2 to give pTA2/*dcsG*. After confirmation of its DNA sequence, *dscG* was removed from pTA2/*dcsG* by digestion with NdeI/XbaI and inserted into the same sites of pIJ8600 (3), a *Streptomyces* expression vector, to yield pIJ8600/*dcsG*. In this construction, *dcsG* is under the control of the *tipA* promoter, and its expression is induced by the addition of thiostrepton (18). pIJ8600/*dcsG* was introduced into the Δ*dcsG* strain using conjugal transfer.

Assay of DCS production. DCS production was analyzed by high-performance liquid chromatography (HPLC) (15). After the culture supernatant fluid was filtrated with a membrane filter (0.2-μm pore size; Advantec), aliquots (10 μl) were applied in HPLC (Senshu Pak; Senshu Scientific Co., Ltd.) with 10 mM ammonium acetate (pH 5.0 adjusted with acetate) for elution and detected at 210 nm by comparison with authentic DCS (Wako). Alternatively, the amount of DCS contained in the culture filtrate was colorimetrically estimated with a sodium nitroprusside reagent according to a previously described method (12).

MALDI-TOF mass analysis. The eluate from HPLC was analyzed using matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry. MALDI-TOF mass analysis was performed using a Voyager RP-3 mass spectrometer (PerSeptive Biosystems) operated in the reflector mode. Mass spectra were obtained under the following conditions: accelerating voltage (ACV), 25 kV; grid voltage, 57.5% of ACV; guide wire voltage, 0.05% of ACV; pulse delay time, 100 ns. CHCA was used for the matrix.

Expression in *E. coli* and purification of DcsB and DcsE proteins. The gene *dcsB* was amplified by PCR using a sense primer, 5′-CATATGATTGATCTGA TCGTCTCCCAG-3′ (NdeI site underlined), and an antisense primer, 5′-CTC GAGTCAGGGCCGTGCGGCGGC-3′ (XhoI site underlined). The amplified gene was cloned into pTA2 to generate pTA2/*dcsB*. After the nucleotide sequence of *dcsB* was analyzed for confirmation, the *dcsB* fragment was cut off from pTA2/*dcsB* by digestion with NdeI/XhoI and inserted into the same sites of pET-28a(+) to yield pET-28a/*dcsB*. *E. coli* BL21(DE3) harboring pET-28a/*dcsB* was grown in Luria broth at 28°C. The expression of *dcsB* was induced by the addition of 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation and disrupted by sonication. *N*-terminal histidine-tagged DcsB was purified by nickel affinity chromatography using His-Bind resin (Novagen) according to the supplier's instruction manual.

The gene *dcsE* was amplified by PCR using a sense primer, 5′-GGAATTCC ATATGAGGGAATTCATACCCCCG-3′ (NdeI site underlined), and an antisense primer, 5′-CCGCTCGAGTCAGGCGACGATCGCCAGGAACTT-3′ (XhoI site underlined). The amplified gene was digested with NdeI/XhoI, gel purified, and inserted into pET-28a(+) to give pET-28a/*dcsE*. *E. coli* BL21(DE3) harboring pET-28a/*dcsE* was grown in Luria broth at 28°C. Expression of *dcsE* was induced by the addition of 1 mM IPTG. *N*-terminal histidine-tagged DcsE was purified by His-Bind resin as described above.

The *dcsB* and *dcsE* gene products were purified to near-homogeneity, which was judged by SDS-PAGE analysis. Each protein concentration was determined by measuring the absorbance at 280 nm using the molar extinction coefficients 34,500 M^{-1} cm^{-1} and 38,700 M^{-1} cm^{-1}, respectively.

Analysis of enzymatic activity of DcsE. The reaction mixture (200 μl), consisting of 4 mM Tris-HCl (pH 7.6), 40 mM NaCl, 1 mM dithiothreitol (DTT), 0.2 mM EDTA, 1 mM L-serine, 1 mM acetyl-coenzyme A (CoA), and 15 μM DcsE, was incubated at 37°C for 2 h. As a control, the same reaction mixture without DcsE was also used. The formation of enzymatic product was analyzed by thin-layer chromatography (TLC) using silica gel 60 F$_{254}$ (Merck). The solvent for the chromatogram was 2-butanone-pyridine-water (20:5:8). L-Serine and *O*-acetyl-L-serine were visualized by the acid ninhydrin regent as described previously (26).

Analysis of enzymatic activity of DcsB. The reaction mixture (90 μl), consisting of 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 1 mM N^G-hydroxy-L-arginine or 1 mM L-arginine, was preincubated at 37°C for 10 min. The reaction was initiated by the addition of 10 μl of DcsB at given concentrations. After incubation at 37°C for 10 min, the reaction was terminated by the addition of 100 μl of glacial acetic acid. The amount of ornithine liberated by the catalysis of DcsB was determined by the previously described method (4).

Effect of nitric oxide synthase (NOS) inhibitor on DCS productivity. *S. lavendulae* ATCC 11924 was grown in medium B at 28°C for 24 h in the presence
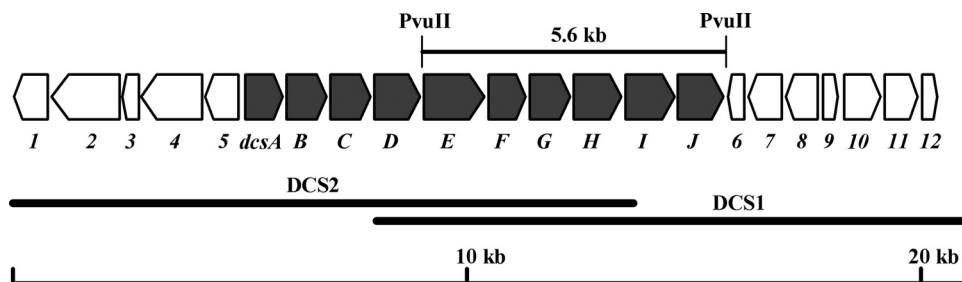
FIG. 1. Gene organization in a 21-kb DNA fragment from *S. lavendulae* ATCC 11924. The entire nucleotide sequences were determined on the basis of information from two successive clones, DCS1 and DCS2. ORFs were predicted by FramePlot analysis. The 10 genes (*dcsA* to *dcsJ*) involved in a putative DCS biosynthetic cluster are indicated by shaded arrows. A bar drawn above ORFs indicates a 5.6-kb DNA fragment used for the gene disruption analysis of *dcsG*.

or absence of 1 mM nitro-L-arginine methyl ester, known as a NOS inhibitor (13). The inhibitor was added to the medium at the time of inoculation. The amount of DCS produced was determined colorimetrically as described above. The data were confirmed by carrying out three independent experiments.

Nucleotide sequence accession number. The DNA sequence data for the entire DCS biosynthetic gene cluster of *S. lavendulae* ATCC 11924 were deposited in the DDBJ database under accession number AB516431.

RESULTS AND DISCUSSION

Cloning of a DCS biosynthetic gene cluster from *S. lavendulae* ATCC 11924. We previously found that a DCS resistance gene (*orfB*), which encodes a putative membrane protein responsible for the excretion of DCS, is harbored in DCS-producing *S. garyphalus* (CSH) 5-12 (17) and *S. lavendulae* ATCC 25233 (20). Furthermore, we have shown that a gene (*ddlS*) encoding D-alanyl-D-alanine ligase, located just upstream of *orfB*, also plays a role as a self-resistance determinant for DCS in the strain ATCC 25233 (20). A gene corresponding to *ddlS* is also just upstream of *orfB* in *S. garyphalus* but is not complete (17). These results suggest that the chromosomal locus responsible for DCS resistance is conserved between two DCS producers, suggesting the possibility that the biosynthetic genes for DCS are present in close proximity to these genes. To evaluate this possibility, testing by the Southern hybridization technique was undertaken using *orfB* and *ddlS* as the probes to determine whether another DCS-producing *S. lavendulae* strain, ATCC 11924, also harbors these two genes and to confirm that these genes are conserved (data not shown).

We cloned DNA regions flanking the two DCS resistance genes from the genomic library of *S. lavendulae* ATCC 11924. By cloning of two successive DNA fragments (DCS1 and DCS2) (Fig. 1), the nucleotide sequences, of approximately 21 kb, were determined. FramePlot analysis (11) suggests the presence of 22 ORFs in the cloned DNA fragment (Fig. 1). Table 1 summarizes the characteristics of ORFs seen in the cloned region. Considering the putative functions of these ORFs and their transcriptional direction, 10 genes, designated *dcsA* to *dcsJ*, may be responsible for the DCS biosynthesis (Fig. 1). This hypothesis was confirmed by heterologous expression

TABLE 1. Summary of ORFs in the cloned DNA

Protein	No. of aa[a]	Proposed function	Sequence homolog (protein name, origin)	Identity (%)	Protein accession no.
ORF1	247	Glycine-sarcosine methyltransferase	SIAM614_20226, *Stappia aggregata* IAM12614	37	EAV43187
ORF2	515	Peptide synthase	Ppro3117, *Pelobacter propionicus* DSM2397	31	ABL00711
ORF3	156	Cyanate hydratase	MAB0054c, *Mycobacterium abscessus*	77	CAM60154
ORF4	273	Unknown	SSCG01501, *Streptomyces clavuligerus* ATCC 27064	39	EDY48220
ORF5	459	Proline permease	SAV5161, *Streptomyces avermitilis* MA-4680	61	BAC72873
DcsA	265	Unknown	Csal1088, *Chromohalobacter salexigens* DSM3043	50	ABE58444
DcsB	273	Arginase	RocF, *Erwinia tasmaniensis*	43	CAO97798
DcsC	287	Diaminopimelate epimerase	DapF, *Xanthomonas campestris* pv. *campestris*	60	AAM42769
DcsD	324	O-Acetylserine sulfhydrylase	Csal1086, *Chromohalobacter salexigens* DSM3043	73	ABE58442
DcsE	374	Homoserine O-acetyltransferase	Smal2708, *Stenotrophomonas maltophila* R551-3	63	ACF52408
DcsF	267	Short-chain dehydrogenase	XCV1542, *Xanthomonas campestris* pv. *vesicatoria*	59	CAJ23174
DcsG	299	Unknown	Csal1083, *Chromohalobacter salexigens* DSM3043	39	ABE58440
DcsH	337	Unknown	Haur2023, *Herpetosiphon aurantiacus* ATCC 23779	30	ABX04666
DcsI	345	D-alanyl-D-alanine ligase	DdlS, *Streptomyces lavendulae* ATCC 25233	93	BAD60786
DcsJ	299	Membrane protein	OrfB, *Streptomyces garyphalus* (CSH) 5-12	97	BAD05865
ORF6	147	Acetyltransferase	OrfIII, *Streptomyces lavendulae* ATCC 25233	89	BAD60788
ORF7	261	Aminoglycoside phosphotransferase	Sare1186, *Salinispora arenicola* CNS-205	40	ABV97094
ORF8	217	Methyltransferase	OrfD, *Streptomyces garyphalus* (CSH) 5-12	93	BAD05867
ORF9	85	Unknown	OrfE, *Streptomyces garyphalus* (CSH) 5-12	94	BAD05868
ORF10	260	Lipoprotein	OrfF, *Streptomyces garyphalus* (CSH) 5-12	88	BAD05869
ORF11	242	Transcriptional regulator	SSAG06618, *Streptomyces* sp. Mg1	49	EDX26827
ORF12	197	Unknown	SSEG02386, "*Streptomyces sviceus*" ATCC 29083	47	EDY55086

[a] aa, amino acids.

in *S. lividans* 66 as a DCS-nonproducing host cell. Computer analysis suggested that the expression of the gene cluster from *dcsA* to *dcsJ* may be controlled by only one putative promoter just upstream of *dcsA*. But at this time, we cannot neglect the possibility that another promoter might be in the intergenic regions. Judging from the high sequence similarity (Table 1), *dcsI* and *dcsJ*, contained in the cluster, correspond to *ddlS* and *orfB*, respectively. These genes must function as the self-resistance determinants for DCS-producing microorganisms.

Heterologous expression of DCS biosynthetic gene cluster. To clarify whether the putative gene cluster consisting of the 10 genes is responsible for DCS biosynthesis, the DNA fragment, including the region from *dcsA* to *dcsJ*, was PCR amplified, subcloned into an integrating vector, pSET152 (2), and introduced into *S. lividans* 66 by the protoplast transformation method. To facilitate its expression in the regenerated cells, a putative promoter located upstream of *dcsA* was included in the process of PCR amplification. *S. lividans* 66 cells transformed with pSET152/*dcs*, which contains the cluster integrated in the chromosome, clearly produced DCS, while the same host transformed with pSET152 did not (Fig. 2). The molecular mass of DCS was also confirmed by MALDI-TOF mass analysis (data not shown), indicating that the cluster is actually responsible for DCS biosynthesis.

Loss of DCS production by *dcsG* disruption and rescue by its complementation. To clarify further that the gene cluster is responsible for the biosynthesis of DCS, the *dcsG* gene located chromosomally in *S. lavendulae* ATCC 11924 was disrupted by the homologous recombination method (Fig. 3A). The function of the *dcsG* product could not be annotated by the homology search, but it seemed to catalyze the final step of DCS biosynthesis, because DcsG has the ATP-grasp fold motif. Thus, we selected *dcsG* as a candidate for gene disruption analysis. Since *dcsG* seems to be transcribed polycistronically with other genes, the in-frame deletion method (14) was adopted for the creation of the *dcsG* mutant to avoid the polar effect. Through two steps of homologous recombination, a desired in-frame deletion mutant, designated the Δ*dcsG* strain, was obtained. In this experiment, the authenticity of the Δ*dcsG* mutant was confirmed by Southern blot analysis. As estimated in Fig. 3A, by Southern analysis, 5.6-kb and 5.0-kb fragments were detected in the wild-type and the Δ*dcsG* mutant, respectively. (Fig. 3B).

As expected, an in-frame deletion mutant, the Δ*dcsG* strain, did not produce DCS, whereas the wild-type strain clearly produced it (Fig. 3C). To determine whether this disappearance of DCS productivity is due to the loss of *dcsG* function, the gene was complemented in *trans* using an expression vector, pIJ8600. The *dcsG* gene, located under the control of the *tipA* promoter in pIJ8600, was introduced into the Δ*dcsG* mutant cell. The complemented cell restored the production of DCS in the presence but not in the absence of thiostrepton (20 μg/ml) as an inducer (data not shown). These results show that the loss of DCS production in the Δ*dcsG* mutant is due to the disruption of *dcsG* and suggest that *dcsG* is necessary for DCS biosynthesis.

Putative pathway for DCS biosynthesis. Based on the sequence information of ORFs found in the DCS biosynthetic gene cluster (encoding DcsA to DcsJ; Table 1) and previous reports (23, 24) by another group, we propose a putative bio-
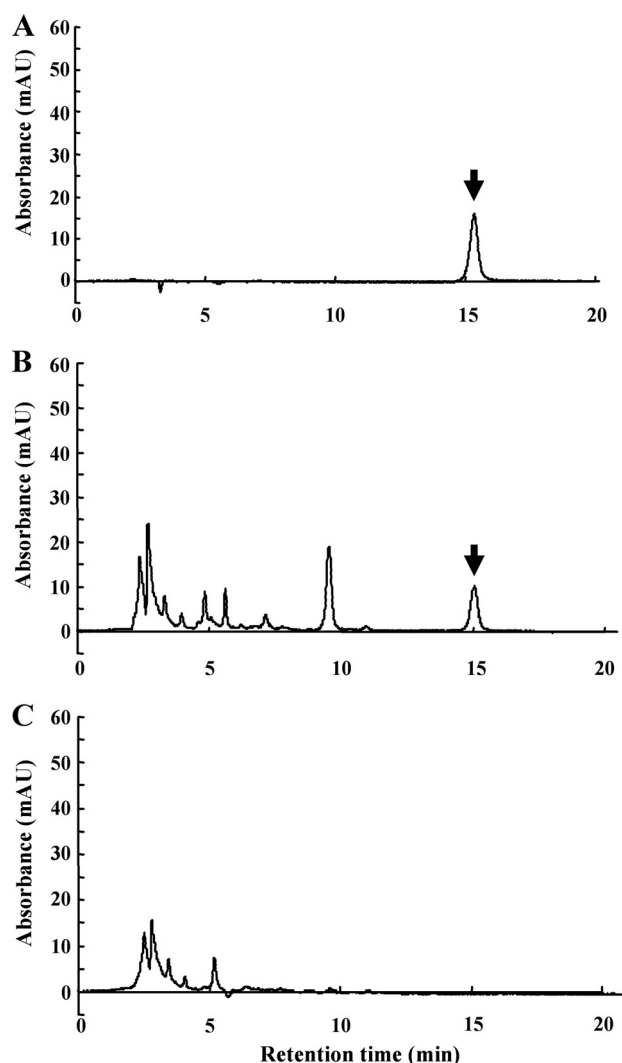

FIG. 2. Heterologous expression of a putative DCS biosynthetic gene cluster. *S. lividans* 66 transformed with pSET152/*dcs* or pSET152 was cultivated at 28°C in liquid medium B for 48 h. The amount of DCS produced in the culture supernatant of the transformant was analyzed by HPLC. (A) Authentic DCS. (B) *S. lividans* 66 transformed with pSET152/*dcs*. (C) *S. lividans* 66 transformed with pSET152. An arrow indicates a peak corresponding to DCS.

synthetic pathway for DCS in *S. lavendulae* ATCC 11924, as shown in Fig. 4.

L-Serine and hydroxyurea have been proposed previously to be components of DCS (23, 25). In order for L-serine to react with hydroxyurea, it must first be activated. Indeed, in *S. garyphalus*, O-acetyl-L-serine has been suggested to be the activated form of L-serine in DCS biosynthesis (24). The O acetylation of L-serine may be catalyzed by DcsE (Fig. 4), which is a homolog of homoserine O-acetyltransferase (Table 1), as shown in a report that a homolog of homoserine O-acetyltransferase in *Aspergillus nidulans* can contribute to the formation of O-acetyl-L-serine (7). In bacteria, O-acetyl-L-serine is used in cysteine biosynthesis and its formation is generally catalyzed by serine O-acetyltransferase. To investigate the function of DcsE, the protein was expressed in *E. coli* and purified. As
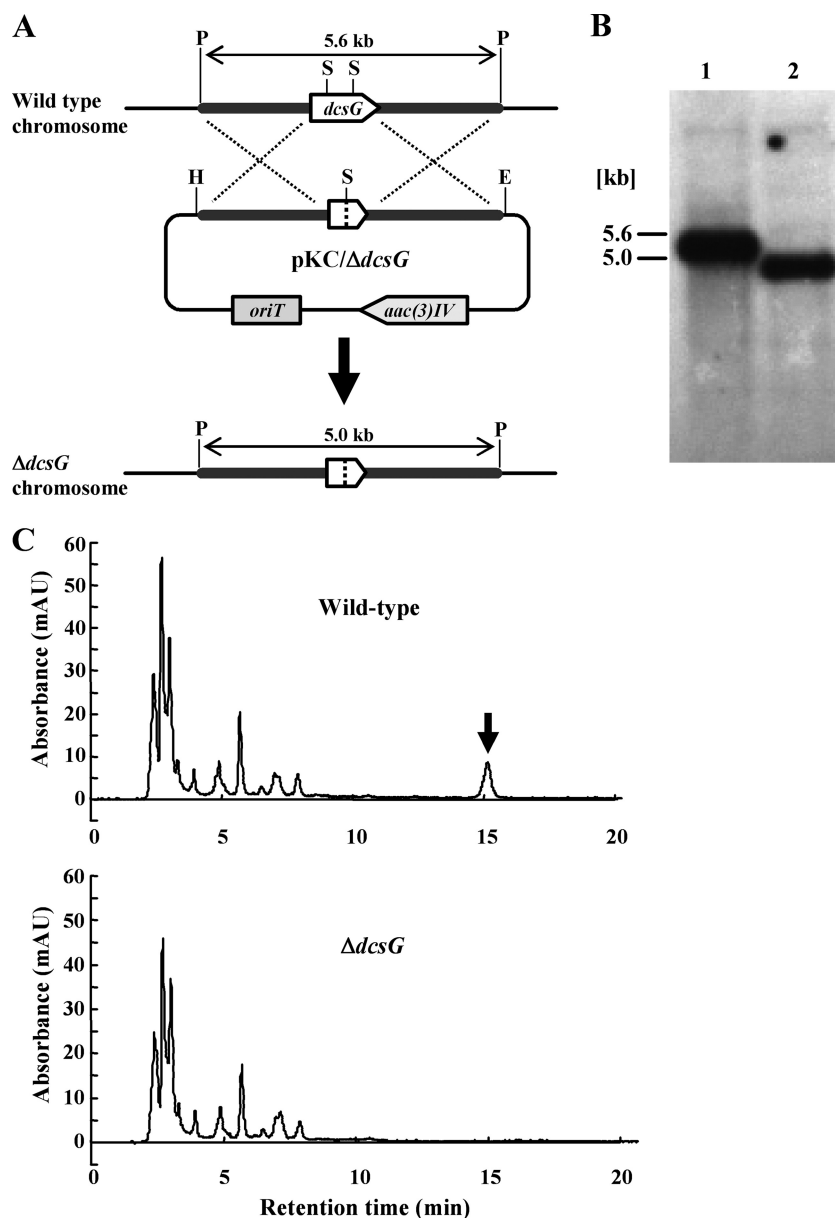
FIG. 3. Strategy for creation of a *dcsG*-disrupted mutant by an in-frame deletion method and analysis of DCS productivity. (A) Schematic representation of the construction of a *dcsG* mutant *via* chromosomal gene disruption. The restriction sites are as follows: E, EcoRI; H, HindIII; P, PvuII; S, SalI. (B) Southern blot analysis of wild-type and Δ*dcsG* genomes. The probe used was a PCR-amplified *dcsG* gene. Lane 1, PvuII-digested wild-type genome; lane 2, PvuII-digested Δ*dcsG* genome. (C) DCS production in the Δ*dcsG* mutant. The wild type and Δ*dcsG* mutant of *S. lavendulae* ATCC 11924 were cultured at 28°C in liquid medium B for 48 h. The detection of DCS produced in the culture supernatant was confirmed by HPLC analysis. Upper chart, wild type; lower chart, Δ*dcsG* mutant. An arrow shows a peak for DCS.

shown in Fig. 5A, the chromatographic profile in TLC indicates that *O*-acetyl-L-serine is formed when L-serine is incubated with DcsE in the presence of acetyl-CoA. This result demonstrates that DcsE functions as a serine *O*-acetyltransferase, suggesting that DcsE contributes to the formation of *O*-acetyl-L-serine in DCS biosynthesis.

O-Acetyl-L-serine is suggested to be converted to *O*-ureido-L-serine when incubated with hydroxyurea (24). Although we have not yet obtained the experimental evidence, this reaction may be catalyzed by a homolog of *O*-acetylserine sulfhydrylase encoded by *dcsD* (Table 1 and Fig. 4), judging from a report

that *O*-ureido-L-serine is generated by the catalysis of *O*-acetylserine sulfhydrylase when *O*-acetyl-L-serine and hydroxyurea were used as the substrates (10). Generally, the *O*-acetylserine sulfhydrylase catalyzes the formation of L-cysteine using *O*-acetyl-L-serine and H$_2$S as the substrates. To investigate the function of DcsD *in vitro*, the expression of recombinant DcsD in *E. coli* is now under way.

Hydroxyurea, a supplier of the nitrogen in the isoxazolidone structure of DCS, was suggested to be derived from L-arginine (23). It is thought that the DcsB protein, which is similar to arginase (Table 1), may be involved in the formation of hy-
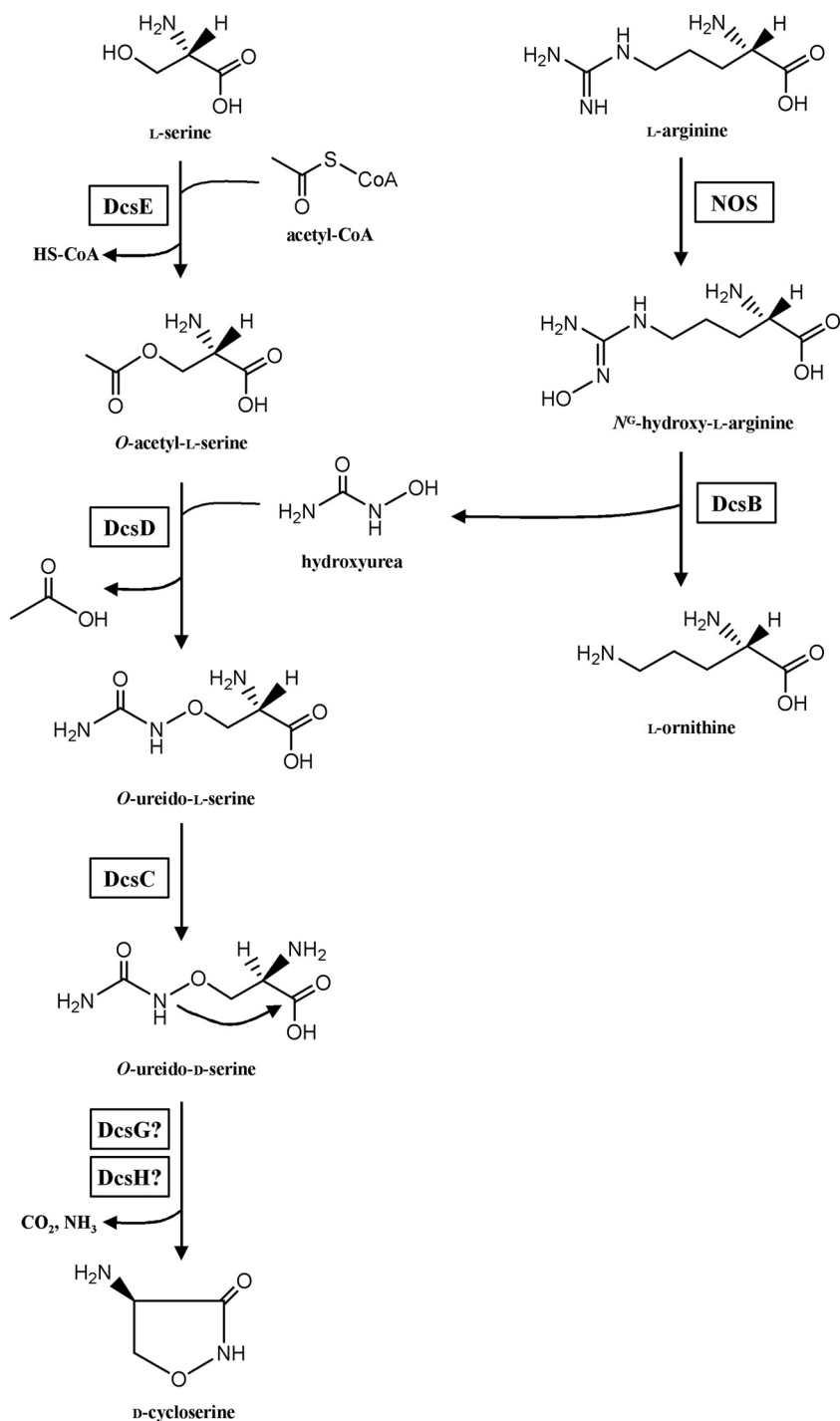
FIG. 4. Predicted biosynthetic pathway of DCS in *S. lavendulae* ATCC 11924.

droxyurea from L-arginine (Fig. 4) because arginase hydrolyzes L-arginine to yield urea and L-ornithine. There are two possibilities for the generation of hydroxyurea: (i) DcsB forms urea and L-ornithine by hydrolyzing L-arginine, and the resulting urea is converted to hydroxyurea; (ii) after L-arginine is first hydroxylated to N^G-hydroxy-L-arginine, the hydroxylated substance is hydrolyzed by DcsB to form hydroxyurea and L-ornithine. The former case may be less likely, because urea did

not stimulate the production of DCS in the washed cells of *S. garyphalus* but L-arginine did (23). Therefore, the latter case is likely to be involved in hydroxyurea formation. To support this hypothesis, we tested whether the recombinant DcsB protein, which was produced in *E. coli* and purified, hydrolyzes N^G-hydroxy-L-arginine. Figure 5B shows that DcsB actually hydrolyzes N^G-hydroxy-L-arginine. However, L-arginine was not a substrate of the enzyme. This result strongly suggests that
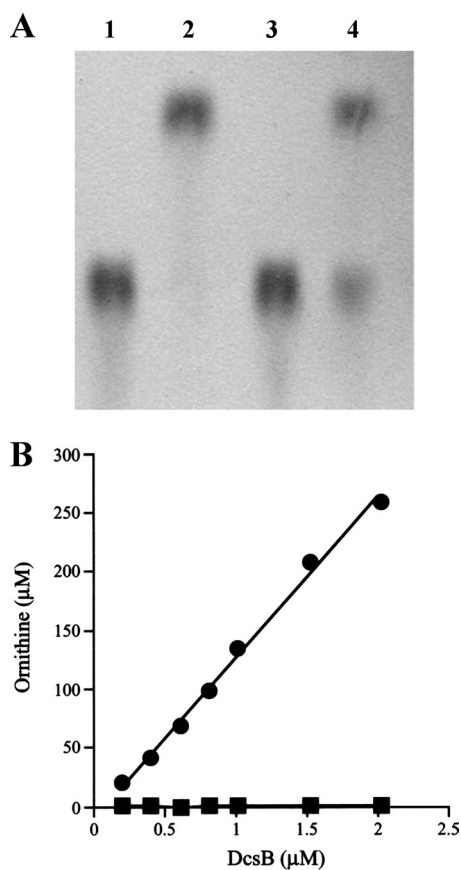
FIG. 5. Analysis of the recombinant DcsE and DcsB proteins. (A) Serine O-acetyltransferase activity of DcsE. After the reaction mixture containing L-serine (1 mM) was incubated with or without DcsE (15 μM) in the presence of acetyl-CoA (1 mM), an aliquot of the mixture was analyzed by TLC. Lane 1, L-serine; 2, O-acetyl-L-serine; 3, the reaction mixture without DcsE; 4, the reaction mixture with DcsE. (B) Hydrolysis of N^G-hydroxy-L-arginine by DcsB. N^G-hydroxy-L-arginine (1 mM) or L-arginine (1 mM) was incubated with the given concentration of DcsB for 10 min at 37°C. After the incubation, the amount of ornithine liberated by the catalysis of DcsB was determined. Closed circles or squares indicate the data obtained with N^G-hydroxy-L-arginine or L-arginine, respectively.

hydroxyurea is formed from N^G-hydroxy-L-arginine by the enzymatic reaction of DcsB (Fig. 4). This finding is surprising, because N^G-hydroxy-L-arginine is known as an inhibitor of arginase (6). In the latter case, we hypothesize that N^G-hydroxy-L-arginine is formed by the action of NOS from L-arginine (Fig. 4), because NOS is known to catalyze the generation of NO from L-arginine through forming N^G-hydroxy-L-arginine as an intermediate. Indeed, we observed that 1 mM nitro-L-arginine methyl ester, a NOS inhibitor, prevents the production of DCS by *S. lavendulae* ATCC 11924 (75% ± 2.9% of the control). Since the candidate gene encoding NOS is not seen in the DCS biosynthetic gene cluster (Table 1), the corresponding gene may be located on another region of the chromosome. It has been reported that some *Streptomyces* strains possess the NOS gene (8, 13).

O-Ureido-L-serine, presumably generated by the catalysis of DcsD, should be racemized to its D form, O-ureido-D-serine (Fig. 4). A homolog of diaminopimelate epimerase encoded by

dcsC (Table 1) could participate in this reaction. Although DcsC exhibits a similarity to diaminopimelate epimerase, which catalyzes the interconversion of L,L-diaminopimelate and its *meso* isomer, it could function as racemase when O-ureido-L-serine is used as a substrate. This hypothesis should be confirmed by *in vitro* analysis using the recombinant DcsC protein.

Finally, O-ureido-D-serine is cyclized to form DCS with the release of ammonia and carbon dioxide (Fig. 4). Indeed, when O-[^{14}C]ureido-D-serine was incubated with the cell extract of *S. garyphalus*, the formation of DCS was observed with the release of $^{14}CO_2$ (24). The cyclization of O-ureido-D-serine could be catalyzed by DcsG or DcsH (Fig. 4), because both proteins have an ATP-grasp fold motif and belong to the ATP-grasp fold family of proteins, which activate the substrate by phosphorylating its carboxyl group using ATP and form a C-N bond. This hypothesis may be supported by an observation by another research group that ATP is required to form DCS from O-ureido-L-serine (24). At this moment, DcsG may be a more potent candidate than DcsH since it is necessary for DCS biosynthesis as revealed by gene disruption analysis. The question of when the hydrolysis of the carbamoyl derivative occurs remains to be answered. It may occur at the time of cyclization catalyzed by DcsG or DcsH or after it. *In vitro* analysis using recombinant DcsG and DcsH with the substrate O-ureido-D-serine will clarify which protein catalyzes the cyclization of O-ureido-D-serine and when the hydrolysis of carbamoyl derivative occurs. We are currently attempting to express DcsG and DcsH in *E. coli*.

Based on our proposal (Fig. 4), the *dcsA* and *dcsF* products might not be involved in DCS biosynthesis. To investigate this possibility, the creation of in-frame deletion mutants for both genes is now ongoing. Furthermore, the creation of the *dcsB*, *dcsC*, *dcsD*, *dcsE*, and *dcsH* in-frame deletion mutants is also in progress. These mutants will contribute to a near-perfect understanding of the DCS biosynthetic pathway.

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