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High-Level Heterologous Production of D-Cycloserine by *Escherichia coli*

Takanori Kumagai,[a] Tomoki Ozawa,[b] Momoko Tanimoto,[b] Masafumi Noda,[a] Yasuyuki Matoba,[a] Masanori Sugiyama[a]

Department of Molecular Microbiology and Biotechnology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan[a]; Department of Pharmaceutical Sciences, Hiroshima University, Hiroshima, Japan[b]

Previously, we successfully cloned a D-cycloserine (D-CS) biosynthetic gene cluster consisting of 10 open reading frames (designated *dcsA* to *dcsJ*) from D-CS-producing *Streptomyces lavendulae* ATCC 11924. In this study, we put four D-CS biosynthetic genes (*dcsC*, *dcsD*, *dcsE*, and *dcsG*) in tandem under the control of the T7 promoter in an *Escherichia coli* host. SDS-PAGE analysis demonstrated that the 4 gene products were simultaneously expressed in host cells. When L-serine and hydroxyurea (HU), the precursors of D-CS, were incubated together with the *E. coli* resting cell suspension, the cells produced significant amounts of D-CS (350 ± 20 μM). To increase the productivity of D-CS, the *dcsJ* gene, which might be responsible for the D-CS excretion, was connected downstream of the four genes. The *E. coli* resting cells harboring the five genes produced D-CS at 660 ± 31 μM. The *dcsD* gene product, DcsD, forms *O*-ureido-L-serine from *O*-acetyl-L-serine (OAS) and HU, which are intermediates in D-CS biosynthesis. DcsD also catalyzes the formation of L-cysteine from OAS and H_2S. To repress the side catalytic activity of DcsD, the *E. coli* chromosomal *cysJ* and *cysK* genes, encoding the sulfite reductase α subunit and OAS sulfhydrylase, respectively, were disrupted. When resting cells of the double-knockout mutant harboring the four D-CS biosynthetic genes, together with *dcsJ*, were incubated with L-serine and HU, the D-CS production was 980 ± 57 μM, which is comparable to that of D-CS-producing *S. lavendulae* ATCC 11924 (930 ± 36 μM).

The cyclic structural analog of the amino acid D-cycloserine (D-CS) (Fig. 1) is a broad-spectrum antibiotic produced by *Streptomyces lavendulae* and *Streptomyces garyphalus* (1). The antibiotic inhibits both alanine racemase and D-alanyl–D-alanine ligase, which are necessary for the biosynthesis of peptidoglycan in the bacterial cell wall (2, 3). Rifampin and isoniazid have been clinically used for the treatment of tuberculosis caused by infection with *Mycobacterium tuberculosis* (4). However, *M. tuberculosis* that is resistant to these drugs has recently occurred. Presently, D-CS is clinically used as a second-line-of-defense drug against these antibiotic-resistant *M. tuberculosis* strains (4). In this connection, it has been shown that *M. smegmatis* overproducing alanine racemase is resistant to D-CS (5, 6). Recently, D-CS has been shown to function as a partial agonist for the *N*-methyl-D-aspartate receptor. As a result, the application of D-CS for the treatment of some psychological dysfunctions has been extensively studied (7–9).

Our group has successfully cloned a D-CS biosynthetic gene cluster from the chromosomal DNA of D-CS-producing *S. lavendulae* ATCC 11924, which is composed of 10 open reading frames, designated *dcsA* to *dcsJ* (10). The functions of *dcsI* and *dcsJ* had previously been analyzed using the corresponding genes cloned from other D-CS-producing strains, i.e., *S. lavendulae* ATCC 25233 (11) and *S. garyphalus* (CSH) 5-12 (12), demonstrating that both gene products are responsible for self-resistance in the D-CS producer. Gene disruption and recombinant protein analyses have demonstrated that the revised D-CS biosynthetic pathway is as follows. L-Serine is *O*-acetylated by DcsE to generate *O*-acetyl-L-serine (OAS) (10, 13). The resultant OAS reacts with hydroxyurea (HU) to yield *O*-ureido-L-serine by use of DcsD, which is a pyridoxal phosphate-dependent enzyme (14). *O*-Ureido-L-serine is racemized by DcsC (14, 15), followed by cyclization with DcsG, which is a member of the ATP-grasp fold family of proteins (10, 14) (Fig. 1).

We have previously hypothesized that L-arginine, as a precursor in the D-CS biosynthetic pathway, must be hydroxylated by nitric oxide synthase (NOS) expressed in D-CS-producing *S. lavendulae* (10). However, we have corrected the hypothesis as follows: DcsA as a heme protein, but not as an NOS protein, contributes to the formation of N^{ω}-hydroxy-L-arginine (16). As shown in Fig. 1, HU is generated by the hydrolysis of N^{ω}-hydroxy-L-arginine with DcsB (10).

In recent years, the heterologous expression of secondary metabolic pathways using a surrogate host, such as *Escherichia coli*, has emerged as an effective way of producing natural products. However, practical antibiotics have not yet been successfully produced using *E. coli* as a host. Our goal is to realize high production of D-CS by expressing its biosynthetic genes (*dcsA* to -*dcsE* and *dcsG*) in *E. coli* as a host cell. In this study, we tried to introduce the four D-CS biosynthetic genes (*dcsC*, *dcsD*, *dcsE*, and *dcsG*) into *E. coli* cells to express these gene products and to construct a D-CS production system by incubating resting cells with precursors of D-CS. We show that coexpression of the four D-CS biosynthetic genes and a self-resistance gene, *dcsJ*, which encodes a putative D-CS efflux protein from D-CS-producing *S. lavendulae*, in combination with metabolic engineering of the *E. coli* host, is effective for the high production of the antibiotic.

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Address correspondence to Masanori Sugiyama, sugi@hiroshima-u.ac.jp.

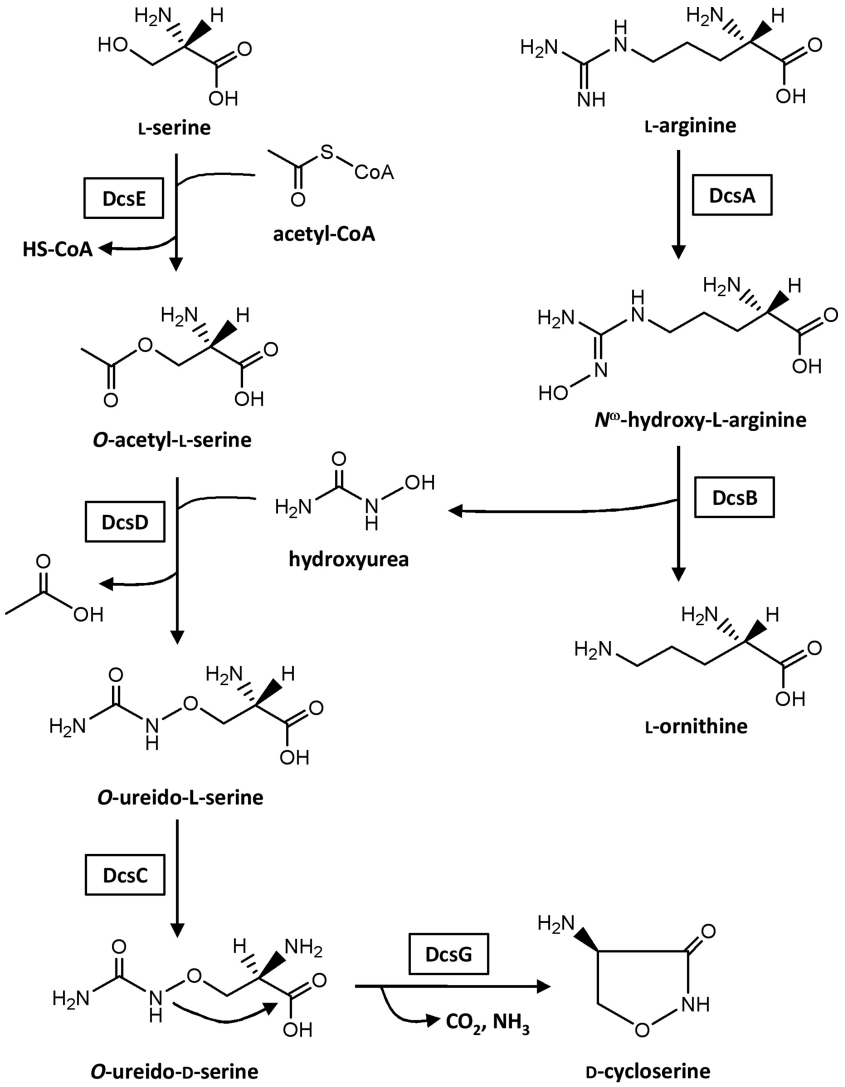

FIG 1 Biosynthetic pathway for D-CS.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *S. lavendulae* ATCC 11924 was maintained at 28°C on FB agar medium (17). To produce D-CS, the ATCC 11924 strain was cultivated at 28°C in liquid medium B (18). *E. coli* DH5α (19) and plasmids pUC19 (20), pBR322 (21), pTA2 (Toyobo, Japan), and pGEM-T (Promega, USA) were used for cloning, sequencing, and creating gene disruption constructs. Plasmid pRedET (Gene Bridges, Germany) was used for gene disruption by Red/ET recombination (22). pET-28a(+) (Merck, Germany) and pBR322 were used as the sources of kanamycin and tetracycline resistance genes, respectively. *E. coli* BL21(DE3) and plasmid pET-21a(+) (Merck) were used for protein expression. All *E. coli* strains were cultivated in Luria broth or on Luria agar supplemented with the appropriate antibiotics when necessary (23).

DNA manipulations. Plasmid DNA was isolated from *E. coli* using the Wizard Plus Minipreps DNA purification system (Promega). DNA fragments separated in an agarose gel were extracted using a GeneClean III kit (MP Biomedicals, USA). Genomic DNA of *Streptomyces* was isolated as described previously (24).

Construction of expression vectors for bioconversion. The primers used in this study are listed in Table 1. *dcsE* was amplified by PCR using KOD-Plus DNA polymerase (Toyobo) and genomic DNA of the ATCC 11924 strain as a template with primers dcsE_F and dcsE_R. The amplified *dcsE* was cloned into a pGEM-T vector to generate pGEM/*dcsE*. After confirmation of the nucleotide sequence, *dcsE* was cut off from pGEM/*dcsE* by digestion with NdeI and HindIII, followed by ligation to the same sites of pET-21a(+) to yield pET-21a(+)/*dcsE*. The *dcsD* gene was amplified by PCR with primers dcsD_F and dcsD_R and inserted into pGEM-T to generate pGEM/*dcsD*. The *dcsD* gene obtained from pGEM/*dcsD* after digestion with NdeI and HindIII was ligated to the same sites of pET-21a(+) to yield pET-21a(+)/*dcsD*. The *dcsD* fragment was cut off from pET-21a(+)/*dcsD* by digestion with XbaI and HindIII and ligated to pET-21a(+)/*dcsE* previously digested with SpeI and HindIII to generate pET-21a(+)/*dcsED*. Each *dcsC* or *dcsG* gene was amplified by PCR with the primer sets listed in Table 1 and subcloned into pET-21a(+) to yield pET-21a(+)/*dcsC* and pET-21a(+)/*dcsG*, respectively. Subsequently, *dcsC* and *dcsG* were successively connected to pET-21a(+)/*dcsED* using the same procedure as described for *dcsD* to generate pET-21a(+)/*dcsEDCG*. A D-CS self-resistance gene, designated *dcsJ* (10), was amplified by PCR with primers dcsJ_F and dcsJ_R; it was then inserted into pET-21a(+)/*dcsEDCG* in the same way to yield pET-21a(+)/*dcsEDCGJ*.

Protein analysis. Whole-cell proteins of *E. coli* BL21(DE3) that expressed D-CS biosynthetic genes were analyzed by SDS-PAGE (25).

TABLE 1 Primers used in this study

Name	Sequence (5′→3′)
dcsE_F	CATATGAGGGAATTCATACCCCCGGCCTCC
dcsE_R	AAGCTTGGACTAGTTCAGGCGACGATCGCCAG GAACTTCG
dcsD_F	CATATGCCTCTGTTTAACAGCATCCTCGACACCATC
dcsD_R	AAGCTTGGACTAGTCTAGAGGCCGGAGCCGGTGTC
dcsC_F	CATATGATACGCATGAGAACGCCGAGCACG
dcsC_R	AAGCTTGGACTAGTTTAAACAGAGGCATGGAG GAAGGTTCCTTCG
dcsG_F	CATATGGGCATCCTCGCCTTGGTCACC
dcsG_R	AAGCTTGGACTAGTCTAGGGCTTGAGCCGTTCGGC
dcsJ_F	CATATGGATGCACGACACAGCACGATCACGTTGA
dcsJ_R	AAGCTTGGACTAGTCTACGCTCTCGCCCGCTGCGT
cysJ_U_F	GAATTCTTTACCCACCAGGCCCACTTCCGCCA
cysJ_U_R	TCTAGAGAGCGCCGTTATCAGCGAGATGTCTAC
cysJ_D_F	CTGCAGCGGAAGGTGGGACCTGTGTCGTCAT
cysJ_D_R	GCATGCCACCAGATGCGCACCATACTCCAGTGATTC
Tet^r_F	TCTAGAAATAAGGGCGACACGGAAATGTTGAAT ACTCATACTCTTCCT
Tet^r_R	CTGCAGGTCGCAGACGTTTTGCAGCAGCAGTC
cysJ_dis_F	ATGATCAGATTCTGGTTCGCCGTAATGCGGA
cysJ_dis_R	CGGAGAATATCGTCACCACAAATAACGCCACA
cysJ_check_F	CATCTCCTTGCATGCACCATTCCTTGC
cysJ_check_R	GCCGTGACGACGTAAATGATGCCAGAAT
cysK_U_F	GAATTCCGAACATATCCTAACTGTCCATTG CGCAATTACCC
cysK_U_R	AAGCTTCTCCGGACCGGTGGTTTTTTCGTG AATTTCAG
cysK_D_F	GCATGCGGGTGAGCGTTATTTAAGCACCG CATTGTTTGC
cysK_D_R	GTCGACCTCTGCACCTTCAACGTCACGAACCGTA
Km^r_F	AAGCTTTTGAAGTGGTGGCCTAACTACGG CTACACTAGAA
Km^r_R	GCATGCTTTTTCGCCCTTTGACGTTGGAGT CCACGTTCTTTAATA
cysK_dis_F	CTAACTGTCCATTGCGCAATTACCCGGT
cysK_dis_R	CGTACCAATGTTAGCGCATACTTCTACCTG
cysK_check_F	CCGATGCGCCAGAGTTGTTTCTGAAACA
cysK_check_R	CGTCGCGCAGTTCCTGTTTGTAGATTTC

Disruption of *cysJ* and *cysK* located on the *E. coli* BL21(DE3) chromosome. A 1.5-kb DNA fragment upstream of *cysJ* was amplified by PCR with primers cysJ_U_F and cysJ_U_R; it was then cloned into pGEM-T to yield pGEM/Δ*cysJ*-U. After confirmation of the nucleotide sequence, a 1.5-kb DNA fragment, which was cut off from pGEM/Δ*cysJ*-U by digestion with EcoRI and XbaI, was inserted into the same sites of pUC19 to generate pUC19/Δ*cysJ*-U. A 1.6-kb DNA fragment downstream of *cysJ* was amplified by PCR with primers cysJ_D_F and cysJ_D_R, inserted into pGEM-T, and subsequently cloned into the PstI/SphI sites of pUC19/Δ*cysJ*-U to yield pUC19/Δ*cysJ*-UD. A pBR322-derived tetracycline resistance gene that includes its promoter and terminator (1.8 kb) was amplified by PCR with primers Tet^r_F and Tet^r_R, cloned into pGEM-T, and inserted into the XbaI/PstI sites of pUC19/Δ*cysJ*-UD to construct pUC19/Δ*cysJ*. Using pUC19/Δ*cysJ* as a template DNA, a disruption cassette for *cysJ* (4.3 kb) was amplified by PCR with primers cysJ_dis_F and cysJ_dis_R. With the *cysJ* cassette, *cysJ*, which is located on the *E. coli* BL21(DE3) chromosome, was disrupted by Red/ET recombination using the Quick and Easy *E. coli* gene deletion kit (Gene Bridges). A *cysJ* disruption mutant (Δ*cysJ*) was selected on an LB agar plate containing tetracycline, and the authenticity of the mutant was confirmed by PCR with primers cysJ_check_F and cysJ_check_R.

DNA fragments upstream (1.5 kb) and downstream (1.6 kb) of *cysK* were amplified by PCR with the primer sets listed in Table 1 and cloned into the EcoRI/HindIII and SphI/SalI sites of pBR322, respectively, to generate pBR322/Δ*cysK*-UD. A kanamycin resistance gene that includes its promoter and terminator (1.4 kb) on pET-28a(+) was amplified by PCR with primers Km^r_F and Km^r_R and inserted into the HindIII/SphI sites of pBR322/Δ*cysK*-UD to construct pBR322/Δ*cysK*. A disruption cassette for *cysK* (4.5 kb) was amplified by PCR with primers cysK_dis_F and cysK_dis_R, and a *cysK*-disrupted mutant (Δ*cysK*) was obtained using the same procedure as described for Δ*cysJ*, with the exception of the selection for kanamycin. The validity of Δ*cysK* was checked by PCR with primers cysK_check_F and cysK_check_R. A *cysJ cysK* double mutant (Δ*cysJ* Δ*cysK*) was created by introducing the *cysK* disruption cassette into Δ*cysJ*, selected with tetracycline and kanamycin, and confirmed by PCR.

Generation of D-CS from L-serine supplemented with HU by bioconversion. *E. coli* BL21(DE3) and its mutants harboring pET-21a(+)/*dcsEDCG* or pET-21a(+)/*dcsEDCGJ* were cultivated in 4 ml of LB supplemented with reagents of the Overnight Express autoinduction system 1 (GE Healthcare, United Kingdom) at 28°C for 24 h. The system allows the induction of T7 RNA polymerase under the control of the *lac* promoter automatically at the stationary phase of growth based on the medium components. Subsequently, the expression of desired proteins under the control of the T7 promoter is induced. The *E. coli* cells were collected by centrifugation, washed once with 10 mM potassium phosphate buffer (pH 7.2), and resuspended in the same buffer to an optical density at 600 nm (OD_{600}) of 1.0. The cell suspension (4 ml), called resting cells here, was incubated with L-serine (2.5 mM) and HU (2.5 mM) (Sigma-Aldrich, USA) at 28°C for 4 h.

Assay of D-CS production. After incubation of the resting cells with the addition of the precursors in the biosynthetic pathway, the cells were centrifuged, and the resulting supernatant fluid was filtered through a 0.2-μm-pore-size filter (Advantech, Japan). An aliquot of the fluid was analyzed by HPLC as described previously (26) to evaluate the amount of D-CS, except that the detection was carried out at 226 nm. Briefly, a solution of authentic D-CS (Wako, Japan) was mixed with L-tryptophan (1 mM) as an internal standard at a volume ratio of 3:1, followed by analysis with high-pressure liquid chromatography (HPLC). A standard curve for quantitation of D-CS was generated by plotting the ratio of peak areas (D-CS to L-tryptophan) versus the concentrations of D-CS. The sample solution was analyzed in the same way as the authentic D-CS. The concentration of D-CS was calculated from the ratio of peak areas and the standard curve. The D-CS titer was expressed as the mean ± standard error (SE) from three independent experiments.

Bioassay. After bioconversion, the reaction mixture was centrifuged to remove the *E. coli* cells. The resulting supernatant fluid was sterilized by filtration. Fifty microliters of the solution was applied on a paper disk, which was put on a bioassay plate overlaid with spores of *Bacillus subtilis* IFO3134 as a test organism. Bioassay was carried out at 37°C for 16 h.

Mass spectrometry. The eluate from the HPLC column was collected and freeze-dried. The resulting residue was dissolved in methanol-water (1:1) and analyzed by electrospray ionization-time of flight (ESI-TOF) mass spectrometry. ESI-TOF mass analysis was done using a TripleTOF 5600 instrument (AB Sciex, USA). Mass spectra were obtained in the negative-ion mode.

Statistical analysis. Statistical analysis was carried out using Student's *t* test. *P* values of <0.05 were used to identify statistically significant differences.

RESULTS AND DISCUSSION

Expression of four D-CS biosynthetic genes in *E. coli* and formation of D-CS. Four genes, *dcsE*, *dcsD*, *dcsC*, and *dcsG* (in order), were located polycistronically under the control of the T7 promoter using an *E. coli* expression vector, pET-21a(+), to create pET-21a(+)/*dcsEDCG* (Fig. 2a). In this case, a ribosome-binding sequence derived from pET-21a(+) was designed to be located in front of each gene. SDS-PAGE analysis of the whole-cell protein of

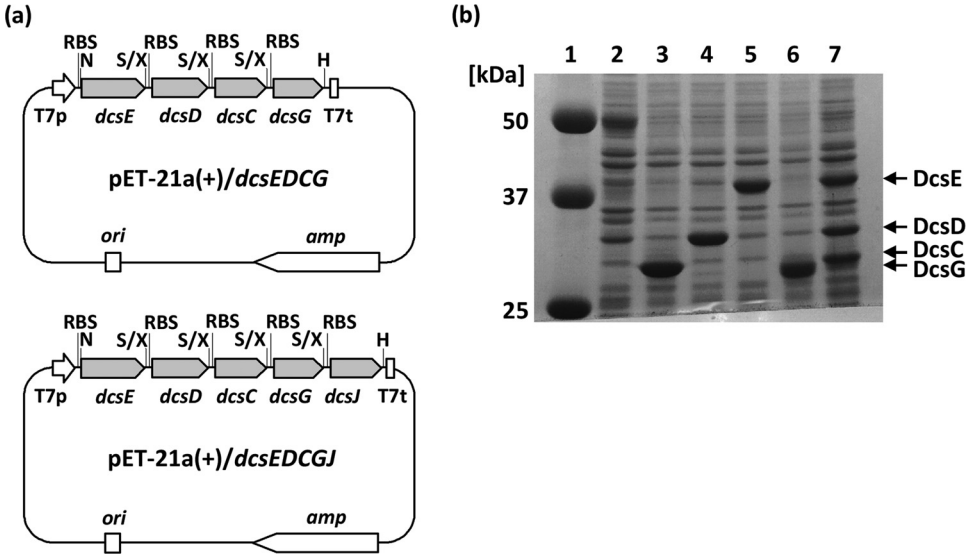

FIG 2 (a) Vectors used for bioconversion in this study. T7p, T7 promoter; RBS, ribosome-binding sequence; T7t, T7 terminator; *amp*, ampicillin resistance gene; *ori*, replication origin; H, HindIII; N, NdeI; S, SpeI; X, XbaI. (b) SDS-PAGE analysis of *E. coli* BL21(DE3) harboring various plasmids. Whole-cell proteins of *E. coli* BL21(DE3) harboring the indicate plasmids and grown at 28°C for 24 h were analyzed by SDS-PAGE (25). Lane 1, molecular mass standards; lane 2, pET-21a(+); lane 3, pET-21a(+)/*dcsC*; lane 4, pET-21a(+)/*dcsD*; lane 5, pET-21a(+)/*dcsE*; lane 6, pET-21a(+)/*dcsG*; lane 7, pET-21a(+)/*dcsEDCG*.

E. coli BL21(DE3) harboring pET-21a(+)/*dcsEDCG* showed that the four proteins were obviously produced, but the expression of DcsG was relatively low (Fig. 2b).

The resting cell suspension of *E. coli* harboring pET-21a(+)/*dcsEDCG* was incubated with L-serine and HU dissolved in potassium phosphate buffer at 28°C. HPLC analysis of the supernatant fluid from the incubation mixture showed that a peak corresponding to D-CS was obtained at the retention time of 17 min (Fig. 3d). When HU was eliminated from the reaction mixture, the peak did not appear (Fig. 3c). The peak also did not appear when L-serine and HU were incubated with cells of *E. coli* harboring pET-21a(+) without the insertion of *dcsEDCG* (Fig. 3b). In addition, the supernatant fluid from the incubation mixture, which is composed of both substrates (L-serine and HU) and the cells of *E. coli* harboring pET-21a(+)/*dcsEDCG*, displayed an antibiotic activity against *Bacillus subtilis* (Fig. 4). Furthermore, a negative-ion peak of *m*/*z* 101.0 (molecular weight [MW] of D-CS, 102.09) was observed by ESI-TOF mass analysis, demonstrating that D-CS is produced by the bioconversion system. To determine the suitable time for bioconversion, L-serine and HU were incubated with *E. coli* cells harboring pET-21a(+)/*dcsEDCG* at 28°C. The D-CS titer was determined every 1 h until 8 h (Fig. 5). Since the D-CS titer was saturated at 4 h, the D-CS titers were evaluated after 4 h of incubation in all of the experiments described below. When L-serine and HU (each at 2.5 mM) were incubated together with the cell suspension, the D-CS production was 350 ± 20 μM (Fig. 6), suggesting that the added substrates are not fully used for the synthesis of D-CS. Because the D-CS production by the mutant *E. coli* BL21(DE3) strain increased, as described below, the saturation of the antibiotic production may be due to the limitation of OAS rather than to the depletion of ATP, which is necessary for the catalytic activity of DcsG (14).

Yield improvement by coexpression of *dcsJ*, which encodes a putative D-CS efflux protein. We have previously cloned a self-resistance gene, designated *orfB*, from a D-CS-producing *S. garyphalus* (CSH) 5-12 (12). The *orfB* gene product deduced from the amino acid sequence was similar to several membrane proteins, suggesting that OrfB functions to efflux D-CS outside the cells (12). Since the *dcsJ* gene product displays a high sequence similarity (97%) with OrfB, the protein is deduced to have the same function as OrfB (10). To increase the production of D-CS, we examined the coexpression of *dcsJ* with *dcsEDCG* by the construction of pET-21a(+)/*dcsEDCGJ* (Fig. 2a). Resting cells of *E. coli* BL21(DE3) harboring pET-21a(+)/*dcsEDCGJ* which were incubated with L-serine and HU produced 650 ± 31 μM D-CS (Fig. 6), demonstrating that the self-resistance gene *dcsJ* increases D-CS production significantly. This increment seems to have occurred due to the high efflux of D-CS toward outside the cells. However, the added L-serine and HU, which were incubated with resting cells harboring pET-21a(+)/*dcsEDCGJ* as well as those harboring pET-21a(+)/*dcsEDCG*, were not completely utilized in the synthesis of D-CS.

High production of D-CS by metabolic engineering of the *E. coli* host. We have previously shown that OAS, an intermediate in the D-CS biosynthetic pathway, is synthesized by the *dcsE* product (10). On the other hand, since *E. coli* cells possess *cysE*, which encodes L-serine-*O*-acetyltransferase, OAS can be supplied by the gene product. However, it is known that the enzymatic activity of CysE is strongly repressed in the presence of L-cysteine (27). In this study, since the *E. coli* cells were grown in LB broth, L-cysteine must be tightly bound to the CysE protein. Therefore, it is likely that OAS is supplied from L-serine and acetyl coenzyme A (acetyl-CoA) by the catalytic activity of DcsE. Because the L-serine-*O*-acetyltransferase activity of DcsE is relatively low (13), there may be a small OAS pool in the *E. coli* cells. Moreover, OAS is consumed by CysK, an OAS sulfhydrylase that forms L-cysteine from OAS and H_2S as substrates. In addition, DcsD catalyzes the same reaction as does CysK, and the OAS sulfhydrylase activity of DcsD is superior to the enzymatic ability to synthesize *O*-ureido-L-serine (14).

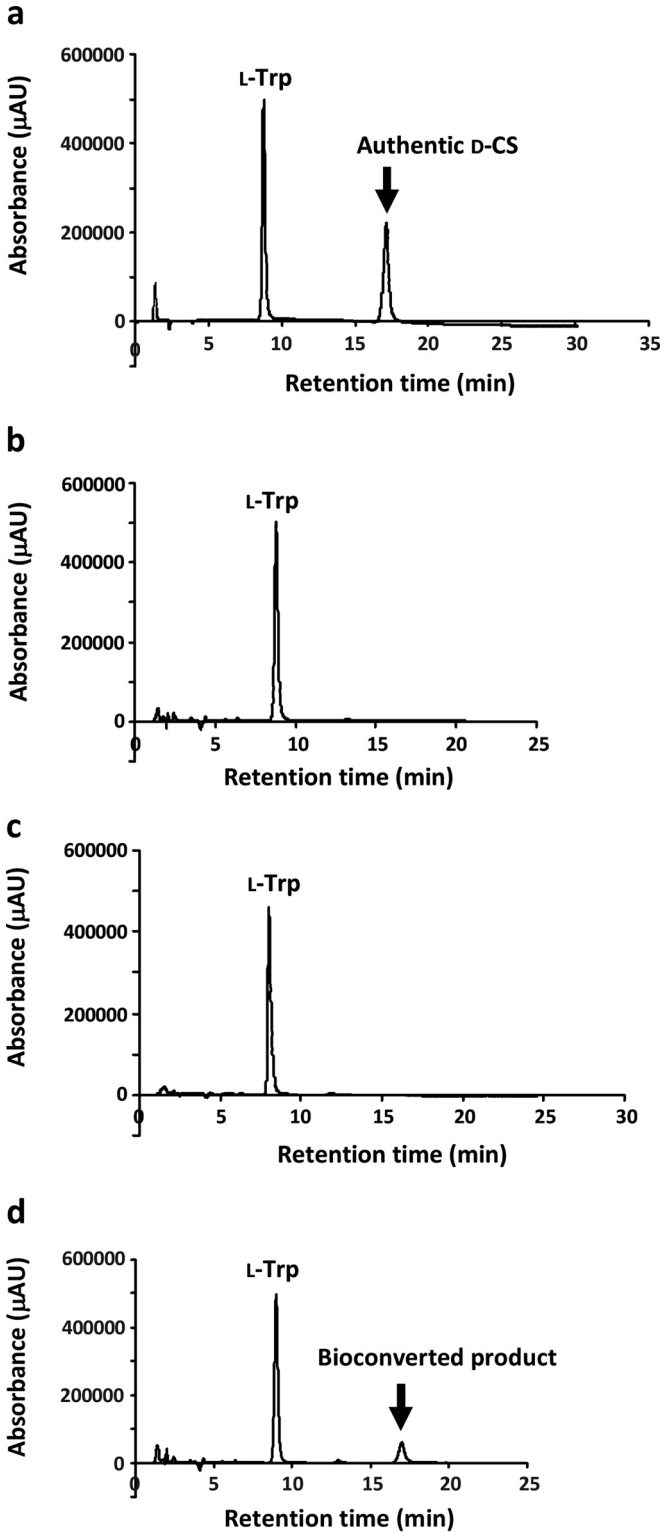

FIG 3 Analysis of bioconverted product by HPLC. The supernatant fluid from the indicated mixtures incubated at 28°C for 4 h was analyzed by HPLC using a Senshu Pak SCX-1251-N column. (a) Authentic D-CS. (b) Cells of *E. coli* BL21(DE3) harboring pET-21a(+) with L-serine and HU (each at 2.5 mM). (c) Cells of *E. coli* BL21(DE3) harboring pET-21a(+)/*dcsEDCG* with L-serine (2.5 mM). (d) Cells of *E. coli* BL21(DE3) harboring pET-21a(+)/*dcsEDCG* with L-serine and HU (each at 2.5 mM).

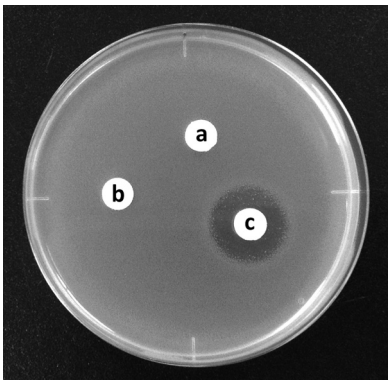

FIG 4 Antibiotic activity of bioconverted mixture. (a) L-Serine and HU (each at 2.5 mM) in 10 mM potassium phosphate (pH 7.2) as a control. (b) Incubation mixture containing both substrates and cells of *E. coli* BL21(DE3) harboring pET-21a(+). (c) Incubation mixture containing both substrates and cells of *E. coli* BL21(DE3) harboring pET-21a(+)/*dcsEDCG*. The supernatant fluid from each mixture was assayed for antibacterial activity using *B. subtilis* IFO3134 as a test organism.

One of the strategies for increasing OAS, which is necessary for D-CS synthesis, is to reduce the above reactions. For this purpose, we disrupted both *cysJ* and *cysK* on the *E. coli* BL21(DE3) chromosome. *cysJ* encodes the α subunit of sulfite reductase, which catalyzes the synthesis of H_2S (28). Using the Red/ET recombination method (22), we made two single-knockout mutants, in which *cysJ* or *cysK* on the chromosome of a parental *E. coli* BL21(DE3) strain was knocked out (designated Δ*cysJ* or Δ*cysK*, respectively), and a *cysJ cysK* double-knockout mutant (designated Δ*cysJ* Δ*cysK*). The authenticity of each mutant was confirmed by PCR. Although the D-CS production by each Δ*cysJ* and Δ*cysK* mutant harboring pET-21a(+)/*dcsEDCG* scarcely increased compared with that by the parental BL21(DE3) strain, the D-CS titer was significantly increased to 820 ± 36 μM (Fig. 6) when using the Δ*cysJ* Δ*cysK* mutant as a host; this shows that the double mutation effectively increases the D-CS yield. This was also observed when using pET-21a(+)/*dcsEDCGJ*, and the D-CS titer was significantly increased to 980 ± 57 μM when the Δ*cysJ* Δ*cysK*

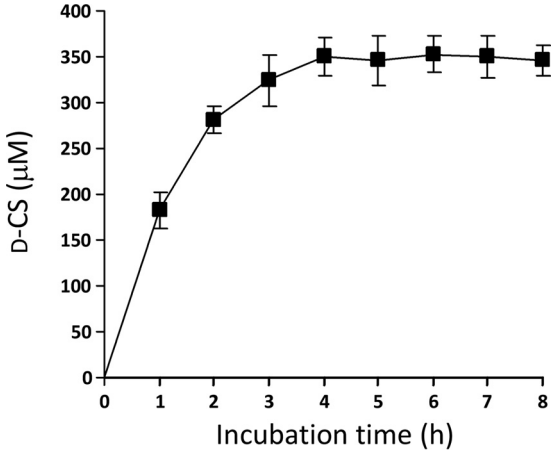

FIG 5 Time course of D-CS production. Cells of *E. coli* harboring pET-21a(+)/*dcsEDCG* were incubated with L-serine (2.5 mM) and HU (2.5 mM) at 28°C for 8 h. The amount of D-CS produced was determined by HPLC analysis every 1 h.

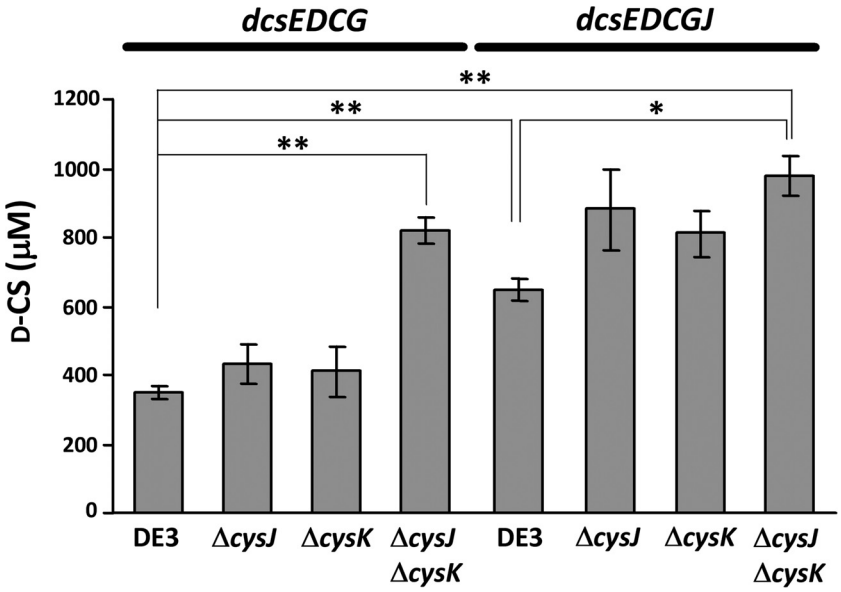

FIG 6 Production of D-CS obtained by bioconversion using a resting cell suspension of *E. coli* BL21(DE3) or its mutants harboring the indicated plasmid. L-Serine (2.5 mM) and HU (2.5 mM) were bioconverted to D-CS by *E. coli* BL21(DE3) and its mutants carrying pET-21a(+)/*dcsEDCG* or pET-21a(+)/*dcsEDCGJ* at 28°C for 4 h; the amount of D-CS was determined by HPLC analysis. *, $P < 0.05$; **, $P < 0.01$.

mutant with pET-21a(+)/*dcsEDCGJ* was used as a biocatalyst (Fig. 6). The D-CS titer obtained by this conversion system is comparable to that for the D-CS-producing microorganism *S. lavendulae* ATCC 11924 (930 ± 36 μM).

In the present study, we established a bioconversion system for the production of D-CS that uses an *E. coli* resting cell suspension and L-serine supplemented with HU as precursors of the antibiotic biosynthesis. The D-CS produced by the system can be easily purified from the incubation mixture using a chromatographic technique. Therefore, the system will be a powerful tool for synthesizing a practical antibiotic. The establishment of a bioconversion system to synthesis HU is in progress. In the near future, a complete bioconversion system for D-CS will be established using the *E. coli* resting cell suspension supplemented with L-serine and L-arginine as substrates.

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