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Identification and Functional Analysis of *Escherichia coli* Cysteine Desulfhydrases

Naoki Awano,[1] Masaru Wada,[1]† Hirotada Mori,[2] Shigeru Nakamori,[1] and Hiroshi Takagi[1]*

Department of Bioscience, Fukui Prefectural University, 4-1-1 Kenjojima, Matsuoka-cho, Fukui 910-1195,[1] and Research and Education Center for Genetic Information, Nara Institute of Science and Technology, Ikoma 630-0101,[2] Japan

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In *Escherichia coli*, three additional proteins having L-cysteine desulfhydrase activity were identified as *O*-acetylserine sulfhydrylase-A, *O*-acetylserine sulfhydrylase-B, and MalY protein, in addition to tryptophanase and cystathionine β-lyase, which have been reported previously. The gene disruption for each protein was significantly effective for overproduction of L-cysteine and L-cystine. Growth phenotype and transcriptional analyses suggest that tryptophanase contributes primarily to L-cysteine degradation.

L-cysteine is an important amino acid in terms of its applications in the pharmaceutical, food, and cosmetic industries. However, due to feedback inhibition by L-cysteine of serine acetyltransferase (SAT; EC 2.3.1.30), which catalyzes the formation of *O*-acetyl-L-serine from acetyl-coenzyme A (CoA) and L-serine (8, 9, 16), high-level production of L-cysteine from glucose has not been successfully achieved in microorganisms. In order to obtain L-cysteine producers, we previously constructed *Escherichia coli cysE* genes that encode altered SATs. These genes were genetically desensitized to the feedback inhibition by L-cysteine through site-directed or random mutagenesis (21, 32). We found that, in the recombinant *E. coli* cells expressing the altered *cysE* gene, there was a marked production of L-cysteine plus L-cystine.

In the same investigation (21), it was demonstrated that proteins with L-cysteine desulfhydrase (CD) activity play an important role in L-cysteine degradation in *E. coli* cells. In order to further improve L-cysteine production, a host strain having a lower level of CD activity must be constructed. CD is known to catalyze the degradation of L-cysteine to pyruvate, ammonia, and sulfide by the following reaction: $HSCH_2CH(NH_2)COOH + H_2O \rightarrow CH_3COCOOH + H_2S + NH_3$. This type of enzyme activity has been demonstrated to be present in several mammalian tissues (15) and in bacteria, such as *Salmonella enterica* serovar Typhimurium (6, 17) and *E. coli* (2, 11). In *E. coli*, cystathionine-β-lyase (CBL) (17) encoded by *metC*, which catalyzes mainly the conversion of cystathionine to homocysteine, pyruvate, and ammonia (9), as well as tryptophanase (TNase) encoded by *tnaA*, which primarily degrades L-tryptophan to indole, pyruvate, and ammonia (22), has been shown to exhibit CD activity in vitro (9, 23). We have previously reported that CBL and TNase catalyzed the CD reaction and acted on L-cysteine degradation in *E. coli* cells by analyses with CD activity staining and gene disruption (2). However, the double CD gene-disrupted mutant still had a low level of CD activity, suggesting that unknown CD proteins remain to be identified. Thus, we report here further identification and characterization of the CDs involved in L-cysteine degradation in *E. coli*.

Identification of CDs in *E. coli*. An *E. coli* cell has 4,388 kinds of open reading frame (ORF), including the function-unknown and -deduced genes. The library was constructed by placing each ORF under the *lacZ* promoter in vector pCA24N (20). *E. coli* wild-type strain JM39 was independently transformed with nine libraries, each consisting of 480 kinds of plasmids, and then approximately 1,200 colonies appeared from each library on Luria-Bertani (LB) medium (27) containing chloramphenicol (100 μg/ml). As a whole, more than 10,000 independent *E. coli* clones were obtained. The transformed cells from each library were mixed and grown at 37°C in 5 ml of LB medium containing chloramphenicol (100 μg/ml). When the absorbance at 600 nm reached 0.5, isopropyl-β-D-thiogalactopyranoside was added to the culture medium to a final concentration of 0.01 mM to induce gene expression. After cultivation for 4 h at 37°C, cell extracts were separated by native polyacrylamide gel electrophoresis (PAGE) and the gel was stained by gently shaking in the solution containing L-cysteine, pyridoxal 5′-phosphate (PLP), and $BiCl_3$ as described previously (2, 35). When the gel was kept at 4°C for 1.5 h, which was a shorter time than in the previous condition (3 h), three CD proteins were newly detected in JM39 in addition to CBL and TNase (Fig. 1): *O*-acetylserinesulfhydrylase-A (OASS-A), encoded by *cysK* (lane 2), *O*-acetylserinesulfhydrylase-B (OASS-B), encoded by *cysM* (lane 3), and MalY, encoded by *malY* (lane 4). OASS-A primarily catalyzes the synthesis of L-cysteine from *O*-acetylserine and sulfide along the L-cysteine biosynthetic pathway (3, 7, 19). OASS-B is considered to be an isomer of OASS-A (30, 31), but its function(s) is not yet clearly understood. Because both OASS-A and -B require PLP for CD activity in a similar manner as CBL, we think that these enzymes can catalyze the analogous reaction. MalY protein is also a PLP-dependent enzyme with the activity of the carbon-sulfur bond cleavage

* Corresponding author. Mailing address: Department of Bioscience, Fukui Prefectural University, 4-1-1 Kenjojima, Matsuoka-cho, Fukui 910-1195, Japan. Phone: 81-776-61-6000. Fax: 81-776-61-6015. E-mail: hiro@fpu.ac.jp.

† Present address: Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan.

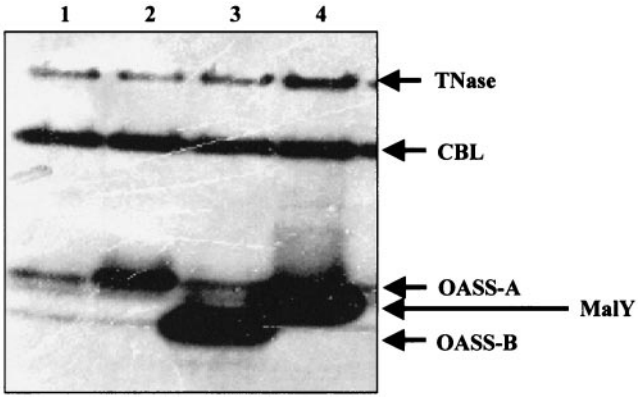

FIG. 1. Detection of OASS-A, MalY, and OASS-B by CD activity staining. Preparation of native PAGE gel, cell extracts, and CD activity staining were carried out according to the method described previously (2). Lane 1, wild-type JM39 harboring pBluescript II SK+ (vector control; Toyobo Biochemicals, Osaka, Japan); lane 2, JM39 harboring p*cysK* (*cysK* plasmid; OASS-A is overexpressed); lane 3, JM39 harboring p*cysM* (*cysM* plasmid; OASS-B is overexpressed); lane 4, JM39 harboring p*malY* (*malY* plasmid; MalY is overexpressed).

(βC-S lyase) of cystathionine (35), as well as a transcriptional regulator in *mal* gene expression (5, 26, 28). The secondary and tertiary structures of MalY are highly homologous to those of CBL (4, 5, 9, 18). Consequently, the five CD proteins in *E. coli* were identified as TNase, CBL, OASS-A, OASS-B, and MalY.

Effect of gene disruption on total CD activities and L-cysteine production in *E. coli*. For the construction of *cysK*-, *cysM*-, and *malY*-disrupted strains, internal fragments of the *cysK*, *cysM*, and *malY* genes of strain JM39 were amplified by PCR and cloned in pEL3, which has a thermosensitive replicon (1). The primers used were 5′-CGC CGC GGA TCC CAA TCT ACC GGT TAT TTT GAT AAC C-3′ and 5′-CGC CGC GGA TCC CAA GCT GGC ATT ACT GTT GCA ATT C-3′ for *cysK*, 5′-GCG GCG GGA TCC TAG GTT GAG TGA ATG TTA AAC GCC C-3′ and 5′-GCG GCG GGA TCC ATA CTG CAT TTG TCG GCA GCA ACA-3′ for *cysM*, and 5′-ATC CAG TCG ATG ATC GAT ACC GGG ATC C-3′ and 5′-CGC GGG ATC CTT AAC GAA CAG CGC GGA TGG CGT TA-3′ for *malY*. Gene disruption was performed as described previously (2). The ampicillin-sensitive strains obtained were characterized and designated as JM39Δ*cysK*, JM39Δ*cysM*, and JM39Δ*malY*. JM39Δ*tnaA* Δ*metC* Δ*cysM* Δ*malY* and JM39Δ*tnaA* Δ*metC* Δ*cysK* Δ*cysM* Δ*malY* were constructed from JM39Δ*tnaA* Δ*metC* (2) in the same manner as described above. CD gene disruption was confirmed by PCR and by CD activity staining. In each CD gene-disrupted mutant, the corresponding CD protein bands disappeared on the activity staining gel. No CD bands were detected in the quintet mutant, JM39Δ*tnaA* Δ*metC* Δ*cysK* Δ*cysM* Δ*malY* (data not shown). Total CD activities of the cell extracts prepared from wild-type JM39 and CD gene-disrupted strains cultured in LB medium were measured as described previously (2) (Table 1). CD activity was measured by colorimetric assay of the sulfide formed from L-cysteine by the enzyme sources (2, 29). The total CD activities of all mutants were lower than that of wild-type JM39. Interestingly, even the quintet mutant still had a low level of CD activity. The reason for the residual activity remains unclear; however, the presence of another CD protein(s), which fails to be separated by native PAGE or is inactivated by oxygen during native PAGE, is possible.

In order to confirm whether CDs were involved in L-cysteine degradation in *E. coli* cells, we analyzed the L-cysteine productivities of wild-type JM39 and each CD gene-disrupted mutant harboring pEAS-m (24, 33), which carries the cDNA encoding feedback-insensitive SAT from *Arabidopsis thaliana*. It should be noted that two mutants, JM39Δ*cysK* and JM39Δ*tnaA* Δ*metC* Δ*cysK* Δ*cysM* Δ*malY*, were not tested, because OASS-A encoded by *cysK* is cysteine synthetase, which is essential for L-cysteine biosynthesis. In contrast, it was found that OASS-B encoded by *cysM* was not an isomer of OASS-A based on the fact that JM39Δ*cysM* grew on a medium lacking L-cysteine (data not shown). For the production of L-cysteine and L-cystine, a loopful of cells cultured for 24 h on LB solid medium containing ampicillin (50 μg/ml) at 30°C was inoculated into 20 ml of C1 plus TS medium in a 500-ml flask and cultured at 30°C on a reciprocal shaker maintained at 120 strokes per min (33). Growth was measured by optical density at 562 nm (OD_{562}) of culture broth after appropriate dilution with 0.1 N

TABLE 1. Effect of CD gene disruption on total CD activities and L-cysteine plus L-cystine production in *E. coli*

Strain	Growth (OD_{610})[a] after 15 h of cultivation in LB plus 30 mM L-cysteine	CD activity (mU/mg of protein)[b] in the absence and presence of L-cysteine		Concn of L-cysteine plus L-cystine after 72 h of cultivation in C1 + TS medium (mg/liter/OD_{562})[c]
		No L-cysteine	10 mM L-cysteine	
JM39 (wild type)	4.0	20.6 ± 0.1	27.6 ± 0.1	590 ± 160
JM39Δ*tnaA*	2.2	15.7 ± 0.1	14.1 ± 0.1	1,290 ± 70
JM39Δ*metC*	3.5	15.0 ± 0.1	27.6 ± 0.5	1,240 ± 100
JM39Δ*cysK*	3.5	18.2 ± 0.5	29.9 ± 0.1	NT[d]
JM39Δ*cysM*	3.6	17.9 ± 0.5	27.8 ± 0.1	1,100 ± 70
JM39Δ*malY*	3.6	15.3 ± 0.1	27.1 ± 0.5	1,360 ± 90
JM39Δ*tnaA* Δ*metC* Δ*cysM* Δ*malY*	NT	9.1 ± 0.5	19.6 ± 0.1	1,080 ± 10
JM39Δ*tnaA* Δ*metC* Δ*cysK* Δ*cysM* Δ*malY*	NT	8.7 ± 0.5	11.5 ± 0.1	NT

[a] The values are means from three independent experiments. The variations in the values were less than 10%.
[b] One unit of activity was defined as the amount of enzyme required to produce 1 μmol of sulfide per min from L-cysteine at 37°C. The values are means ± standard deviations from three independent experiments.
[c] The values are means ± standard deviations from three independent experiments.
[d] NT, not tested since *cysK*-disrupted cells showed L-cysteine auxotrophy.

HCl. The amounts of L-cysteine and L-cystine were determined by microbioassay with *Pediococcus acidilactici* IFO3076, as described by Tsunoda et al. (34). As L-cysteine in the culture fluid was easily oxidized to L-cystine, which was slightly soluble in water, the culture fluids were assayed after L-cystine was dissolved with 0.5 N HCl. As shown in Table 1, the production of L-cysteine and L-cystine by these mutants was higher than that observed in the case of JM39. The amounts of L-cysteine and L-cystine produced after 72 h of cultivation increased by a factor of 1.8 to 2.3. These results clearly indicate that CD proteins played important roles in L-cysteine degradation in *E. coli* cells and that the corresponding gene disruption was effective in the production of L-cysteine and L-cystine by *E. coli* cells. However, the amounts of L-cysteine and L-cystine decreased significantly after 96 h of cultivation in all the strains, probably because of the remaining CD enzyme(s) (data not shown).

TNase contributes primarily to L-cysteine degradation in *E. coli*. Although the mechanisms are not yet understood, it has previously been observed that the growth of *E. coli* cells is inhibited by excess L-cysteine (12–14, 25). Therefore, the effect of CD gene disruption on the growth of *E. coli* cells in the presence of L-cysteine was examined. All of the strains were grown in LB plus 30 mM L-cysteine at 37°C, and cell growth was measured by optical density at 610 nm. As shown in Table 1, the growth of the *tnaA* disruptant JM39Δ*tnaA* was significantly inhibited, while all of the strains showed the same level of growth when cultured in LB medium (data not shown). This result suggests that TNase is a key enzyme in L-cysteine degradation in *E. coli* cells.

We examined the role of CD enzymes on L-cysteine degradation. Wild-type JM39 and CD gene-disrupted mutants were cultivated in LB plus 10 mM L-cysteine, and total CD activities were measured (Table 1). All of the single mutants, except for JM39Δ*tnaA*, showed a prominent increase in CD activity, ranging from 38% to 84% of that observed in the absence of L-cysteine. The CD activity from the *tnaA*-disrupted strain was virtually unchanged in the presence or absence of L-cysteine. Northern blot analysis for *tnaA* was carried out by using a Gene Images Random-Prime Labeling and Detection System (Amersham Pharmacia Biotech, Buckinghamshire, England). Strain JM39 was cultivated in LB medium or LB plus 10 mM L-cysteine, and total RNA was prepared using an RNeasy Protect Bacteria Mini kit (QIAGEN, Valencia, Calif.). As a DNA probe, the DNA fragment of *tnaA* was prepared by PCR with oligonucleotide primers 5′-CCG TTC CGC ATT CGT GTT AT-3′ and 5′-TGC GGT GAA GTG ACG CAA TA-3′. As shown in Fig. 2, the addition of L-cysteine resulted in an elevated level of the specific transcript (ca. 1.7 kb), while the basal level of expression in LB medium was fairly low. Transcription of the *E. coli tna* operon, consisting of two major structural genes, *tnaA* encoding tryptophanase and *tnaB* encoding tryptophan permease, have been studied in detail (10). This operon also contains a 319-bp transcribed leader regulatory region, *tnaC*, preceding *tnaA* and specifying a 24-residue leader peptide, TnaC, and expression of the *tna* operon is induced by L-tryptophan. Interestingly, the *tnaA* DNA probe detected a *tnaC-tnaA* transcript of ca. 1.7-kb in the presence of L-cysteine (lane 2), because no transcript was observed when the DNA fragment of *tnaB* was used as a probe (data not shown). These results indicate that TNase synthesis is induced by L-cysteine, in agreement with previous data on native PAGE (2). Our results may suggest that TNase contributes mainly to L-cysteine degradation and that a novel transcriptional regulation system is involved in *tnaA* expression.

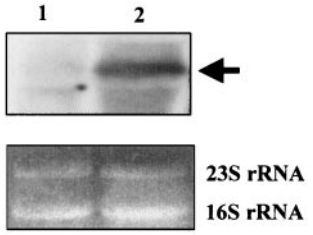

FIG. 2. TNase induction by the addition of L-cysteine. Total RNA of JM39 was prepared from cells cultivated in LB medium (lane 1) and LB plus 10 mM L-cysteine (lane 2). Each lane was loaded with 20 μg of total RNA. The arrow indicates a *tnaC-tnaA* transcript of ca. 1.7 kb. The 23S and 16S rRNAs are total RNA-loading controls detected by UV spectrometer.

In conclusion, five CD enzymes were identified in *E. coli* cells, and the gene disruption for each protein was significantly effective for overproduction of L-cysteine and L-cystine. However, it is noteworthy that the quintet mutant JM39Δ*tnaA* Δ*metC* Δ*cysK* Δ*cysM* Δ*malY* in the presence of L-cysteine showed higher CD activity than that observed in the absence of L-cysteine (Table 1). It appears that other CDs, in addition to the five proteins identified, could be induced by L-cysteine in *E. coli*. We must further analyze the quintet mutant in order to investigate the mechanism. Through the CD activity staining described here, some faint bands were still seen in the gel and some proteins appeared not to be migrated into the gel. We therefore think that unidentified proteins with CD activity, which may be induced by L-cysteine, are still present. Development of alternative methods to detect the remaining CDs, including enzyme purification and CD activity staining, is necessary and is currently in progress. We will also analyze the genome-wide expression profile in each CD gene-disrupted mutant using DNA microarray technology. It is possible that one gene disruption would affect the expression of other CD proteins and the whole metabolic profile in *E. coli*.

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