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Note

N-Carbamoyl-L-Cysteine as an Intermediate in the Bioconversion from D,L-2-Amino-Δ^2-Thiazoline-4-Carboxylic Acid to L-Cysteine by *Pseudomonas* sp. ON-4a

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We investigated the conversion of D,L-2-amino-Δ^2-thiazoline-4-carboxylic acid (D,L-ATC) to L-cysteine with *Pseudomonas* sp. ON-4a, an ATC-assimilating bacterium. Cysteine and *N*-carbamoylcysteine (NCC), but not *S*-carbamoylcysteine (SCC), were produced from D,L-ATC by a cell-free extract from the strain. These products were isolated from the reaction mixture and then identified as the L-form. Similar results were obtained with *P. putida* AJ3865 and unidentified strain TG-3, an ATC-assimilating bacteria. It became clear that L-NCC is an intermediate in the conversion of D,L-ATC to L-cysteine in these *Pseudomonas* strains. Furthermore, it was suggested that these bacteria have L-ATC hydrolase and L-NCC amidohydrolase.

Key words: *N*-carbamoyl-L-cysteine (L-NCC); bioconversion; 2-amino-Δ^2-thiazoline-4-carboxylic acid (ATC); L-cysteine; *Pseudomonas* species

The bioconversion of a chemically synthesized precursor, D,L-2-amino-Δ^2-thiazoline-4-carboxylic acid (D,L-ATC), to L-cysteine by some bacteria that assimilate D,L-ATC for the growth was investigated.[1-3] Sano *et al.* demonstrated that *S*-carbamoyl-L-cysteine (L-SCC) was formed from D,L-ATC by a crude enzyme solution of *Pseudomonas thiazolinophilum*, but neither L-cysteine nor L-cystine was formed from *N*-carbamoyl-L-cysteine (L-NCC) or *N*-carbamoyl-D-cysteine (D-NCC).[4] Ryu and Shin reported the same result with *Pseudomonas* sp. CU6.[5] They suggested that the bioconversion of D,L-ATC to L-cysteine might consist of two steps and L-SCC was probably an intermediate in the steps (Fig. 1-I).[4,6]

In this work, we investigated in detail the process of conversion of D,L-ATC to L-cysteine with newly selected *Pseudomonas* species, ATC-assimilating bacteria and found that L-NCC is an intermediate of the metabolic pathway from D,L-ATC to L-cysteine in these strains. A microorganism, strain ON-4a, isolated from a hot-spring, was a Gram-negative rod, and motile. The bacterium was grown aerobically in an nutrient broth at pH 4–9 and used citrate. MR and V-P tests were negative, and the bacterium denitrified nitrate and produced pigments. The GC content of the DNA was 62.7 mol%. According to Bergeys Manual of Systematic Bacteriology, Vol. 1, the bacterium was classified as a *Pseudomonas* species.[7]

The bacterium, strain ON-4a, was inoculated into a test tube (18 × 180 mm) containing 5 ml of ATC medium which was composed of 0.2% D,L-ATC·$3H_2O$, 2% glucose, 0.5% yeast extract, 0.5% peptone, 0.25% sodium chloride, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0007% $MnSO_4 \cdot 4H_2O$ and deionized water (pH 7.0). The bacterium was incubated aerobically at 30°C for 1 day and transferred to a 500 ml flask containing 100 ml of ATC medium, then incubated at 30°C for 36 h with shaking. The cells were collected by centrifugation and suspended in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 mM $MnSO_4$. The cell-concentration of the suspension was adjusted to 0.3 ~ 0.4 g wet cell weight per milliliter and treated with a Tomy ultrasonic disruptor (UD-201, 10 min, 20 KHz). A cell-free extract was obtained by centrifugation at 12,000 rpm for 20 min. The reaction mixture (1.0 ml), which contained 10 mM D,L-ATC, 20 mM hydroxylamine hydrochloride, 300 mM Tris-HCl (pH 8.0), 0.3 ml of cell-free extract (12 mg protein/ml), and with or without EDTA (10 mM), was incubated at 37°C for 3 h. The reaction was stopped by boiling in a water bath for 1 min. After cooling and adding 0.1 ml of 0.1 M dithiothreitol (DTT), the supernatant obtained by centrifugation was used for thin-layer chromatography (TLC). Portions were put on a TLC plate (DC-Plastikfolien Cellulose, Merck), and developed with a solvent of butanol-acetic acid-water (2:1:1). The products formed were compared with authentic samples of L-cysteine, L-cystine, *N*-carbamoyl-L-cysteine (L-NCC), and *S*-carbamoyl-L-cysteine (L-SCC) on the TLC plates. As shown in Fig. 2-C, NCC was newly produced from D,L-ATC in the reaction mixture with EDTA. Cysteine also was produced from D,L-ATC in the reaction mixture without EDTA (Fig. 2-D). When L-NCC was used as a substrate, L-NCC was converted to cysteine in the absence of EDTA, but not converted to cysteine in the presence of EDTA (data not shown). From these results, it was found that EDTA inhibits the conversion of NCC to cysteine, and it is suggested that L-NCC is the intermediate in the biosynthetic

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Abbreviations: L-NCC, *N*-carbamoyl-L-cysteine; SCC, *S*-carbamoyl-cysteine; ATC, 2-amino-Δ^2-thiazoline-4-carboxylic acid

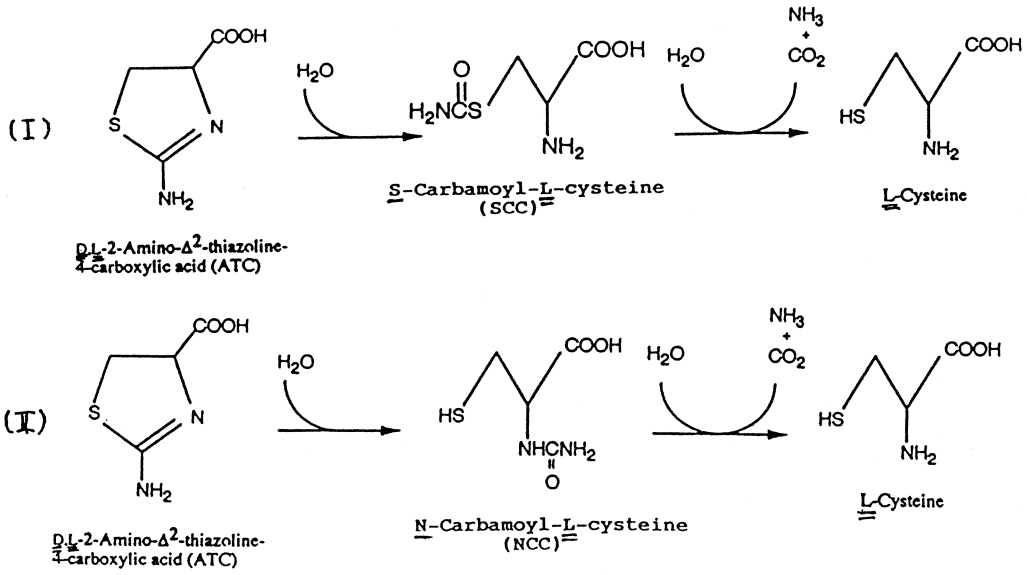

Fig. 1. Possible Metabolic pathways of 2-Amino-Δ^2-Thiazoline-4-Carboxylic acid to L-Cysteine in *Pseudomonas* species.

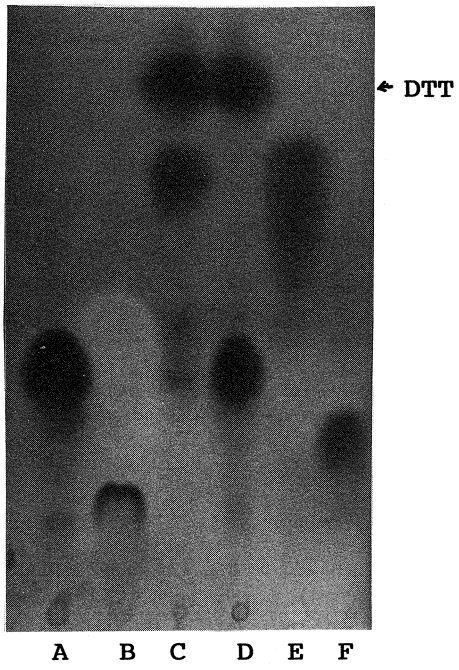

Fig. 2. Thin-Layer Chromatograms of the Products from D,L-ATC with *Pseudomonas* sp. ON-4a.

Line A; L-Cysteine. B; L-Cystine. C; Products from D,L-ATC by cell-free extract in the presence of EDTA. D; Product from D,L-ATC by cell-free extract. E; *N*-Carbamoyl-L-cysteine (L-NCC). F; *S*-Carbamoyl-L-cysteine (L-SCC).

pathway of cysteine from D,L-ATC in this strain (Fig. 1-I). However, SCC was not detected in any case tested (Fig. 2, lines C, D).

To discover from which intermediates cysteine can be formed, L-, D-NCC or L-, D-SCC was used as a substrate for cysteine-forming reactions with cell-free extracts of *Pseudomonas* sp. ON-4a. The cysteine produced was measured by the Gaitonde method[8] and HPLC (Shimazu LC-9A).

Table 1 shows that only L-NCC was converted to cysteine, thus indicating that L-NCC was used as a substrate in the reaction. Moreover, it was shown that the strain ON-4a had an ATC hydrolase activity induced only by ATC amoung the compounds tested, while it showed an L-NCC amidohydrolase activity under any conditions tested. These results suggest that these enzymes differ in the regulation of enzyme formation, as well as ATC hydrolase and SCC hydrolase in *Pseudomonas* sp. CU6.[6]

We tried to isolate the NCC produced from D,L-ATC in the reaction mixture with EDTA. The reaction was done with a 300 ml flask containing 100 ml of reaction mixture [10 mM D,L-ATC, 20 mM hydroxylamine hydrochloride, 300 mM Tris-HCl buffer (pH 8.0), 30 ml of cell-free extract (120 mg protein), 10 mM EDTA] at 37°C for 3 h. After incubation, the reaction was stopped by boiling and preciptates were removed by centrifugation. Then, the white preciptates formed by adjusting the pH of the solution to 2.0 were removed, and the evaporation of the filtrate resulted in the formation of a white residue. After dissolving this in hot methanol, the residue formed was removed by filtration. The filtrate was taken to dryness, and then the residue was washed with ether. The resulting white solid was dissolved in a minimum volume of hot water. After this was left overnight at 4°C, the crystals formed were washed with cold water, then ether and the solvent were evaporated to dryness over P_2O_5 under reduced pressure. The product was obtained in a 33% yield. Also the cysteine formed was isolated by an ordinary method in a yield of 45% from the reaction mixture without EDTA. The NCC and cysteine isolated were analyzed with infrared spectrometer by comparing the patterns of authentic cysteine and chemically synthesized NCC,[9] which was identified by ^{13}C-NMR, ^{1}H-NMR, mass spectrum, and elementary analysis. The optical rotations of

Table 1. Cysteine-Forming Activity of Cell-free Extract from *Pseudomonas* sp. ON-4a

Substrates[a]	Cysteine-forming activity[b]			
	Inducers[c]			
	D,L-ATC	L-NCC	L-SCC	None
D,L-2-Amino-Δ^2-thiazoline-4-carboxylic acid (D,L-ATC)	100	0	0	0
N-Carbamoyl-L-cysteine (L-NCC)	204	140	98	108
N-Carbamoyl-D-cysteine (D-NCC)	0	0	0	0
S-Carbamoyl-L-cysteine (L-SCC)	0	0	0	0
S-Carbamoyl-D-cysteine (D-SCC)	0	0	0	0

[a] ATC, 30 mM; other substrates, 15 mM.
[b] The reaction mixture (1 ml) composed of 5 mM NH_2OH, 300 mM Tris-HCl (pH 8.0), cell-free extract (100 μl, 2.2 mg) and substrate as indicated (a), was incubated for 1 h at 37°C. The reaction was stopped by boiling in a water bath for 1 min. The amount of cysteine formed was measured by the Gaitonde method. Relative activity is expressed in 100 for D,L-ATC (85 mU/mg protein).
[c] Cells were grown in the ATC-medium containing 0.2% L-NCC or L-SCC instead of ATC, respectively.

Table 2. Activity of Cysteine-Forming Enzyme of Various Bacterial Strains

Substrates[a]	Activity of Cysteine-forming enzymes[b]		
	Enzyme sources[c]		
	Pseudomonas sp. ON-4a	*Pseudomonas putida* AJ3865	Unidentified bacterium TG-3
D,L-2-Amino-Δ^2-thiazoline-4-carboxylic acid	100	250	60
L-2-Amino-Δ^2-thiazoline-4-carboxylic acid	91	200	52
D-2-Amino-Δ^2-thiazoline-4-carboxylic acid	86	170	48
N-Carbamoyl-L-cysteine	180	350	100
N-Carbamoyl-D-cysteine	0	0	0
S-Carbamoyl-L-cysteine	0	0	0
S-Carbamoyl-D-cysteine	0	0	0

[a] ATC, 30 mM; NCC and SCC, 15 mM.
[b] The reaction mixture (1 ml) composed of 5 mM NH_2OH, 300 mM Tris-HCl (pH 8.0), cell-free extract (2–3 mg) and substrate as indicated (a), was incubated for 1 h at 37°C and the reaction was stopped by boiling in a bath for 1 min. The amount of cysteine formed was measured by the Gaitonde method. The cysteine forming activity for D,L-ATC in ON-4a was taken as 100 (85 mU/mg protein).
[c] Cells were grown in the ATC-medium and cell-free extracts were prepared.

the NCC and cysteine isolated were measured by a polarimeter and agreed with these of authentic L-NCC ($[\alpha]_D^{20} = -30.83$) and L-cysteine ($[\alpha]_D^{20} = -225.2$), respectively. The cysteine was analyzed by HPLC using a CROWNPAK CR(+). The retention time of cysteine isolated was also agreed with that of authentic L-cysteine (4 min 50 sec.). From these results, it was found that the NCC and cysteine isolated were L-form. The results indicated that, in *Pseudomonas* sp. ON-4a, L-NCC is produced as an intermediate in the biosynthetic pathway of L-cysteine from D,L-ATC, which consists of two steps, i.e., from D,L-ATC to L-NCC, and L-NCC to L-cysteine (Fig. 1-II). This is the first report that L-NCC is hydrolyzed to L-cysteine by a bacterial cell-free extract. From these results, it was thought that some ATC-assimilating bacteria could synthesize L-NCC from D,L-ATC. Then, with cell-free extracts of *Pseudomonas putida* AJ 3865[10] grown in the ATC medium, it was confirmed that L-NCC and L-cysteine was produced from D,L-ATC. A similar result was obtained with unidentified strain TG-3, an ATC-assimilating bacterium (data not shown). Furthermore, Table 2 shows that these three bacteria have ATC hydrolase and L-NCC amidohydrolase activities but not SCC hydrolase activity. In spite of many reports of *N*-carbamoyl-L-amino acid amidohydrolases from various bacteria,[11–14] thus far there has been no observation that these bacterial enzymes showed the activity toward *N*-carbamoyl-L-cysteine as a substrate. Therefore, further ezymatic studies are needed to discover the role of L-NCC amidohydrolase in the ATC metabolism. Thus, we showed with some ATC-assimilating bacteria that L-NCC is the intermediate in the conversion of D,L-ATC to L-cysteine. It also became clear that ATC-assimilating bacteria have either pathway I or II in the conversion of D,L-ATC to L-cysteine (Fig. 1-I, II).

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