Identification, Cloning, and Sequencing of the Genes Involved in the Conversion of D,L-2-Amino-2-thiazoline-4-carboxylic Acid to L-Cysteine in Pseudomonas sp. Strain ON-4a

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The newly isolated strain Pseudomonas sp. ON-4a converts D,L-2-amino-2-thiazoline-4-carboxylic acid to L-cysteine via N-carbamoyl-L-cysteine. A genomic DNA fragment from this strain containing the genes encoding enzymes that convert D,L-2-amino-2-thiazoline-4-carboxylic acid into L-cysteine was cloned in Escherichia coli. Transformants expressing cysteine-forming activity were selected by growth of an E. coli mutant defective in the cysB gene. A positive clone, denoted CM1, carrying the plasmid pCM1 with an insert DNA of approximately 3.4 kb was obtained, and the nucleotide sequence of a complementing region was analyzed. Analysis of the sequence found two open reading frames, ORF1 and ORF2, which encoded proteins of 183 and 435 amino acid residues, respectively. E. coli DH5α harboring pTrCM1, which was constructed by inserting the subcloned sequence into an expression vector, expressed two proteins of 25 kDa and 45 kDa. From the analyses of crude extracts of E. coli DH5α carrying deletion derivatives of pTrCM1 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by enzymatic activity, it was found that the 25-kDa protein encoded by ORF1 was the enzyme 2-amino-2-thiazoline-4-carboxylic acid hydrolase, which catalyzes the conversion of L-2-amino-2-thiazoline-4-carboxylic acid to N-carbamoyl-L-cysteine, and that the 45-kDa protein encoded by ORF2 was the enzyme N-carbamoyl-L-cysteine amidohydrolase, which catalyzes the conversion of N-carbamoyl-L-cysteine to L-cysteine.

Key words: Pseudomonas species; gene cloning; D,L-2-amino-2-thiazoline-4-carboxylic acid (D,L-ATC); N-carbamoyl-L-cysteine (NCC); L-cysteine

L-Cysteine is an important amino acid used as a food additive, a component of nutritional infusions and cosmetics, and a chemical reagent. Its derivatives are also used as medicine. L-Cysteine production has been done by extraction from hydrolysates of human hair. However, this method has several problems, such as the bad smell occurring during hydrolysis, a low yield, and a heavy cost borne for waste treatment. A microbial method for L-cysteine production has been investigated using a chemically synthesized substrate, D,L-2-Amino-2-thiazoline-4-carboxylic acid (D,L-ATC).

The bioconversion process from D,L-ATC to L-cysteine by some bacteria such as Pseudomonas thiazolinophilium and Pseudomonas desmolytica was developed and industrialized.1–3) The bioconversion of D,L-ATC is an effective method for the production of L-cysteine. In this conversion, Sano et al. reported that S-carbamoyl-L-cysteine (L-SCC) was formed from D,L-ATC by crude enzymes from Pseudomonas thiazolinophilium,3) and also Ryu and Shin showed a similar result with Pseudomonas sp. CU6.6) They supposed that L-SCC might be probably an intermediate in the bioconversion of D,L-ATC to L-cysteine.

On the other hand, we investigated in detail the process of the conversion of D,L-ATC to L-cysteine with a new strain of Pseudomonas sp. ON-4a isolated by us,20) and found that L-cysteine and N-carbamoyl-L-cysteine (L-NCC) were produced from D,L-ATC by crude enzymes from this strain. Similar results were obtained with ATC- assimilating bacteria, such as Pseudomonas putida AJ3865.21) It therefore appears that L-NCC is an intermediate in the conversion of
d,L-ATC to L-cysteine in these *Pseudomonas* strains. Furthermore, Shiba and Shiba\(^6\) recently reported the isolation of a gene encoding an ATC racemase from *Pseudomonas ovalis* strain BS, which catalyzes the interconversion reaction between d- and l-forms of ATC. These findings suggest that the conversion process of d,L-ATC to L-cysteine in these strains may consist of the following three steps: 1) racemization of d-ATC to L-ATC, 2) hydrolysis of L-ATC to L-NCC by a L-specific ATC hydrolase, and 3) hydrolysis of L-NCC to L-cysteine by a L-specific NCC amidohydrolase.

To investigate the molecular mechanism of this conversion in strain ON-4a, we cloned a DNA fragment containing the genes for the enzymes involved in the conversion, analyzed its nucleotide sequence, and confirmed the participation of ATC hydrolase and NCC amidohydrolase in the conversion of d-ATC to L-cysteine.

**Materials and Methods**

**Bacterial strains, media, and vectors.** *Pseudomonas* sp. ON-4a, isolated previously in our laboratory, was cultivated at 30°C for 20 h in ATC medium containing 0.2% d,L-ATC, 2% glucose, 0.5% yeast extract, 0.5% peptone, 0.25% NaCl, 0.1% K2HPO4, and 0.05% MgSO4・7H2O.\(^7\) The *E. coli* W3110 cysB\(^-\) mutant was obtained from the National Institute of Genetics, Genetic Stock Research Center (Shimizu, Japan). For the complementation test of this mutant, an agar plate of minimal medium, containing 0.7% K2HPO4, 0.2% KH2PO4, 0.01% MgSO4・7H2O, 0.1% (NH4)2SO4, 0.05% sodium citrate・2H2O, and 0.2% glucose and supplemented with 0.3% d,L-ATC and 50 μg/ml of ampicillin (Amp), was used as a selective plate. *E. coli* strains, DH5α and XL-1 Blue, were used for subcloning and expression, and were incubated in Luria broth (LB) medium at 37°C. Strains of *E. coli* carrying the plasmid were grown at 37°C in LB medium containing 50 μg/ml of Amp.

**DNA manipulations.** Plasmid DNA preparation, restriction enzyme digestion, separation of DNA fragments by agarose gel electrophoresis, and DNA ligation were done by standard methods as described previously.\(^7\) Transformation of *E. coli* strains was done by the method of Hanahan.\(^8\)

**Construction of genomic library and screening.** Chromosomal DNA from *Pseudomonas* sp. ON-4a was prepared by the method of Wilson.\(^9\) The DNA was partially digested with Sau3A1 and separated by agarose gel electrophoresis. The 3 to 8-kb DNA fragments were purified from the gel, and ligated into the BamHI site of pUC18 to construct a mini genomic library. *E. coli* W3110 cysB\(^-\) cells were transformed with this library, and spread on selective minimal medium agar plates containing 0.3% d,L-ATC and 50 μg/ml of Amp, and then incubated at 37°C for 3 days. Transformants with the ability to convert d,L-ATC to L-cysteine can form colonies on the plates.

**Nucleotide sequencing and analysis.** The recombinant plasmid pCM1, isolated from a complemented transformant, contains a 3.4-kb Sau3AI fragment that is inserted into the BamHI site of pUC18 and carries the cysteine-forming enzyme genes. A DNA fragment containing the relevant genes was subcloned by double digestion of pCM1 with XbaI and BamHI, single sites for which were present in the pUC18 and in the 3.4-kb insert, respectively. This fragment was treated with SI nuclease to create blunt ends and was then inserted into the Smal site of pBluescript SKII-. The unidirectional deletions of the insert DNA were generated with exonuclease III by the method of Henikoff.\(^10\) *E. coli* XL-1 Blue was transformed with the resulting recombinants. Template DNAs for sequencing were purified by the standard method.\(^7\) The nucleotide sequences of the clones were analyzed for both strands by the dideoxy chain termination method,\(^13\) using a Sequenase Ver. 2.0 kit. Sequences were analyzed with MacDNASIS (Hitachi Software Engineering, Japan). Protein sequence similarity searches of the GenBank and Swissprot databases were performed with the BLAST program of the National Center for Biotechnology Information (NCBI), National Library of Medicine.

**Construction of deletion derivatives.** The blunt-ended 3.4-kb XbaI/BamHI fragment, prepared from pCM1 as described above, was inserted into the Smal site downstream of the trc promoter of the expression vector pTrc99A to construct the plasmid pTrcCM1. As shown in Fig. 1, pTrcCM1 had two SacI and two XhoI sites in the insert DNA. pTrcCM1 was digested with SacI to produce a small fragment of 930 bp and a large fragment containing some vector sequence and parts of the insert sequence. The large fragment DNA was self-ligated to give a deletion plasmid, pTrcCM1S. pTrcCM1X was prepared from pTrcCM1 by deleting the 910-bp XhoI fragment within the insert. Another deletion plasmid, pTrcCM1Bg, was prepared from pTrcCM1 by deletion of the 530-bp BglII/BamHI fragment. The physical structure of these plasmids was verified by restriction enzyme analysis.

**Identification of the gene products.** *E. coli* DH5α harboring the plasmid pTrcCM1 or either of its deletion derivatives was incubated with shaking in 2 ml of LB medium containing 50 μg/ml of Amp at 37°C for...
4 h. The cells were collected by centrifugation, suspended in 2 ml of fresh LB medium and inoculated into 50 ml of LB medium containing 50 μg/ml of Amp. Incubation was continued with shaking at 37°C. Isopropyl-1-thio-β-D-galactoside (IPTG) was added to the culture at a final concentration of 1 mM at an A600 of about 0.6 (after incubation for 3 h), and incubation was continued for another 3 h. The cells were harvested by centrifugation at 5,000 × g for 5 min at 4°C, and then washed twice with cold 50 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA. The washed cells were suspended in 2.0 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM MnSO4 and 10% glycerol, and disrupted by sonication on ice with an Ultrasonic Disrupter UD-201 (TOMY Seiko Co. Tokyo, Japan). The cell debris was removed by centrifugation at 12,000 × g for 10 min. The resulting supernatants were used as the crude extracts. The crude extracts were put on a SDS-polyacrylamide gel according to the method of Laemmli. After electrophoresis, the gel was stained with Coomassie brilliant blue. Standard protein markers used for molecular mass estimation were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (LMW electrophoresis calibration kit, Amersham Pharmacia Biotech, England).

**Partially purification of NCC amidohydrolase and ATC hydrolase.** The crude extract of E. coli DH5α harboring pTrCM1 prepared from 500 ml of the culture broth as described above was used for the purification of NCC amidohydrolase and ATC hydrolase. All procedures were done at 0–4°C. The crude extract (about 530 mg protein) was dialyzed against buffer A (20 mM Tris-HCl (pH 8.0)-10% glycerol) overnight, and put on a DEAE-Cellulofine A-500 (Seikagaku Corp. Tokyo, Japan) column (2.5 × 12.0 cm) equilibrated previously with the same buffer. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of 10–200 mM sodium phosphate buffer (pH 7.5). The enzyme activity was detected in the flowthrough. That was concentrated by ammonium sulfate precipitation (80% saturation), and was used as the purified NCC amidohydrolase (17.0 U/mg protein of specific activity). The ATC hydrolase fraction eluted from the DEAE-Cellulofine A-500 column described above was dialyzed against 10 mM sodium phosphate buffer (pH 7.5)-10% glycerol overnight. The dialyzed solution was put on a Bio-Gel HP column (1.5 × 7.0 cm) equilibrated previously with the same buffer. Elution was done with a linear gradient of 10–200 mM sodium phosphate buffer (pH 7.5). The enzyme activity was detected in eluate with 70 to 100 mM sodium phosphate. The active fraction was pooled and concentrated by ammonium sulfate precipitation (0.89 U/mg protein of specific activity).

**Measurement of enzyme activity.** The activities of cysteine-forming enzymes in the crude extracts were assayed by the measurement of l-cysteine formed from D,L-ATC or L-NCC. The reaction mixture (1 ml) contained crude extract (about 0.1 mg protein), 0.3 M Tris-HCl (pH 8.5), 2.5 mM NH2OH, and 5 mM substrate. It was incubated at 37°C for 10 min, and the reaction was stopped by the addition of 5% trichloroacetic acid. The l-cysteine formed was measured by the Gaitonde method with a modification. One unit of cysteine-forming enzyme activity was defined as the amount of enzyme that produces 1 μmol of l-cysteine/min at 37°C. NCC amidohydrolase activity was measured by formation of l-cysteine from L-NCC. The assay of ATC hydrolase activity was done in the presence of an excess of partially purified NCC amidohydrolase (ATC hydrolase-free), which allowed complete conversion of L-NCC to l-cysteine.

**Analytical methods.** The protein concentration was measured by the method of Lowry et al. using bovine serum albumin as a standard. To analyze the N-terminal amino acid sequences, the 25-kDa (ATC hydrolase) and 45-kDa (NCC amidohydrolase) proteins partially purified as described above were put through SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes (ATTO, Osaka, Japan). The protein bands corresponding to ATC hydrolase and NCC amidohydrolase on the membranes were cut out and ana-
lyzed on a SHIMADZU protein sequencer (Model PPSQ-10) with an on-line phenylthiohydantoin amino acid analyzer (Model LC-10AS) according to the manufacturer's instructions.

Chemicals and enzymes. Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA, USA), Takara Shuzo (Kyoto, Japan), and Nippon Gene (Toyama, Japan). A Sequenase Ver. 2.0 sequencing kit and \([\alpha-^{32}P]dCTP\) were purchased from U.S. Biochemical Corp. (Cleveland, OH, USA) and Amersham Pharmacia Biotech (Buckinghamshire, England), respectively. D,L-ATC and L-NCC were prepared by the method described previously.5 Other biochemicals were obtained from Wako Pure Chemical Co. (Osaka, Japan) and Sigma (St. Louis, MO, USA).

Nucleotide sequence accession number. The nucleotide sequence data described in this paper has been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases and assigned the accession number AB029899.

Results

Cloning of the genes for cysteine-forming enzymes

For the cloning of the genes involved in the conversion of D,L-ATC to L-cysteine in \(Pseudomonas\) sp. ON-4a, we used the \(E. coli\) W3110 cysB\(^-\) mutant, which could not grow on a minimal medium containing D,L-ATC. A partial Sau3AI genomic library of strain ON-4a was constructed as described in “Materials and Methods”. Approximately 30,000 recombinant colonies were screened for the ability to form a colony on a selective plate with D,L-ATC. One positive clone, denoted CM1, was obtained. Crude extracts of CM1 expressed 6–18 mU/mg protein of cysteine-forming enzyme activity, but no activity was detected in crude extracts from \(E. coli\) W3110 cysB\(^-\) carrying the pUC18 vector only (data not shown).

Nucleotide sequence analysis

Clone CM1 had a plasmid, pCM1, containing an insert DNA of 3.4 kb. The complete nucleotide sequence of the 2,488-bp HindIII-BamHI fragment subcloned from the original insert was analyzed. Sequence analysis showed two open reading frames (ORFs), designated ORF1 and ORF2. ORF1 started with an ATG (nucleotides 406 to 408) downstream of a potential ribosome-binding site (nucleotides 393 to 397, GGAGG), and encoded a protein of 183 amino acids with a calculated molecular mass of 20,273. ORF2 was preceded by a possible ribosome-binding site (nucleotides 939 to 942, GGAG) and was predicted to encode a protein of 435 amino acids with a calculated molecular mass of 47,347.

Identification of ATC hydrolase and NCC amidohydrolase

We then examined whether ATC hydrolase and NCC amidohydrolase were indeed encoded by either of the two ORFs. \(E. coli\) DH5\(^{\alpha}\) harboring pTrCM1 containing the XbaI/BamHI fragment of the original 3.4-kb insert had both enzyme activities (Table 1). \(E. coli\) DH5\(^{\alpha}\) harboring no plasmid or pTrc99A (the expression vector with no insert) expressed no activity. These results show that the XbaI/BamHI insert DNA contains cysteine-forming enzyme genes, ATC hydrolase, and NCC amidohydrolase genes.

To identify the gene products of the ORFs 1 and 2, the deletion derivatives of pTrCM1 shown in Fig. 1 were constructed. These deletion derivatives were transferred to \(E. coli\) DH5\(^{\alpha}\), and ATC hydrolase and NCC amidohydrolase activities were measured in crude extracts of these transformants (Table 1). \(E. coli\) harboring pTrCM\(\Delta\)S expressed NCC amidohydrolase activity and no ATC hydrolase activity,
and *E. coli* harboring pTrCM\(D_{\text{G}}\) expressed only ATC hydrolase activity. Neither enzyme activity was detected in crude extracts of *E. coli* harboring pTrCM\(D_{\text{X}}\). A deletion derivative, which deleted an 880-bp *HindIII* fragment of the upstream region of pTrCM1, did not influence the expression of either enzyme activity (data not shown). From these results, it was concluded that the ATC hydrolase and NCC amidohydrolase genes are located in the *HindIII*/*BamHI* fragment, as indicated in Fig. 1.

The molecular masses of the expressed proteins were measured by SDS-polyacrylamide gel electrophoresis (Fig. 2). *E. coli* harboring pTrCM1 had two additional protein bands with apparent molecular masses of 45 kDa and 25 kDa (lane 4, arrows), compared with those harboring no plasmid and pTrc99A (lanes 2 and 3). *E. coli* harboring pTrCM\(D_{\text{S}}\) expressed only the 45-kDa protein (lane 5) and that harboring pTrCM\(D_{\text{G}}\) expressed the 25-kDa and 45-kDa proteins, respectively.

**Comparison of the sequence of NCC amidohydrolase with other N-carbamoyl-L-amino acid amidohydrolases**

It was found with a protein similarity search done using the BLAST program that the predicted amino acid sequence of ORF2 (NCC amidohydrolase) had a significant similarity with six N-carbamoyl-L-amino acid amidohydrolases from other microorganisms. These are the enzymes from *A. aurescence DSM3747*, *B. stearothermophilus NS714A*, *Pseudomonas* sp. NS671, *B. subtilis* (GenBank accession number Z99120), *E. coli* (U82664), and *H. influenzae* (U32740). The enzymes from *A. aurescence, B. stearothermophilus* and *Pseudomonas* sp. NS671 were enzymatically demonstrated to be N-carbamoyl-L-amino acid amidohydrolases involved in the production of L-amino acid from the corresponding hydantoins, whereas the sequences from *B. subtilis, E. coli*, and *H. influenzae* were obtained in the genome sequence projects, and the function of these enzymes was only suggested by sequence comparisons. An alignment of NCC amidohydrolase and six enzymes is shown in Fig. 3. NCC amidohydrolase showed 23.7–28.5% (32.2–39.2%) sequence identity (similarity) to the enzymes from these bacteria. The latter enzymes were more similar to each other (28.5–42.9% identity and 42.0–52.1% similarity) than to the enzyme we studied, indicating that it may have diverged from the others. On the other hand, no significant similarity was found between the deduced amino acid sequence of ORF1 (ATC hydrolase) and that of any known protein in the databases.

**Discussion**

As shown in our previous work, in the conversion of d,L-ATC to L-cysteine in *Pseudomonas* sp. ON-4a and other ATC-assimilating bacteria, L-NCC was detected as an intermediate and L-cysteine was formed from L-NCC. It is thus considered that only L-ATC in d,L-ATC is converted to L-cysteine by two L-specific enzymes of ATC hydrolase and NCC amidohydrolase in these bacterial strains. In this study, we cloned a 3.4-kb DNA fragment from strain ON-4a containing the genes for these two enzymes involved in the conversion of d,L-ATC to L-cysteine, ATC hydrolase, and NCC amidohydrolase.

In the bioconversion of d,L-5′-substituted hydantoins to the corresponding d- and/or L-amino acids, d-amino acids are formed from N-carbamoyl-d-amino acids by N-carbamoyl-d-amino acid amidohydrolase, and L-amino acids are from N-carbamoyl-L-amino acids by N-carbamoyl-L-amino acid amidohydrolase.
amidohydrolase. Several microorganisms producing N-carbamoyl-D- and/or N-carbamoyl-L-amino acid amidohydrolases have been isolated, and some of these enzymes were purified and characterized. These results suggest that there are 2 classes of N-carbamoyl amino acid amidohydrolases, N-carbamoyl-D- and N-carbamoyl-L-amino acid amidohydrolases. NCC amidohydrolase hydrolyzed only the L-form of NCC, and showed significant similarity to the known N-carbamoyl-L-amino acid amidohydrolases (Fig. 3), suggesting that NCC amidohydrolase is a member of the N-carbamoyl-L-amino acid amidohydrolase class. The sequences of three regions, indicated as RI, RII, and RIII in Fig. 3, were very highly conserved, suggesting that these sequences may be involved in enzyme activity and substrate specificity. At present, however, we have no further information on the active site and the substrate specificity of NCC amidohydrolase. We tried to purify NCC amidohydrolase from the E. coli transformant carrying pTrCM1, but the purified fraction contained several minor proteins, and the purification was not successful. We are working on the complete purification and characterization of NCC amidohydrolase.

Although ATC is structurally different from 5'-substituted hydantoins, the action of ATC hydrolase may be similar to that of the enzyme hydantoinase.
which hydrolyzes 5′-substituted hydantoinos to the corresponding N-carbamoyl-L-amino acids. However, ATC hydrolase had no significant similarity to hydantoinases from Pseudomonas sp. NS671 \(^{26}\) and \(P.\) putida \(^{27}\) and crude extracts from the transformant expressing ATC hydrolase activity did not hydrolyze hydantoinos (data not shown). What kind of substrates except for ATC would be hydrolyzed by ATC hydrolase? We have a great interest in the substrate specificity of this enzyme. Although ATC hydrolase was purified from the transformant carrying pTrCM1 to characterize, this enzyme was very unstable and a large amount of the activity was lost during the purification steps. Therefore, we are currently examining the stability of the enzyme and further purification.

Since both D- and L-ATC were converted only to L-cysteine via NCC, \(^9\) it is suggested that ATC racemase may be present in ATC-assimilating bacteria. ATC racemase is also an important enzyme in the production of L-cysteine from D,L-ATC, as well as ATC hydrolase and NCC amidohydrolase. We have not yet tested whether ATC racemase activity is present or not in strain ON-4a. Because the comparison with the sequence of ATC racemase gene from \(P.\) ovalis strain BS\(^\circ\) showed that the sequence corresponding to the ATC racemase gene is absent in our 3.4-kb insert DNA, it seems important to isolate the ATC racemase gene from strain ON-4a.

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