Functional analysis of l-serine O-acetyltransferase from Corynebacterium glutamicum

Yutaka Haitani, Naoki Awano, Mizue Yamazaki, Masaru Wada, Shigeru Nakamori & Hiroshi Takagi

Department of Bioscience, Fukui Prefectural University, Fukui, Japan

Correspondence: Hiroshi Takagi, Department of Bioscience, Fukui Prefectural University, 4-1-1 Kenjojima, Matsuoka-cho, Fukui 910-1195, Japan. Tel.: +81 776 61 6000; fax: +81 776 61 6015; e-mail: hiro@fpu.ac.jp

Present address: Masaru Wada, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan.

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Abstract
We report here the function of l-serine O-acetyltransferase (SAT) from the glutamic acid-producing bacterium Corynebacterium glutamicum. Based on the genome sequence of C. glutamicum and the NH2-terminal amino-acid sequence, the gene encoding SAT (cysE) was cloned and expressed in C. glutamicum. Deletion analysis of the 5’-noncoding region showed a putative −10 region (−27TAAAGT−22 or −26TAAGTC−21) and a possible ribosome-binding site (−12AGA−10) just upstream from the start codon. We found that the SAT activity was sensitive to feedback inhibition by l-cysteine, and that SAT synthesis was repressed by l-methionine. Further, cysE-disrupted cells showed l-cysteine auxotrophy, indicating that C. glutamicum synthesizes l-cysteine from l-serine via O-acetyl-l-serine through the pathway involving SAT and O-acetyl-l-serine sulfhydrolase in the same manner as Escherichia coli.

Introduction
l-Cysteine is an important amino acid in terms of its applications in the pharmaceutical, food, and cosmetic industries. However, owing to feedback inhibition by l-cysteine of l-serine O-acetyltransferase (SAT) (EC 2.3.1.30), which catalyzes the formation of O-acetyl-l-serine from acetyl-coenzyme A (CoA) and l-serine (Kredich, 1983; Soda, 1987), high-level production of l-cysteine from glucose had not been successfully achieved in microorganisms. Recently, a fermentation process for the production of l-cysteine in high amounts by an Escherichia coli strain containing the deregulated SAT enzyme was described in a patent application (Leinfelder & Heinrich, 1997). To obtain l-cysteine producers, we previously constructed E. coli cysE genes that encode altered SATs. These genes were genetically less sensitive to the feedback inhibition by l-cysteine through site-directed or random mutagenesis (Nakamori et al., 1998; Takagi et al., 1999b). We found marked production of l-cysteine plus l-cystine from glucose (1000 mg L−1) in recombinant E. coli cells expressing the altered cysE gene (Nakamori et al., 1998; Takagi et al., 1999b). Further, the expression of two cDNAs encoding feedback inhibition-insensitive SATs of Arabidopsis thaliana significantly increased productivity (1700 mg L−1) (Takagi et al., 1999a).

Corynebacterium glutamicum, Brevibacterium lactofermentum, and related bacteria are Gram-positive and are called coryneform glutamic acid-producing bacteria. Their derivatives have been used in industry for the production of various amino acids by fermentation (Leuchtenberger, 1996). In our previous study, we found that l-cysteine was produced by C. glutamicum having an altered cysE gene of E. coli (290 mg L−1) (Wada et al., 2002). The whole-genome sequencing project was completed in 2002 (Ikeda & Nakagawa, 2003), and two l-cysteine biosynthetic genes, cysE and cysK, are registered in the database as the genes encoding putative SAT and O-acetylsereine sulfhydrolase (OASS) (EC 4.2.99.8), respectively. Although there had been no report on the l-cysteine biosynthesis enzyme(s) of C. glutamicum, we recently cloned and expressed the cysK gene encoding OASS of C. glutamicum, and also purified and characterized the gene product, OASS (Wada et al., 2004).
Hence, in this study, the putative gene (cysE) encoding SAT of *C. glutamicum* was cloned based on its NH₂-terminal amino-acid sequence and then expressed in *C. glutamicum* cells. We also report here the characterization and the physiological role of *C. glutamicum* SAT.

**Materials and methods**

**Microorganisms and plasmids**

The *Corynebacterium glutamicum* wild-type strain used in this study was ATCC 13032 (obtained from the Institute of Molecular and Cellular Biosciences, Tokyo, Japan). Plasmid pBCT (supplied by Ajinomoto Co. Inc., Tokyo, Japan), which contains the bacterial kanamycin-resistant *sacB* gene, was used to express the cysE gene in *C. glutamicum*. High-copy-number plasmid pBCT was constructed by combining the 3.0 kb Sau3AI fragment, which has a thermosensitive replicon from pHM1519 (Miwa et al., 1984) found in *C. glutamicum*, with BamHI-digested pHSG398 (Takara Shuzo, Kyoto, Japan) by ligation. The *E. coli* vector pK18mobscarB (supplied by A. Schäfer) (Schäfer et al., 1994), which contains the bacterial kanamycin-resistant gene and the sacB gene encoding levansucrase of *Bacillus subtilis*, was used for disrupting the cysE gene.

*Escherichia coli* strain DH5α [F λ − Φ80lacZΔM15 Δ(lacZYA argF)U169 deoR recA1 endA1 hsdR17 (rk M15) supE44 thi-1 gyrA96] and plasmid pBluescript II SK+ (Toyobo Biochemicals, Osaka, Japan), which has the bacterial ampicillin-resistant gene, were used to subclone and sequence the cysE gene.

**Culture media**

The media used for growth of *C. glutamicum* were Luria–Bertani (LB) medium containing 1% tryptone, 0.5% yeast extract, and 1% NaCl (pH 7.0), M-CM2G which contained (per liter of distilled water) 5 g of glucose, 10 g of peptone, 10 g of yeast extract, 5 g of NaCl, and 0.2 g of l-methionine (pH 7.0), which contains the bacterial kanamycin-resistant gene and the sacB gene encoding levansucrase of *Bacillus subtilis*, was used for disrupting the cysE gene.

**DNA manipulation and transformation**

The enzymes used for DNA manipulation were obtained from Takara Shuzo. *Corynebacterium glutamicum* cells were transformed by electroporation essentially as described previously (Liebl et al., 1989). Southern blot analysis was carried out using ECL direct nucleic acid labelling and detection systems based on enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

**Assay of SAT activity**

*Corynebacterium glutamicum* cells were grown in 5 mL of LB, M-CM2G or MM medium at 30 °C with shaking to the stationary phase, washed with 200 mM Tris-HCl (pH 8.0) containing 1 mM ethylenediamine tetra-acetic acid (EDTA), and resuspended in the same buffer. The supernatants obtained after disruption of the cells by sonic oscillation and centrifugation at 20,000 g for 20 min were used as enzyme sources. The enzyme activity of SAT was assayed at 30 °C by monitoring the cleavage of the thioester bond of acetyl-CoA as described previously with some modifications (Denk & Böck, 1987). The initial rate of the decrease in absorbance at 232 nm of the reaction mixture (final volume, 1 mL) containing 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 1 mM l-serine, 0.1 mM acetyl-CoA, and enzyme solution was measured, and that obtained for a solution containing all the materials except l-serine (blank) was subtracted. The reaction rate was calculated using the differential extinction coefficient between acetyl-CoA and CoA of 3.2 mM cm⁻¹. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol CoA min⁻¹. Protein concentrations were determined using a Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin as the standard protein.

**NH₂-terminal amino-acid sequence analysis of SAT**

All procedures were performed at 0–4 °C in 200 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. Starting from 40 g (wet weight) *C. glutamicum* cells, the enzyme having SAT activity was partially purified after two successive purification steps, including Q-Sepharose (Amersham Biosciences) and MonoQ (Amersham Biosciences) column chromatography (Fig. 1). The NH₂-terminal amino-acid sequence of the enzyme was determined by automated Edman degradation using a model 476A protein sequencer (Applied Biosystems, Foster City, CA).

**Construction of plasmids for expression of the cysE gene in *Corynebacterium glutamicum***

The DNA fragments containing different lengths of the 5'-flanking region and the coding region of the cysE gene were prepared by polymerase chain reaction (PCR) with genomic DNA of *C. glutamicum* ATCC 13032 and oligonucleotide primers. The forward primers were 5'-CTG CGT CGA CGA CTG TTA ACC ACT CAA GCT C-3', 5'-ATG CAA GTC GAC GTTT TCA TGT CTC AAG GTC GG-3', 5'-AGA TCT


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positions of the SalI sites), and the reverse primer was 5' - ACC AAT CTA GAT TCT TAA ATG TAA TAG TCC GGA TGC AC-3' (the underlined sequence is the position of the XbaI site). The unique amplified bands corresponding to 752, 708, 641, 630, 602, 582, and 567 bp were digested with SalI and XbaI, then ligated to into the SalI and XbaI sites of pBCT to construct the expression plasmids (pBCTsp-0, pBCTsp-1, pBCTsp-2, pBCTsp-3, pBCTsp-4, pBCTsp-5, and pBCTsp-7) (Fig. 2).

**Disruption of the cysE gene in *Corynebacterium glutamicum***

The internal DNA fragment of the cysE gene was prepared by PCR with genomic DNA of *C. glutamicum* ATCC 13032 and oligonucleotide primers 5' - CGC GGA TTC GCC GAT-3', 5' - CGC ACG TGC AGT TAA GTC ACG CT-3', and 5' - CTG CGT CTA CAT GCT GCC ATG CA-3'(the underlined sequence are the KEGG accession number for the cysE gene and the termina-

The GenBank accession numbers for the *C. glutamicum* cysE gene, the *E. coli* cysE gene, and plasmid pHM1519 are AX405283, M15745, and AB027714, respectively. The KEGG accession number for the *Mycobacterium*

**Data deposition**

The GenBank accession numbers for the *C. glutamicum* cysE gene, the *E. coli* cysE gene, and plasmid pHM1519 are AX405283, M15745, and AB027714, respectively. The KEGG accession number for the *Mycobacterium*
_tuberculosis_ cysE gene and PIR™ accession number for the _Bacillus stearothermophilus_ cysE genes are MT2398 and E53402, respectively.

**Results and discussion**

**Amino-acid sequence analysis of the _Corynebacterium glutamicum_ SAT**

A basic local alignment search tool search (Altschul et al., 1990) of protein databases revealed that _Corynebacterium glutamicum_ contains one open reading frame consisting of 183 amino acids that is 54% identical to the SATs from _M. tuberculosis_ and _B. stearothermophilus_. In addition, the deduced sequence shared 37% identity to the _E. coli_ SAT, within the overlapping region of 182 amino acids (Ser80-Asn261). However, another possible start codon (ATG), found 18 bp upstream of the predicted start codon, was capable of encoding a polypeptide of 189 amino acids.

After two chromatographic steps (Fig. 1), the NH₂-terminal amino-acid sequence of the enzyme having SAT activity was determined as Met-Leu-Ser-Thr-Ile-Lys-Met-(Ile-Arg-Glu-Asp-Leu). This sequence was the same as the NH₂-terminal sequence of the open reading frame consisting of 189 amino acids. We therefore concluded that the gene (cysE), containing an open reading frame of 570 bp, encodes a SAT enzyme of _C. glutamicum_.

**Characteristics of the _Corynebacterium glutamicum_ cysE Gene**

To examine the function of the _C. glutamicum_ cysE gene, expression plasmid pBCTsp-0, harboring the complete coding sequence with a putative promoter region (177 bp upstream of the start codon) was introduced into _C. glutamicum_ ATCC 13032. As shown in Fig. 2, the SAT activity of _C. glutamicum_ carrying pBCTsp-0 was 12-fold higher than that of _C. glutamicum_ having the vector used (pBCT), and this difference was attributable to the gene dosage effect. This result indicates that the cysE gene encodes the _C. glutamicum_ SAT.

Next, we attempted to identify the promoter region responsible for expression of the cysE gene. Seven plasmids containing different lengths of the 5'-flanking region and the coding region of the cysE gene (pBCTsp-1–pBCTsp-7) (Fig. 2) were introduced into _C. glutamicum_ ATCC 13032, and the SAT activity in the cell extracts was then examined by culturing the transformants obtained (Fig. 3). The cells carrying pBCTsp-1 and pBCTsp-5 had high levels of SAT activity, equivalent to that of the cells carrying pBCTsp-0. In the case of pBCTsp-2, pBCTsp-3, and pBCTsp-4, the SAT activity was slightly higher than that of the cells carrying pBCTsp-0. However, little SAT activity was detected in the transformant carrying plasmid pBCTsp-6 (deletion up to −15); the level was almost the same as those of pBCTsp-7 (deletion from −177 to −1) and of the vector only. These results suggest that the promoter sequences for the cysE gene were located between −63 and −15 upstream from the start codon.

In the consensus _C. glutamicum_ promoter, the prominent feature is a conserved extended −10 region tggntA(c/t)aaTgg, while the −35 region is much less conserved (Pátek et al., 2003). Thus, the −35 region seems to play a marginal role in the function of a considerable proportion of the _C. glutamicum_ promoters. Instead, an extended −10 region appears more responsible for the recognition of the promoter by RNA polymerase. Although transcriptional analysis of the cysE gene was not carried out because of the technical problem, deletion analysis of the 5'-noncoding region showed a putative −10 region (−27TAAAGT−22 or −26TAAGTC−21) and a possible ribosome-binding site (−12AGA−10) just upstream from the start codon (Fig. 2).

**Regulation of the _Corynebacterium glutamicum_ SAT**

L-cysteine biosynthesis in wild-type strains of _E. coli_ and _Salmonella typhimurium_ is strictly regulated through feedback inhibition of SAT by l-cysteine (Kredich, 1983; Soda, 1987). We analyzed the feedback inhibition of the _C. glutamicum_ SAT activity by l-cysteine using cell extracts (Fig. 4a). Activity decreased significantly in the presence of l-cysteine. The concentration of l-cysteine needed to achieve 50% inhibition was 2.0 μM, the same order of magnitude as with the _A. thaliana_ SAT-c (1.8 μM) (Noji et al., 1998) and the _E. coli_ SAT (6.0 μM) (Takagi et al., 2003).
in the presence of either L-methionine or L-cysteine (Fig. 4b). The activity was low in cells grown in M-CM2G medium (about 22% and 20% of the level in the absence of L-methionine or L-cysteine, respectively). However, the addition of L-cysteine to the medium did not affect the expression of the C. glutamicum SAT. These results indicate that L-methionine repressed the expression of C. glutamicum SAT.

In E. coli and S. typhimurium, L-cysteine down-regulates the sulfate reduction pathway by inhibiting the synthesis of O-acetyl-L-serine, which isomerizes to N-acetyl-L-serine. N-acetyl-L-serine, together with the transcriptional activator CysB, acts as an inducer of the gene for enzymes in the sulfate reduction pathway (Kredich, 1983). To examine the regulation of the C. glutamicum cysE gene at the transcriptional level, C. glutamicum ATCC 13032 harboring pBCTsp-0 was cultivated in M-CM2G or MM liquid medium, and the SAT activities in the cell extracts were then examined (Fig. 4b). The activity was low in cells grown in M-CM2G medium (5.7 mU mg\(^{-1}\)) and high in cells grown in MM medium (33.6 mU mg\(^{-1}\)). This finding was in accordance with our previous results that the SAT activities from C. glutamicum and C. glutamicum cysE, which, respectively, encode an aporpressor and a methionine adenosyltransferase (the corepressor), mediate the enzyme repression of the L-methionine regulon. However, SAT gene repression by L-methionine has not yet been discovered in any organisms. Recently, the McbR protein (Rey et al., 2003, 2005) and an open reading frame Ncgl2640 (Mampel et al., 2005) were identified as the putative transcriptional regulator that controls the synthesis of sulfur containing amino acids in C. glutamicum. In particular, the transcriptional repressor McbR, a member of the TetR-family, directly regulates the expression of a set of genes involved in L-cysteine biosynthesis such as the cysK gene, and sulfur reduction, such as the cysN, cysD, cysH, cysI, and cysJ genes (Rey et al., 2005). Bioinformatic analysis also identified the inverted repeat motif as a potential McbR-binding site in front of the target genes (Rey et al., 2005). As shown in Fig. 4b, 1-methionine was shown to repress cysE expression in a strain carrying pBCTsp-0. However, the SAT activity of strains carrying pBCTsp-0 and pBCsp-1 is lower than that of strains carrying pBCTsp-2, pBCTsp-3, and pBCTsp-4 (Fig. 3), raising the possibility that the DNA region between −141 and −91 upstream from the start
codon might be inhibitory for transcription, e.g. by containing the McbR repressor-binding site. We therefore tested the effect of L-methionine in strains carrying pBCTsp-1, pBCTsp-2, pBCTsp-3, pBCTsp-4, and pBCTsp-5. In a similar manner as pBCTsp-0 (7.4 mU mg⁻¹), 0.5 mM L-methionine repressed cysE expression in these strains (6.6, 7.1, 6.6, 6.4, and 6.4 mU mg⁻¹, respectively). The predicted McbR binding motif (5'-[T/C][A/G][G/T/A]AC-N(6)-GT[C/A/T][T/C][A/G]-3') (Rey et al., 2005) was not found within the sequence between -141 and -91 upstream of the start codon. Although the transcription initiation and termination sites should be accurately mapped in vivo, our results suggest that a novel transcriptional regulation system, not the system controlled by McbR, is involved in the C. glutamicum cysE gene expression. From these observations, it appears that the C. glutamicum SAT is regulated at both the transcriptional and posttranslational levels.

**Physiological role of the Corynebacterium glutamicum SAT**

To further examine the role of the C. glutamicum SAT in L-cysteine biosynthesis, we disrupted the cysE gene by a single crossover, as described in Materials and methods. The putative disruptant KCP01 from several kanamycin-resistant transformants was used for genomic Southern blot analysis to confirm the cysE disruption. Genomic DNAs prepared from the cysE-disrupted and wild-type strains (KCP01 and ATCC 13032, respectively) were completely digested with HindIII and analyzed by Southern hybridization (Fig. 5). When the PCR product containing the cysE gene was used as a probe, a 2.0 kb fragment from the intact cysE gene was detected in the wild-type strain. Meanwhile, two bands, corresponding to 7.0 and 1.0 kb fragments, were observed for the disruptant. Disruption of the cysE gene was also confirmed by the measurement of SAT activity in cell
extracts (Fig. 6a). The wild-type strain, ATCC 13032, exhibited SAT activity at a significant level, while SAT activity was hardly detected in the cysE-disrupted strain, KCP01.

As shown in Fig. 6b, strain KCP01 failed to grow on MM only or MM supplemented with L-methionine, although it was capable of growing on MM medium in the presence of 0.5 mM L-cysteine. When high-copy-number plasmid pBCTsps harboring the cysE gene was introduced into strain KCP01, the transformants showed restored activity of the L-cysteine-auxotrophic phenotype (data not shown). These results obviously indicate that C. glutamicum cells synthesize L-cysteine from L-serine through the pathway constituted with SAT and OASS in a manner similar to that of enteric bacteria (E. coli and S. typhimurium) and of higher plants (Kredich, 1996; Hell, 1997). Some microorganisms, such as S. cerevisiae and Pseudomonas aeruginosa, have been reported to synthesize L-cysteine from L-methionine through reverse trans-sulfuration from L-homocysteine to L-cysteine (Brzywczy & Paszewski, 1993; Foglino et al., 1995). It is unlikely that this microorganism catalyzes this reverse trans-sulfuration.

The L-methionine biosynthetic pathway in C. glutamicum has been reported in considerable detail (Lee & Hwang, 2003). To our knowledge, the present study is the first to report the functional analysis of the cysE gene coding for SAT of C. glutamicum. In the course of our study of the construction of E. coli overproducing l-cysteine, we demonstrated that proteins with L-cysteine desulfhydrase (CD) activity play an important role in L-cysteine degradation in E. coli cells (Nakamori et al., 1998; Awano et al., 2003, 2005). To further improve L-cysteine production, a host strain having a lower level of CD activity must be constructed. We recently identified the C. glutamicum CD as the accD gene product, a C-S lyase with \( \alpha, \beta \)-elimination activity (Wada et al., 2002). When the E. coli cysE gene encoding altered SAT, which was less sensitive to feedback inhibition, was expressed in the accD-disrupted strain, the transformed cells produced L-cysteine plus L-cystine (290 mg L\(^{-1}\)) (Wada et al., 2002). The altered C. glutamicum SAT, which is desensitized to feedback inhibition by L-cysteine and is constitutively expressed with derepression by L-methionine, would be necessary for overproduction of L-cysteine in C. glutamicum. Such engineering of the C. glutamicum cysE gene is currently under way.

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