PCR random mutagenesis into *Escherichia coli* serine acetyltransferase: isolation of the mutant enzymes that cause overproduction of L-cysteine and L-cystine due to the desensitization to feedback inhibition

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Abstract PCR random mutagenesis in the *cysE* gene encoding *Escherichia coli* serine acetyltransferase was employed to isolate the mutant enzymes that, due to a much less feedback inhibition by L-cysteine, cause overproduction of L-cysteine and L-cystine in the recombinant strains. The L-cysteine auxotrophic and non-utilizing *E. coli* strain was transformed with plasmids having the altered *cysE* genes. Then, several transformants overproducing L-cysteine were selected by detecting the halo formation of the L-cysteine auxotroph. The production test of amino acids and analysis of the catalytic property on the mutant enzymes suggest that the carboxy-terminal region of serine acetyltransferase plays an important role in the desensitization to feedback inhibition and the high level production of L-cysteine and L-cystine.

Key words: L-Cysteine overproduction; Feedback inhibition; Random mutagenesis; Serine acetyltransferase

1. Introduction

L-Cysteine is an important amino acid in terms of its industrial application for pharmaceuticals, foods and cosmetics. Due to feedback inhibition by L-cysteine of serine acetyltransferase (SAT: EC 2.3.1.30), which consists of 273 amino acid residues and catalyzes the formation of *o*-acetyl-L-serine from acetyl-CoA and L-serine and to repression of a series of enzymes involved in sulfide reduction from sulfate by L-cysteine [1], a high level microbial production of L-cysteine from glucose has not been successfully achieved. To obtain L-cysteine producers, we recently constructed *Escherichia coli cysE* genes encoding altered SAT, which was genetically desensitized to feedback inhibition by L-cysteine, by replacing the methionine residue at position 256 with 19 other amino acid residues using site-directed mutagenesis [2]. We found that, in the recombinant strains carrying the mutant *cysE* gene, there was a marked production of L-cysteine plus L-cystine (maximum 790 mg/l for the Met-256-Ala mutant SAT) and we demonstrated that stable expression of feedback inhibition-insensitive SAT is necessary for overproduction of L-cysteine. However, the mutant SAT was maximally desensitized by a factor of 15 compared to that of the wild-type (concentration for 50% inhibition: 100 μM for the mutant SAT versus 6 μM for the wild-type). Further improvement of the L-cysteine production was therefore expected by use of the mutant SAT with much less feedback inhibition by L-cysteine. Although SATs from bacteria and higher plants have been purified and characterized [3–12], the three-dimensional structure which is useful for engineering SAT has not yet been determined.

In the present study, we attempted to isolate the mutant SATs that produce large amounts of L-cysteine plus L-cystine by using error-prone PCR random mutagenesis into the *cysE* gene and found that several amino acid residues in the carboxy-terminal region showed higher levels of desensitization and of overproduction of these amino acids.

2. Materials and methods

2.1. Materials

An *E. coli* L-cysteine auxotrophic and non-utilizing strain JM39-8 (F^+ *cysE51 tfr-8*) [2] was used as a host for transformations of plasmids having altered *cysE* genes and also as an indicator for screening the L-cysteine-producing transformants. The plasmid vector pUC19 (Takara Shuzo, Kyoto, Japan) was used for cloning and expression of the *cysE* gene. A plasmid pCE harboring the wild-type *cysE* gene was used as the template DNA for PCR random mutagenesis. All enzymes for DNA manipulations were obtained from Takara Shuzo and used under conditions recommended by the supplier.

2.2. PCR random mutagenesis

Fig. 1 shows the strategy used to construct the plasmid carrying the altered *cysE* gene. Random mutagenesis of the *cysE* gene was carried out using the PCR technique [13]. To introduce random mutagenesis into the wild-type gene containing the putative promoter and terminator regions by PCR, plasmid pCE was prepared and forward and reverse primers were designed based on the nucleotide sequences outside of the gene, as determined by Denk and Bock [5]. The forward primer was 5′-GGGAATTCATCGCTTCGGCGTTGAAA-3′ (the underlined sequence shows the position of a *Eco*RI site) and the reverse primer was 5′-GGCTCTAGAAGCGGTATTGAGAGA-GATTA-3′ (the underlined sequence shows the position of a *Xba*I site). 10 ng of pCE DNA was added as a template to a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 10 mM β-mercaptoethanol, 10 μl DMSO, 1 mM each dGTP, dCTP and dTTP, 0.2 mM dATP, 0.5 μM each of the two primers and 1 μl of Ex Taq DNA polymerase (5 U/μl) and enough distilled water to bring the total volume to 100 μl. 25 Cycles (94°C for 1 min, 55°C for 1 min, 72°C for 3 min) of PCR were carried out with a Gene Amp PCR system 2400 (Perkin-Elmer Applied Biosystems). The unique amplified band of 1117 bp was digested with *Eco*RI and *Xba*I and ligated to the *Eco*RI and *Xba*I sites of pUC19.

2.3. Isolation of L-cysteine-producing transformants

The ligated DNA was used to transform *E. coli* JM39-8 on LB solid medium containing 50 μg/ml ampicillin and the ampicillin resistant colonies were then transferred on M9 agar plates supplemented with 50 μg/ml ampicillin and seeded with the L-cysteine auxotrophic indicator strain *E. coli* JM39-8 carrying pUC19 at 1×10^7 cells/ml. After the plates were incubated at 37°C overnight, L-cysteine-excreting transformants were isolated on the plate by detecting halo formations of *E. coli* JM39-8 cells. The plasmid DNA was purified from each transformant and the nucleotide sequence of the *cysE* gene was confirmed with a model 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) by using the dideoxy chain termina-

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tion method. C1 medium was used for the production of L-cysteine and L-cystine and contained in 1 l of distilled water 30 g glucose, 2 g KH_2PO_4, 10 g $(NH_4)2SO_4$, 1 g $MgSO_4{\cdot}7H_2O$, 10 mg $FeSO_4{\cdot}7H_2O$, 10 mg $MnCl_2{\cdot}4H_2O$, 20 g $CaCO_3$ (added after sterilizing separately) and 100 mg each of L-isoleucine, L-leucine, L-methionine and glycine. Adding these amino acids was found to effectively reverse the growth inhibition caused by L-cysteine [2]. The pH was adjusted to 7.0 with KOH. When appropriate, ampicillin (50 μg/ml) was added to the medium. For the production of the amino acids, a loopful of cells cultured for 24 h on LB solid medium containing ampicillin at 30°C was inoculated into 20 ml of C1 medium in 500 ml flasks and cultured at 30°C for 72 h on a reciprocal shaker at 120 strokes per min.

2.4. Determination of L-cysteine and L-cystine

The amounts of L-cysteine and L-cystine were determined by a microbioassay using *Pediococcus acidilactici* (formerly classified as *Leuconostoc mesenteroides*) IFO 3076 [14]. The strain was confirmed to respond equally to L-cysteine and L-cystine. 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 dissolving L-cystine with 0.5 N HCl.

2.5. Assay of SAT activity

For the determination of SAT (EC 2.3.1.30) activities, cells were grown in 20 ml of C1 medium at 30°C for 72 h to the stationary phase, washed with 50 mM Tris-HCl buffer (pH 7.5) and resuspended in 4 ml of the same buffer containing 2 mM dithiothreitol. The supernatants obtained after the disruption of the cells by ultrasonication and centrifugations at $30\,000\times g$ for 30 min were used as enzyme sources. SAT activity was assayed by following the decrease in A_{232} of the reaction mixture in a final volume of 1 ml containing 50 μmol of Tris-HCl (pH 7.6), 1 μmol of L-serine, 0.1 μmol of acetyl-CoA and enzyme solution, at 30°C, as described by Denk and Bock [5]. Protein concentrations were determined using a Bio-Rad Protein Assay kit (Hercules, CA, USA) and bovine serum albumin as the standard.

3. Results

3.1. Isolation of mutant SATs that cause L-cysteine production by PCR random mutagenesis

To introduce random mutagenesis into the wild-type *cysE* gene containing the putative promoter and terminator regions, error-prone PCR was carried out with pCE as a template and with two primers as described in Section 2. From approximately 400 transformants exhibiting ampicillin resistance, 280 colonies were found to grow on M9 agar plates, indicating that the mutant *cysE* gene in the transformant complemented the cysteine auxotrophy of host strain JM39-8. Among them, 37 halo-forming colonies caused by excretion of L-cysteine into the medium were then isolated on the same plates containing L-cysteine auxotrophic indicator (JM39-8 cells carrying pUC19). The cells carrying the wild-type *cysE* gene, due to the feedback inhibition of SAT protein by L-cysteine, did not form a halo. Recombinant plasmids were extracted from these halo-forming colonies and the DNA sequences of the insert fragments were determined. A total of 25 types of amino acid substitutions in SAT were then independently identified (Table 1). Multiple amino acid substitutions due to hypermutagenic PCR were also found. It is worth noting that in one of the mutants, Val was substituted for Met at position 256, where amino acid residues could be replaced to obtain a higher level of desensitization to feedback inhibition [2].

3.2. Selection of the L-cysteine-overproducing strains

We then compared the abilities of 24 mutants, except the Met-256-Val, to form halos on a M9 agar plate containing L-cysteine auxotrophic indicator, as shown in Fig. 1. The halo sizes are considered to be related to the ability of cells to produce L-cysteine, which accumulates large amounts of L-cysteine in the cell and, subsequently, through excretion outside the cell. Selecting for a halo size almost equivalent to that of the Met-256-Ala mutant, which accumulated a maximum of 790 mg/l L-cysteine and L-cystine [2], seven transformants carrying the Asn-51-Lys/Arg-91-His/His-233-Tyr, Glu-166-Gly/Met-201-Val, Thr-167-Lys, Met-201-Arg, Met-201-Thr, Pro-252-Arg and Ser-253-Leu mutant SAT, respectively, were selected as the L-cysteine-overproducing strains. It

Table 1
Summary of amino acid and DNA substitutions in the *E. coli* mutant SAT, obtained by PCR random mutagenesis and screening for L-cysteine production

Mutant	Amino acid substitution (base substitution)			
1	E-7-V (A→T)	L-27-P (T→C)	S-43-R (T→A)	D-271-G (A→G)
2	E-7-D (A→T)	F-131-L (T→A)	P-232-L (C→T)	
3	N-12-I (A→T)			
4	N-12-I (A→T)	R-197-H (G→A)		
5	A-17-D (C→A)	Q-258-P (A→C)		
6	T-19-A (A→G)	C-23-W (T→G)		
7	E-24-K (G→A)	L-36-F (C→T)		
8	S-29-C (A→T)			
9	N-40-S (A→G)	L-120-W (T→G)		
10	M-48-V (A→G)			
11	N-51-K (C→A)	R-91-H (G→A)	H-233-Y (C→T)	
12	E-68-V (A→T)			
13	W-119-X (G→A)			
14	A-127-T (G→A)	V-130-G (T→G)		
15	V-138-M (G→A)			
16	E-166-G (A→G)	M-201-V (A→G)		
17	T-167-K (C→A)			
18	D-173-N (G→A)			
19	D-173-G (A→G)	G-270-R (G→A)		
20	M-201-R (T→G)			
21	M-201-T (T→C)			
22	Q-228-P (A→C)			
23	P-252-R (C→G)			
24	S-253-L (C→T)			
25	M-256-V (A→G)			

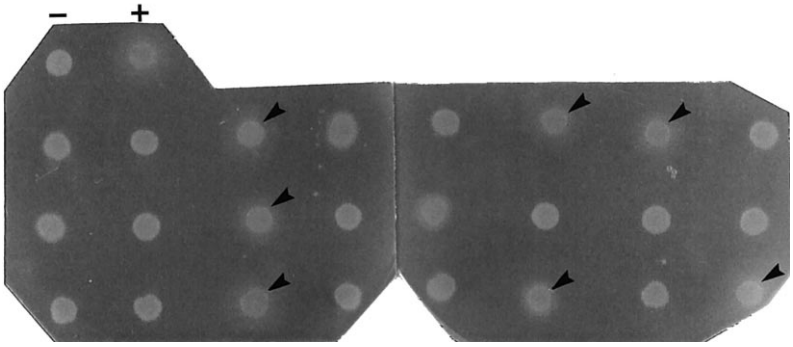

Fig. 1. Halo formation of the L-cysteine auxotroph by the recombinant strain carrying the altered *cysE* gene. Each strain was inoculated on a M9 agar plate containing the L-cysteine auxotrophic strain as described in Section 2. The plate was incubated at 37°C overnight. Minus is a negative control with cells harboring the wild-type *cysE* gene and plus is a halo-forming positive control with cells expressing the Met-256-Ala mutant SAT. Arrows represent the L-cysteine-overproducing strains carrying the mutant *cysE* gene.

should be emphasized that most of the amino acid substitutions in the L-cysteine-overproducing strains were located in the carboxy-terminal region of SAT.

3.3. L-Cysteine and L-cystine production and catalytic properties by the strains carrying the mutant cysE genes

To determine production of L-cysteine plus L-cystine and SAT activity, seven transformants carrying the mutant *cysE* gene were cultured in C1 medium at 30°C for 72 h (Table 2). Plasmids pCE and pCEM256A [2], which harbor the *E. coli* wild-type and Met-256-Ala mutant *cysE* gene, respectively, were used as control enzymes. It was found that in the recombinant strains carrying the mutant *cysE* gene, except that harboring pHC252R, there was a marked production of L-cysteine and L-cystine (200–990 mg/ml), while no L-cysteine or L-cystine was detected in JM39-8 harboring the wild-type *cysE* gene. In particular, the Met-201-Arg, Met-201-Thr and Ser-253-Leu mutant SATs showed a L-cysteine and L-cystine production almost equivalent to that of the Met-256-Ala mutant SAT.

We tested SAT activities in the recombinant strains during the stationary phase of growth (Table 2). The enzymes in the soluble fraction obtained from sonicated cells were assayed without further purification. The level of activity of the Met-256-Ala mutant SAT was 24% even in the presence of 100 μM L-cysteine, while the wild-type SAT was markedly inhibited by L-cysteine, as we reported previously [2]. On the other hand, the levels of activity of the mutant enzymes were approximately 20–80%, even in the presence of 100 μM L-cysteine, although significant decreases in enzymatic activity occurred in all cases of mutant SATs. These results indicate that the carboxy-terminal region of SAT plays an important role in the desensitization to feedback inhibition by L-cysteine and in the overproduction of L-cysteine and L-cystine.

We further investigated the degree of contribution of each substitution in the mutants having multiple amino acid substitutions to L-cysteine plus L-cystine production and feedback inhibition by L-cysteine. Two plasmids, pHC51K/91H/233Y and pCE, were digested with *Eco*RI and *Bst*PI and with *Bst*PI and *Xba*I and then, a small fragment of pHC51K/91H/233Y and a large fragment of pCE were mixed, ligated and introduced into *E. coli* JM39-8 to construct pHC51K/91H (carrying the Asn-51-Lys and Arg-91-His mutations) and pHC233Y (carrying the His-233-Tyr mutation), respectively. It should be noted that the Asn-51-Lys and Arg-91-His mutations were not separated because these substitutions were located in the amino-terminal region of SAT. In a manner similar to that above, pHC166G/201V and pCE were digested with *Eco*RI and *Bst*PI and with *Bst*PI and *Xba*I and then, a small fragment of pHC166G/201V and a large fragment of pCE were mixed, ligated and introduced into JM39-8 to construct pHC166G (carrying the Glu-166-Gly mutation) and pHC201V (carrying the Met-201-Val mutation), respectively. Transformants harboring these resultant plasmids were cultivated in C1 medium

Table 2
Production of L-cysteine plus L-cystine and catalytic properties of the *E. coli* mutant SATs

Plasmid	Amino acid substitution	L-Cysteine plus L-cystine produced (mg/l)	SAT activity (U/min/mg)	Activity remaining in the presence of 100 μM L-cysteine (%)
pCE	Wild-type	ND[a]	2.76 ± 0.49	0.9
pCE M256A	M-256-A	790 ± 380	0.38 ± 0.19	24.1
pHC 51K/91H/233Y	N-51-K/R-91-H/H-233-Y	210 ± 170	0.16 ± 0.04	51.9
pHC 166G/201V	E-166-G/M-201-V	330 ± 70	0.06 ± 0.03	78.2
pHC 167K	T-167-K	260 ± 50	0.43 ± 0.14	37.1
pHC 201R	M-201-R	990 ± 200	0.41 ± 0.06	20.9
pHC 201T	M-201-T	740 ± 120	0.38 ± 0.08	16.3
pHC 252R	P-252-R	50 ± 20	0.37 ± 0.10	33.2
pHC 253L	S-253-L	960 ± 460	0.41 ± 0.12	28.9

The data shown are means ± S.D. from three independent experiments.
Each plasmid was introduced into JM39-8 with a defective chromosomal *cysE* gene.
Transformants were cultivated in C1 medium at 30°C for 72 h for amino acid production and soluble fractions from the cells were used as enzyme sources.
[a]ND, not detected.

Table 3
Effect of an individual amino acid substitution in the *E. coli* SAT on production of L-cysteine plus L-cystine and feedback inhibition by L-cysteine

Plasmid	Amino acid substitution	L-cysteine plus L-cystine produced (mg/l)	SAT activity remaining in the presence of 100 μM L-cysteine (%)
pHC 51K/91H/233Y	N-51-K/R-91-H/H-233-Y	210 ± 170	51.9
pHC 51K/91H	N-51-K/R-91-H	ND[a]	0.0
pHC 233Y	H-233-Y	ND	2.5
pHC 166G/201V	E-166-G/M-201-V	330 ± 70	78.2
pHC 166G	E-166-G	ND	47.6
pHC 201V	M-201-V	810 ± 150	33.3

The data shown are means ± S.D. from three independent experiments.
[a]ND, not detected.

to examine the amino acid production and SAT activity (Table 3). It was found that the substitution at position 201 was greatly involved in the production of L-cysteine and L-cystine, while double mutations (Glu-166-Gly and Met-201-Val) had a significant influence on the desensitization to feedback inhibition. Interestingly, in the case of triple mutations (Asn-51-Lys, Arg-91-His and His-233-Tyr), an individual substitution was unlikely to make a contribution to both characteristics. These results suggest that several amino acid residues in a position other than position 256 in the carboxy-terminal portion of the *E. coli* SAT protein are responsible for the overproduction of L-cysteine and L-cystine as well as for the feedback inhibition by L-cysteine.

4. Discussion

In this study, we attempted to identify positions in the *E. coli* wild-type SAT where amino acid residues could be replaced to obtain a higher level of L-cysteine plus L-cystine production as well as a desensitization to feedback inhibition by L-cysteine. Through PCR random mutagenesis into the *cysE* gene, the desired amino acid substitutions mainly occurred at various positions including position 256, which was previously found to be involved in feedback inhibition [2,5], in the carboxy-terminal part of the SAT protein. Recently, Mino et al. showed that the truncated SAT deleting 20 amino acid residues, 254–273, was much less sensitive to feedback inhibition than the wild-type enzyme, nor did it form a complex with *o*-acetylserine sulfhydrylase (EC 4.2.99.8), which converts *o*-acetylserine and sulfide to L-cysteine and acetate [15]. The prediction of secondary structures of the carboxy-terminal region of the wild-type SAT also indicated that the region from Met-254 to Gly-262 forms an α-helix and the region from Ile-263 to Tyr-269 forms a β-sheet [15]. On the basis of these observations, the carboxy-terminal region downstream of Met-254 is considered to be involved in feedback inhibition by L-cysteine and in complex formation with *o*-acetylserine sulfhydrylase. The notable finding in this study is that positions 166, 167, 201, 233, 252 and 253 also play an important role in the L-cysteine recognition or the binding site for the feedback inhibition that causes overproduction of L-cysteine and L-cystine. In particular, three independent mutant SATs at position 201 were isolated, suggesting that replacement of the methionine residue at this position in wild-type SAT with other amino acids may bring about uniform desensitization to feedback inhibition. Another surprising feature is that the overproduction as well as the feedback desensitization did not occur when the mutations were divided into two parts (Asn-51-Lys/Arg-91-His and His-233-Tyr). It is feasible to speculate that these residues are closely located in the tertiary structure and that three amino acid substitutions may cause the structural change around the regulatory domain in the *E. coli* SAT protein.

The clear relationship between the degree of feedback desensitization in the mutant SAT protein and the level of L-cysteine plus L-cystine production is not discussed herein. Constructing a potent microorganism for metabolite production requires solving the problem that both the key enzyme, such as SAT, and the subsequent enzyme involved in the biosynthesis, such as *o*-acetylserine sulfhydrylase, need to be elevated by a gene-dosage effect with a good balance in order to optimize the metabolic flow for the formation of the target metabolite. In such a case, the stable and balanced expression of several different genes in the host cells is essential [16]. A stable supply derived from genetically engineered systems of precursors for L-cysteine, such as sulfide and L-serine, might enhance further the L-cysteine production. However, L-cysteine plus L-cystine were only negligibly produced by the *E. coli* strains carrying the Pro-252-Arg and Glu-166-Gly mutant SAT gene, despite the fact that these SAT activities were more resistant than the Met-256-Ala mutant to feedback inhibition by L-cysteine. In fact, pHC252R and pHC166G, the plasmids having these mutant SAT genes, gradually disappeared during cultivation (data not shown). It is therefore probable that the instability of these plasmids in particular would be responsible for very little productivity.

Further, the feedback-insensitive mutant SATs showed a very large decrease in enzymatic activity relative to that of the wild-type enzyme. Although analysis of the mRNA level and purification of each enzyme were not carried out, it is probable that the lower enzymatic activities of the feedback-insensitive SATs were mainly caused by a lower efficiency of transcription relative to that of the wild-type enzyme.

Much more research on the catalytic mechanism of SAT conducted with purified enzymes is needed and a determination of the tertiary structure of the *E. coli* wild-type SAT is also essential to elucidate the mechanism involved in L-cysteine recognition and the binding site for feedback inhibition.

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