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Properties, Sequence, and Synthesis in *Escherichia coli* of 1-Aminocyclopropane-1-Carboxylate Deaminase from *Hansenula saturnus*

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The plant hormone ethylene is generated from a unique precursor, 1-aminocyclopropane-1-carboxylate (ACC). In previous studies, ACC deaminase, which degrades ACC to α-ketobutyrate and ammonia, was found in four strains of *Pseudomonas*, characterized, and sequenced. To verify the wider distribution of ACC deaminase in microorganisms, we purified and sequenced ACC deaminase from the yeast *Hansenula saturnus*. The purified enzyme was active toward ACC, D-serine and *dl*-coronamic acid, indicating the same stereospecificity as the *Pseudomonas* enzyme, but unlike the bacterial enzyme it was not active toward β-chloro-D-alanine and *O*-acetyl-D-serine. Analyses of peptides from proteolytic digests of the purified and modified ACC deaminase covered more than 90% of its amino acid sequence and showed a blocked N-terminal residue as *N*-acetylserine. A cDNA encoding the ACC deaminase was isolated from *H. saturnus* cells incubated in α-aminoisobutyrate medium, and sequenced. The yeast enzyme has 441 amino acid residues, of which 60 to 63% are identical to those of reported *Pseudomonas* enzymes. The open reading frame encoding ACC deaminase was subcloned into pET-11d and expressed in *Escherichia coli* BL21 (DE3) as an active enzyme.

Key words: 1-aminocyclopropane-1-carboxylate (ACC), ACC deaminase, *Hansenula saturnus*.

Cyclopropanoid amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) was isolated from apples and pears (*1*) and cow berry (*2*) and identified as an intermediate in the biosynthesis of ethylene, which acts as a plant hormone regulating many aspects of plant growth and development, especially fruit ripening (*3*). In plant tissues, ACC is generated from *S*-adenosyl-L-methionine and fragmented into ethylene, carbon dioxide, and cyanide. Furthermore, *N*-malonylation of ACC was found as an enzyme reaction that reduces cellular ACC concentration (*4*). On the other hand, the soil microorganisms *Pseudomonas* sp. and *Hansenula saturnus* are known to grow on medium containing ACC as a sole nitrogen source and produce ACC deaminase [1-aminocyclopropane-1-carboxylate endolyase (deaminating), EC 4.1.99.4] as an adaptive enzyme that degrades ACC to α-ketobutyrate and ammonia (*5*). Interest in regulation of fruit ripening led to work on transgenic tomato plants in which expression of the ACC deaminase gene resulted in reduction of ethylene synthesis and delay of fruit ripening (*6, 7*). These works stimulated further screening of ACC deaminase-producing bacteria (*8*) and studies of plant growth-promoting rhizobacteria. The latter showed the occurrence of ACC deaminase in a strain of *Pseudomonas putida* (*9*). ACC deaminase has been found in four strains of *Pseudomonas* and its gene has been cloned from three of these strains (*6, 8, 10*). Three amino acid sequences deduced from the bacterial ACC deaminase genes are equal in size and highly homologous. However, the ACC deaminase of eukaryotes has not been analyzed except for estimation of K_m for ACC and molecular size using the enzyme purified partially from *H. saturnus* (*5*). A key question in the catalysis of ACC deaminase as a pyridoxal phosphate (PLP)-dependent enzyme is mechanism of the cyclopropane-ring-opening process following the Schiff base formation of PLP with ACC. The cloning of ACC deaminase-encoding DNAs from different origins should give better choice to allow detailed characterization of the structure and mechanism of the enzyme reaction. In this paper we present the analytical results on the ACC deaminase purified from *H. saturnus*, cloning of cDNA encoding ACC deaminase, and its expression in *Escherichia coli*.

MATERIALS AND METHODS

Organism and Growth—A yeast, *H. saturnus*, was isolated from soil (*5*). This yeast was grown in the first medium containing 2% D-glucose, 0.5% peptone, and 0.3% dried yeast extract for 24 h at 30°C with shaking. The grown cells were collected by centrifugation, washed with 0.1 M potassium phosphate, pH 7.5, and incubated at 30°C

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Abbreviation: ACC, 1-aminocyclopropane-1-carboxylic acid; PLP, pyridoxal phosphate.

for 30 h in the second medium containing 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.013% $CaCl_2 \cdot 2H_2O$, 0.0013% $FeSO_4 \cdot 7H_2O$, 2% sucrose, and 1% α-aminoisobutyric acid in a half volume of the first medium.

Assay of ACC Deaminase—Activity of ACC deamination was assayed by measurement of α-ketobutyrate formed from 50 mM ACC as described previously (*5*). Protein concentration was determined by method of Bradford (*11*) and by absorbance at 280 nm using bovine serum albumin as a standard.

Purification of ACC Deaminase—Harvested yeast cells were suspended in 0.04 M potassium phosphate, pH 7.5, containing 0.5 M potassium chloride and ruptured with a French Press (Ohtake Works, Tokyo) at 1,800 kg/cm^2. Centrifugation of the homogenate from yeast cells (323 g) gave 1,360 ml of the cell extract. The extract was dialyzed against 10 volumes of water, mixed with an adequate volume of glycerol up to 20%, and put on a DEAE-Sepharose column (3.0×18 cm) equilibrated with 0.04 M potassium phosphate, pH 6.5, containing 0.01 mM PLP and 20% glycerol. The column was washed with the same buffer (1,000 ml), and protein was eluted with a linear gradient of 0 to 0.3 M potassium chloride (1,400 ml). To an active fraction of the eluate, solid ammonium sulfate was dissolved up to 25% saturation and the resultant solution was put on a butyl-Toyopearl column (1.9×25 cm) equilibrated with 0.02 M potassium phosphate, pH 6.5, containing 0.01 mM PLP, 20% glycerol, and 25% saturation of ammonium sulfate, followed by washing with the same buffer (350 ml) and elution with a linear gradient of 25 to 0% saturation of ammonium sulfate (700 ml). The eluted enzyme fraction was rechromatographed with a smaller column (1.0×12 cm) of butyl-Toyopearl. The resultant enzyme solution was concentrated by ultrafiltration and passed through a column of Sephadex G-150 (2.5×42 cm) equilibrated with 0.04 M potassium phosphate, pH 6.5, containing 0.01 mM PLP and 20% glycerol. The effluent of the enzyme activity was put on a DEAE-Toyopearl (1.0×10 cm) equilibrated with the buffer described above. The column was washed with the same buffer (50 ml), and the enzyme was eluted with a linear gradient of 0 to 0.25 M potassium chloride (200 ml). The eluted enzyme fraction was subjected to the gel filtration as described above. ACC deaminase in the effluent was precipitated by addition of saturated ammonium sulfate solution and stored in 3 M ammonium sulfate.

Chemical Modification—ACC deaminase in 0.1 M potassium phosphate, pH 7.5, was reduced with a small amount of solid sodium borohydride until yellow color of the solution disappeared, then dialyzed against the same buffer. Guanidine hydrochloride was dissolved in the enzyme solution to a concentration of 6 M, and 2-mercaptoethanol was added in the proportion of 1 μl per mg protein. The mixture was left to stand under nitrogen overnight at room temperature. Pyridylethylation of cysteine residues was done at room temperature by the addition of 1.5 μl of 4-vinylpyridine per mg protein. The modified enzyme protein was purified by reversed-phase chromatography with Asahipak C4P-50 (4.6×150 mm) and a gradient of 0 to 40% acetonitril in 0.1% trifluoroacetic acid. The absorbance at 216, 280, and 325 nm was monitored. The purified protein fraction was evaporated to dryness under reduced pressure.

Proteolytic Digestions—The modified ACC deaminase protein was digested with one-fiftieth weight of lysylendopeptidase (*Achromobacter lyticus* protease 1, Wako Pure Chemical) in 0.01 M Tris-hydrochloride, pH 9.0, containing 4 M urea at 30°C overnight.

For tryptic digestion, the modified ACC deaminase was citraconated in advance, as follows (*12*). The modified ACC deaminase was dissolved in 286 μl of 0.1 M potassium pyrophosphate, pH 8.0, with 18 mg of urea. Citraconic anhydride was added in an equimolar amount with lysine residues, and the mixture was left at room temperature for 20 min. The addition was repeated three more times with the same interval, then glycine was added in an equimolar amount with the citraconic anhydride used, and the mixture was left for 30 min. The citraconated protein in the resultant solution was incubated with TPCK-treated trypsin (Sigma Chemical) (1:100, w/w) at 25°C for 20 h.

Peptides in these digests were separated by the reversed-phase chromatography with Asahipak C8P-50 (4.6×150 mm) and a gradient of 0 to 40% acetonitril in 0.1% trifluoroacetic acid. The sequence of each peptide was analyzed with an Applied Biosystems Protein Sequencer Model 477A.

Release of Acetyl Group from N-Terminal Acetylserine—The acetyl group blocking the N-terminal serine was released by incubation of an N-terminal peptide in trifluoroacetic acid vapor at 45°C for 45 h according to Wellner *et al.* (*13*).

Molecular Mass of a Blocked N-Terminal Peptide—The peptide was dissolved in 0.1% acetic acid in a concentration of 1 μmol/100 μl and analyzed by high resolution electron spray mass spectrometry. Molecular size of the peptide was also estimated from amino acid analysis of hydrolysate of the peptide with 6 N hydrochloric acid at 110°C for 24 h.

Construction of a cDNA Library—Yeast cells grown in the first medium were incubated in the second medium for 5 h. Total RNA was isolated by the guanidine hydrochloride/phenol method (*14*), and poly(A)$^+$ RNA was prepared from the total RNA with Oligotex-dT30 (Nippon Gousei Gum, Tokyo). The double-stranded cDNA was synthesized with cDNA Synthesis Kit (Takara Shuzo) and inserted into λgt 11 vector (Stratagene). The recombinant DNAs were packaged into bacteriophage particles using a Packaging Kit (Stratagene) and grown on *Escherichia coli* Y1090.

Preparation of a Probe—An oligonucleotide, 5′-CA(A/G)GA(A/G)GA(C/T)TGGGTICCIATICCIGA(A/G)GCIG-A(A/G)AA-3′, was prepared with an Applied Biosystems DNA Synthesizer 380B and labeled with Dig Oligonucleotide Tailing Kit (Boehringer Mannheim).

Construction of Expression Vector—To insert the open reading frame of ACC deaminase into an expression vector, PCR primers were designed to incorporate an *Nco*I site in the N-terminal region: 5′-AATTCCGGCCGCCATGGCCG-GTGTCGCCAAATTC-3′ and 5′-GTGACGGCTTCTATA-TTATGAC-3′. The PCR fragment was digested with *Nco*I and *Sca*I and inserted into the *Nco*I-*Bam*HI (blunt end) site of pET-11d (Takara) to construct the expression vector pHACCD. *E. coli* BL 21 (DE 3) (Takara) was transformed with pHACCD and grown on 1.6% Trypton, 1% yeast extract, 0.8% NaCl, 0.005% ampicillin, and 1 mM pyridoxine (pH 7.0) at 16°C.

RESULTS AND DISCUSSION

Induction of ACC Deaminase—It was previously de-

scribed that ACC deaminase was induced by ACC (*5*) in *Pseudomonas* sp. and *H. saturnus*, and that ACC could be replaced with α-aminoisobutyrate in the case of *Pseudomonas* sp. (*15*). The ACC deaminase of *H. saturnus* was induced in the second medium containing α-aminoisobutyrate within 10 h (Fig. 1). The yeast cells were harvested after 30 h of the second incubation for the enzyme purification and 5 h for the extraction of mRNA.

Purification of ACC Deaminase—As yeast cells were tougher to rupture than bacterial cells, higher pressure (1,800 kg/cm^2) in French Press treatment and the presence of 0.5 M potassium chloride in the cell suspension were required for effective enzyme extraction. ACC deaminase in the extract was purified as detailed in "MATERIALS AND METHODS" and the results are summarized in Table I. In the final step of gel filtration with Sephadex G-150, fractions giving a single band in the polyacrylamide gel electrophoresis (*16*) were collected.

Molecular size of ACC deaminase from *H. saturnus* was estimated to be 40 kDa by SDS polyacrylamide gel electrophoresis (*17*) and 69 kDa by the Hedrick-Smith method (*18*) using 6 to 9% polyacrylamide gel in the system of Davis (*16*). The latter value agreed with that obtained previously from gel filtration (*5*). The ACC deaminase from *Pseudomonas* sp. was deduced to be a trimer from the molecular masses of 36.5 kDa obtained by SDS electrophoresis and 110 kDa obtained by gel filtration and the Hedrick-Smith method (*19*). For the yeast enzyme, the above data suggest it to be a dimer.

Specificity—Previous studies noted that K_m for ACC was 1.6 mM with the ACC deaminase from *Pseudomonas* sp. and 2.6 mM with the enzyme from *H. saturnus* (*5*), and that the *Pseudomonas* enzyme was also active toward *dl*-coronamic acid [(1*S*,2*S*) and (1*R*,2*R*) 2-ethyl ACC], D-serine, β-chloro-D-alanine, and *O*-acetyl-D-serine (*20, 21*). The enzyme of *H. saturnus* had activity toward 50 mM *dl*-coronamic acid, D-serine, and D-cysteine with relative activity of 15.4, 2.9, and 0.7% of that of ACC, respectively. These values were slightly lower than those of the *Pseudomonas* enzyme (*20, 21*), and the activities toward β-chloro-D-alanine and *O*-acetyl-D-serine were not detected. The L-isomers of serine, β-chloroalanine, and *O*-acetylserine,

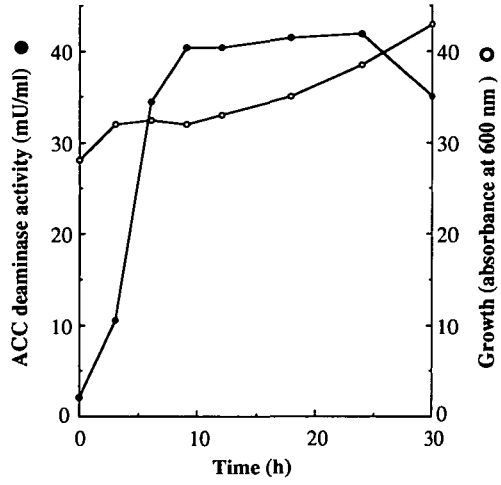


Fig. 1. **Induction of ACC deaminase.** *Hansenula saturnus* cells grown in the first medium for 24 h at 30°C were incubated in a half volume of the second medium containing 1% α-aminoisobutyric acid at 30°C with shaking. Cells in 5 ml of the medium were harvested by centrifugation, suspended in 5 ml of 0.04 M potassium phosphate containing 0.5 M KCl, pH 7.5, and treated with a French Press.

TABLE I. **Purification of ACC deaminase from *Hansenula saturnus*.**

	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	9,280	309	0.033	100
DEAE-Sepharose	3,130	180	0.058	58.3
Butyl-Toyopearl	166	150	0.904	48.5
Sephadex G-150	56.7	131	2.31	42.4
DEAE-Toyopearl	25.9	94.0	3.63	30.4
Sephadex G-150	12.1	62.0	5.12	20.1

TABLE II. **Competitive inhibition constants to ACC deamination.**

Inhibitor	Inhibition constant (mM)
L-Alanine	14.5
D-Alanine	280
L-Serine	2.9
D-Serine	13.5
L-Homoserine	59.0
L-α-Aminobutyrate	148

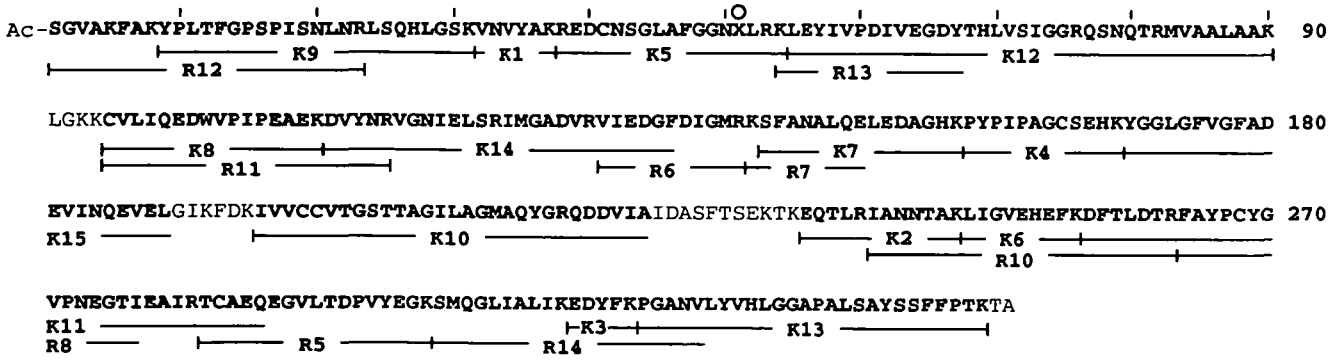


Fig. 2. **Amino acid sequence of the ACC deaminase purified from *Hansenula saturnus*.** Peptides from lysylendopeptidase digests are indicated by K1 to 15, and those from tryptic digestions by R5 to 8 and R10 to 14. Sequencing cycle at X in peptide K5 did not give an assignable amino acid. Residues written in fine letters were deduced from the nucleotide sequence of cDNA, and the N-terminal acetyl group is shown by Ac.

D- and L-threonine, DL-homoserine, DL-allothreonine, D- and L-methionine, and *dl*-allocoronamic acid were inert. These data indicate that *H. saturnus* enzyme has higher specificity for ACC than the *Pseudomonas* enzyme has.

Several amino acids were competitive inhibitors to ACC deamination. As shown in Table II, the L-form of each amino acid was a much stronger inhibitor than the D-form, and the deamination in 2.6 mM ACC was slightly inhibited by 25 mM glycine, L-threonine, and D-aminobutyrate but not by D-threonine and α-aminoisobutyrate. These findings gave the same outline of the specificity as that with the *Pseudomonas* enzyme: that is, *dl*-coronamic acid and D-serine in addition to ACC were active, and the L-form of each amino acid had higher affinity than the D-form. Reactivity of D-serine was supported by proton NMR spectrum of D-alanine incubated with the ACC deaminase in D_2O. Exchange of a proton at the α carbon atom with deuterium was observed with D-alanine but not with L-alanine.

Amino Acid Sequence—The amino acid sequence of ACC deaminase purified from *H. saturnus* is shown in Fig. 2. Reduced and pyridylethylated ACC deaminase was digested with lysylendopeptidase and separated into peptides by reversed-phase chromatography (Fig. 3). Each of 15 peaks in Fig. 3 was analyzed, and the peptide sequences obtained are indicated by K1 to K15 in Fig. 2. The sequence of K5, which had high absorbance at 325 nm, was identical to the sequence around the PLP binding site of *Pseudomonas* ACC

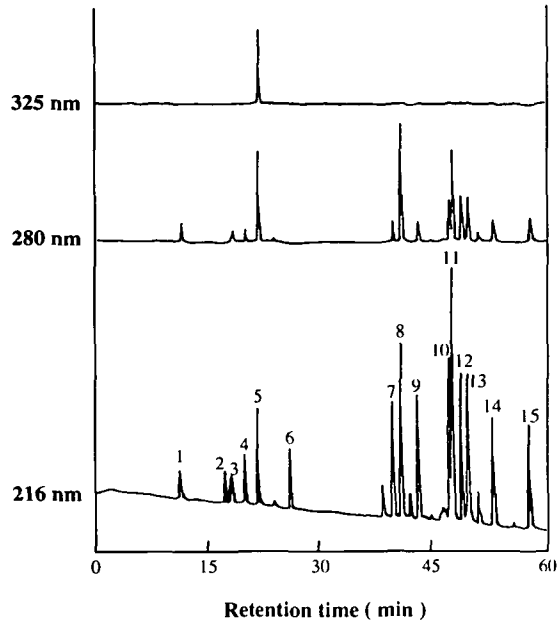
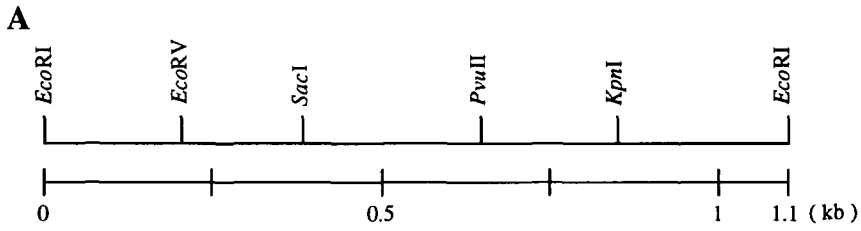


Fig. 3. **Reversed-phase HPLC of lysylendopeptidase digests of modified ACC deaminase.**



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1    aattccggccgaaATGTCCGGTGTCGCCAAATTCGCCAAATACCCCCTTACTTTCGGGCC
61   TTCTCCAATCTCAAACTTGAACCGTCTCTCCCAGCACTTGGGTTCCAAAGTTAACGTCTA
121  TGCTAAAAGAGAGGATTGTAACTCCGGTCTCGCCTTTGGTGGTAATAAGCTCAGAAAGTT
181  GGAATATATCGTTCCAGATATCGTCGAGGGTGACTACACGCACTTGGTCTCCATTGGTGG
241  TAGACAAAGTAACCAAACAAGAATGGTTGCTGCATTGGCTGCTAAATTGGGTAAGAAATG
301  TGTGTTGATTCAAGAAGATTGGGTGCCGATTCCAGAGGCTGAGAAGGATGTTTACAATAG
361  AGTTGGAAACATCGAGCTCTCTAGAATCATGGGAGCTGATGTTCGTGTTATCGAAGATGG
421  ATTTGATATTGGTATGAGGAAGTCATTTGCTAATGCACTTCAAGAGTTGGAAGATGCTGG
481  TCATAAACCGTACCCTATCCCCGCTGGTTGTTCTGAGCACAAGTACGGCGGCTTAGGTTT
541  TGTTGGCTTTGCGGATGAAGTTATCAACCAAGAGGTTGAATTGGGTATCAAGTTTGATAA
601  GATCGTTGTGTGTTGTGTTACGGGGTCCACGACAGCTGGTATTCTTGCTGGTATGGCCCA
661  ATACGGTAGACAAGACGATGTCATTGCCATCGATGCCTCTTTCACATCAGAAAAGACCAA
721  GGAACAAACTTTGAGAATCGCAAACAACACTGCAAAACTGATCGGTGTTGAACATGAGTT
781  CAAAGATTTCACGTTGGATACAAGATTCGCTTATCCATGTTATGGTGTTCCAAATGAGGG
841  TACCATTGAAGCTATTAGAACATGCGCTGAACAAGAAGGTGTTTTGACTGATCCAGTTTA
901  TGAGGGTAAGTCCATGCAAGGCTTGATAGCATTGATCAAAGAGGATTACTTCAAGCCAGG
961  CGCGAATGTGCTCTATGTCCATTTGGGTGGTGCTCCAGCGTTGTCAGCATACTCTTCTTT
1021 CTTCCCAACAAAGACTGCTTGAtgcagtactgctgtgtgtacattgaatagagtcataat
1081 atagaagtcgtcacatttttcccggaatt
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Fig. 4. **Nucleotide sequence of cDNA encoding ACC deaminase.** (A) Restriction map of the isolated cDNA fragment. (B) Nucleotide sequence of the cDNA fragment. The open reading frame corresponding to the ACC deaminase sequence is shown in uppercase letters. Two sequences indicated by single underlines were used to design PCR primers to construct an expression vector. A probe for the screening was designed from the amino acid sequence corresponding to the dotted underline. The double underline indicates the recognition site for *Sca*I used in construction of the expression vector.

deaminase (*10*) with the exception of one residue. Each peptide except for K3, which was finally settled in a sequence of R14, was assigned to a certain part of the sequence of the *Pseudomonas* enzyme. Although peptide K9 gave the closest sequence to the N terminal of the enzyme molecule, the N-terminal structure was obscure. Sequence analysis of the reduced and modified ACC deaminase failed to give the N-terminal sequence. Tryptic digestion of the reduced, pyridylethylated, and citraconated ACC deaminase provided a peptide R12 with a blocked N terminal. Analyses of 8 peptides from the tryptic digestions and 15 peptides from the lysylendopeptidase digestions covered more than 90% of a sequence of *H. saturnus* ACC deaminase.

Analysis of N-Terminal Structure—Failure of N-terminal sequencing suggested that the N terminal of the *H. saturnus* ACC deaminase was blocked, and that peptide R12 was derived from the N terminal. Amino acid analysis of R12 supported the sequence in Fig. 2, which was deduced from cDNA analysis as described in the next paragraph. If the N terminal of a peptide is *N*-acetyl serine, the method of Wellner *et al.* (*13*) is available for deacylation from N terminal. Thus, R12 was treated with trifluoroacetic acid as described in "MATERIALS AND METHODS." Amino acid sequence analysis of the resultant mixture gave two sequences, SGVAKFAKYPL and TFGPSPISNLNR. The results indicated that *N*-acetyl serine was the N terminal of R12 and the treatment with trifluoroacetic acid cleaved a linkage between leucine and threonine residues. Cleavage at threonine was noted by Wellner *et al.* (*13*). From a high resolution mass analysis, the molecular formula of R12 was determined as $C_{116}H_{180}N_{30}O_{32}$ (observed *m/z*: 2,505.3293, calculated *m/z*: 2,505.3308). This formula designated the N-terminal-blocking group of R12 as an acetyl group.

Isolation and Sequence Analysis of an ACC Deaminase cDNA—The amino acid sequence of Q99 to K110 of the ACC deaminase (Fig. 2) was used to design an oligonucleotide fragment described in "MATERIALS AND METHODS" as a hybridization probe in screening. A cDNA library constructed in λgt 11 was prepared from *H. saturnus* incubated for 5 h in the second medium (Fig. 1), and screened with the hybridization probe. Many positive plaques were detected in approximately 2×10^5 phages, and 20 of them were purified and tested for the estimation of size of the insert. The longest clone was isolated and sequenced. Restriction map and nucleotide sequence of an isolated cDNA are shown in Fig. 4. The cDNA contained an open reading frame which included the entire coding region for the ACC deaminase. The deduced amino acid sequence included all the sequences determined chemically from peptides which were separated from the digests of purified ACC deaminase (Fig. 2), and had an additional N-terminal methionine residue. A fragment of about 460 bp including the 3′-noncoding-region was used for analysis of RNA in *H. saturnus* cells, which were incubated in the second medium for 0, 2, 4, 6, and 8 h. Results of the Northern blot analysis

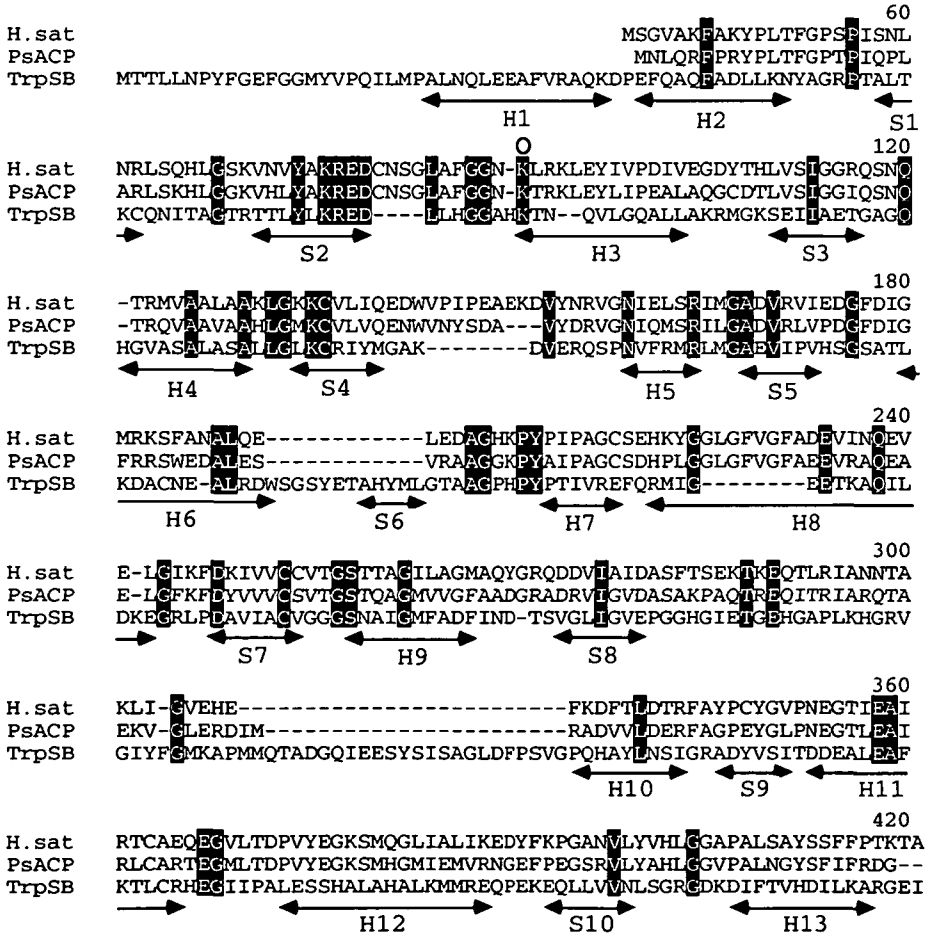

Fig. 5. **Comparison of amino acid sequences of ACC deaminases and tryptophan synthase β-subunit.** Amino acid sequences of ACC deaminase from *Hansenula saturnus* (H. sat) and *Pseudomonas* sp. ACP (PsACP) and tryptophan synthase β-subunit (TrpSB) from *Salmonella* (*22*) were aligned by fitting at PLP binding site (○). H1 to 13 and S1 to 10 indicate the second structure of tryptophan synthase β-subunit reported by Hyde *et al.* (*22*).

(not shown) agreed with that shown in Fig. 1, confirming that this clone was for ACC deaminase which was induced by α-aminoisobutyrate.

Comparison with Sequence of Tryptophan Synthase β-Subunit—ACC deaminases from three strains of *Pseudomonas*, ACP, 6G5, and f17, are equal in size (338 residues) and similar in sequence (97% between 6G5 and f17, 82% between ACP and f17, and 83% betweem ACP and 6G5). The ACC deaminase of *H. saturnus* has 341 residues including insertion of 5 residues and deletion of 2 residues relative to the sequence of the *Pseudomonas* enzymes, and has sequence similarity of 60 to 63% with the *Pseudomonas* ones (Fig. 5). We previously discussed structural similarity between the ACC deaminase of *Pseudomonas* sp. ACP and tryptophan synthase β-subunit (*22*) with respect to enzyme reaction, PLP binding site, and tryptic cleavage site (*23*). When ACC deaminase sequences from *Pseudomonas* sp. ACP and *H. saturnus* are aligned with that of tryptophan synthase β-subunit by fitting the PLP binding lysine residue (Fig. 5), identical residues across three sequences appear frequently in four regions: (i) from mid S2 to N-end of H3 in the structure of tryptophan synthase β-subunit (*22*), (ii) from mid H4 to mid S4, (iii) from H5 C-end to mid S5, and (iv) from S7 N-end to mid H9. The first region is the N-terminal side of the PLP binding site, and the fourth is described as being near the 5′-phosphate group and the pyridyl-ring N atom of PLP in the tryptophan synthase β-subunit (*22*).

Expression in E. coli—The open reading frame of the ACC deaminase cDNA was subcloned into pET-11d using the PCR primers described in "MATERIALS AND METHODS" to form an expression vector pHACCD. A strain BL21 (DE 3) transformed with pHACCD was incubated in the medium containing pyridoxine at lower temperature, in order to obtain the extract with higher enzyme activity. No inducer was required for expression of the ACC deaminase.

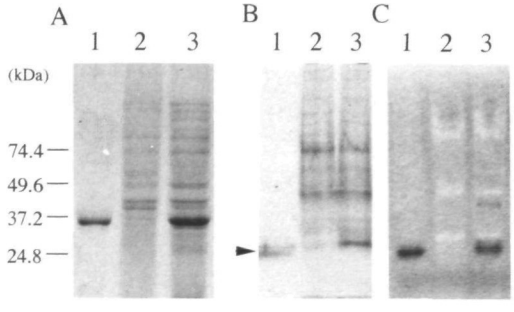

Fig. 6. **Expression of ACC deaminase cDNA in *E. coli*.** Cells of *E. coli* BL 21 (DE 3) containing pHACCD or pET-11d (control) were grown at 16°C for 175 h on 1.6% Trypton, 1% yeast extract, 0.8% NaCl, 0.005% ampicillin, and 1 mM pyridoxine, harvested by centrifugation, and extracted in 0.1 M Tris-0.045 M KH_2PO_4, pH 8.5, by sonication. The homogenate was centrifuged at 11,000×*g* for 5 min, and the supernatant was used for SDS-PAGE (*24*) and PAGE by the system of Davis (*16*). A, SDS-PAGE. Protein was stained with Coomassie Brilliant Blue. B, PAGE of native protein. Protein was stained with Coomassie Brilliant Blue. C, PAGE of native protein. ACC deaminase activity was stained by incubation in 0.1 M Tris-0.045 M KH_2PO_4, pH 8.5, containing 20 mM ACC, 0.1% nitroblue tetrazolium, and 0.01% phenazine methosulfate (*25*). Lane 1, ACC deaminase purified from *H. saturnus*; lane 2, extract from *E. coli* (pET-11d); lane 3, extract from *E. coli* (pHACCD).

Native or SDS polyacrylamide gel electrophoresis of the extract from *E. coli* containing pHACCD showed a major band at around a position corresponding to the purified ACC deaminase (Fig. 6, A and B), and the major band on the native polyacrylamide gel electrophoresis was stained with ACC deaminase activity (Fig. 6C). The major band on SDS polyacrylamide gel electrophoregram (Fig. 6A) was blotted onto a ProBlott (Perkin Elmer) membrane and applied to the protein sequencer. The obtained N-terminal sequence, AGVAKFAKYPLTFGPSPI, agreed with that of *H. saturnus* ACC deaminase (Fig. 2), except for the N-terminal residue, which had been changed serine to alanine for construction of an *Nco*I site at the N-terminal region. Furthermore, N-terminal methionine residue was also found to be cleaved in *E. coli* cells.

Burroughs noted that ACC appeared to be less readily assimilated by yeasts, since ACC was a major amino acid in fermented perry where it was present in pear juice (*1*). In this paper, we showed the yeast *H. saturnus* to have ACC deaminase by the enzyme purification, cloning, and synthesis in *E. coli*.

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