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Gene Cloning and Expression of Cephalosporin-C Deacetylase from *Bacillus* sp. KCCM10143

CHOI, DUK-HO*, YONG-DUK KIM[1], IL-SUN CHUNG[1], SANG-HUN LEE[1], SANG-MO KANG, TAE-JONG KWON, AND KUM-SOO HAN[1]

Department of Microbial Engineering, Konkuk University, 93-1 Mojin-Dong, Kwangjin-Ku, Seoul 143-701, Korea
[1]Pharmaceutical Division, R&D Center of Daesang Co., Ltd., 125-8, Pyokyo-Ri, Majang-Myun, Ichon-City, Kyounggi-Do 467-810, Korea

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Abstract Cephalosporin-C deacetylase (CAH) catalyzes the deacetylation of cephalosporin derivatives. A novel gene encoding the CAH from *Bacillus* sp. KCCM10143 was cloned and sequenced. The nucleotide sequence contained an open reading frame encoding a polypeptide consisting of 217 amino acids and a molecular weight of 24 kDa which was in good agreement with the value obtained by sodium dodecylsulfate-polyacrylamide gel electrophoresis. An expression plasmid was constructed by inserting the CAH gene into the region of the pTrc99A expression vector. An active form of the CAH protein was expressed in the soluble fraction obtained after cell disruption. In fermentation using a 5-l jar fermenter, the transformant *E. coli* JM109 (pDST654) produced 4.12 U of CAH per ml of culture during 16 h of incubation.

Key words: Cephalosporin, deacetylase, *Bacillus*, 7-aminocephalosporanic acid (7-ACA)

Cephalosporin-C deacetylase (CAH:EC 3. 1. 1. 41) is an esterase that catalyzes the deacetylation of cephalosporins such as cephalosporin-C (CPC), 7-aminocephalosporanic acid (7-ACA), and 2-methoxyimino-2-furyl-acetic cephalosporanic acid (MIFACA). The resulting products, deacetyl cephalosporins, are used as starting materials for various semisynthetic cephalosporin antibiotics such as cefuroxime [14]. The enzyme activities have been found in certain strains of *Bacillus subtilis* [1, 2, 11], and other microorganisms [5, 8, 10, 16, 19]. Recently, we found that a strain (KCCM10143) of *Bacillus* sp. isolated from a soil sample produced a novel CAH. This CAH differs from the known CAHs of other strains of *B. subtilis* in the following respects: gene sequence [13], amino acid sequence [13, 16], and molecular weight [1, 2, 11, 16]. These features are advantageous for the industrial production of deacetyl cephalosporins due to its high K_{cat} values to various cephalosporins and negligible product inhibition activity. Therefore, it was intended to breed a strain which could produce large amounts of the CAH by means of genetic engineering with *Escherichia coli*.

Herein, the cloning and nucleotide sequencing of the CAH gene from *Bacillus* sp. KCCM10143 are described, along with its highly efficient expression in *E. coli*.

*Corresponding author
Phone: 82-336-639-2040; Fax: 82-336-638-1500;
E-mail: dukho@mw.co.kr

MATERIALS AND METHODS

Bacterial Strains, Plasmid, and Vector

Bacillus sp. DS1152 (KCCM10143; Korea Culture Collection of Microorganisms), a producer of CAH, was isolated from a soil sample. *E. coli* JM109 was used as the host strain for gene cloning and expression. Plasmid pUC18 (Am^r) was used as the cloning vector. Plasmid pTrc99A (Am^r; purchased from Pharmacia-LKB, Uppsala, Sweden) was used for the gene expression.

Partial Purification of Enzyme

LB medium was placed in a 500-ml flask and sterilized by autoclaving at 121°C for 20 min. The flask was inoculated with *Bacillus* sp. KCCM10143 and the resulting culture was grown at 30°C for 9 h to be used as a seed culture.

Four liters of LB medium was placed in a 7-l jar fermenter and sterilization was carried out by autoclaving at 121°C for 20 min. The flask was inoculated with the seed concentration of 1% and the cultivation was carried out at 30°C for 48 h.

Cephalosporin deacetylase was purified from the culture broth by the following method. The culture broth was

centrifuged (5,000 rpm × 30 min) to collect cells, which were then suspended in 50 mM Tris/HCl buffer (pH 8.0) and disintegrated with an ultrasonic homogenizer. The resulting disintegrated cells were subjected to centrifugation (12,000 rpm × 30 min) to remove insoluble fractions.

Supernatant was fractionated by precipitating with 40–80% of saturated ammonium sulfate solutions ($(NH_4)_2SO_4$) to give an initial purification. The resulting solution was subjected to ultrafiltration (MWCO 30 kDa) to remove salts and to concentrate the proteins. Thus resulting protein solution concentrate was loaded onto a column packed with DEAE-sepharose equilibrated with 10 mM Tris buffer (pH 8.0) at a flow rate of 2.0 ml/min and at an amount of 40 mg protein per ml of gel. Then, the column was washed with the equilibration buffer and eluted by the continuous gradient manner using 0–300 mM NaCl. Most of the Cephalosporin deacetylase was eluted at 150–200 mM NaCl concentrations. They were pooled and concentrated by subjection to ultrafiltration (MWCO 30 kDa). Subsequently, this enzyme solution was applied to a Sephacryl S-300 column (2.4 × 100 cm), equilibrated with Tris-HCl buffer, in a conventional system (flow rate, 5 ml/min; fraction volume, 3 ml). The active fractions were pooled and concentrated.

Measurement of Molecular Weight of Enzyme

Gel chromatography was carried out on Sephacryl S-300 packed in a 1.6×100 cm column (gel volume, 300 ml) to measure the molecular weight of the enzyme. Molecular weight marker proteins of β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), or cytochrome C (12.4 kDa) were eluted using a buffer at a flow rate of 2.5 ml/min. The molecular weight of the enzyme was calculated using a standard curve based on the marker proteins.

The molecular weights of the enzyme subunits were estimated by the SDS-PAGE method [12], using 12% (w/v) acrylamide gels. Staining was carried out with Coomassie brilliant blue. The marker proteins used as the standards were from Pharmacia Biotech. Co. Ltd.

Kinetic Studies

Kinetic analysis of the CAH was carried out at 37°C and pH 7.0, using 100 mM sodium phosphate buffer. K_m values were determined by double-reciprocal plots. The reaction products were analyzed by HPLC analysis. The HPLC was performed using a Waters dual pump and U.V. detector (Milipore, Milford, MA, U.S.A.). A computing integrator was used for monitoring the peak area (at 254 nm) and retention time. The column (250 mm× 4.6 mm I.D.) was Alltech NH_2. The mobile phase consisted of 50 mM $(NH_4)_2HPO_4$ and 55% CH_3CN at pH 3.2. The flow rate was 0.6 ml/min at 45°C.

Bacterial Culture Condition

The *E. coli* strain was grown at 36.5°C in Luria-Bertani (LB) broth or on LB agar [15]. When necessary, ampicillin (50 μg/ml) was added to the medium. For the CAH production at flask level (50 ml of medium per 500 ml flask), the cells were grown for 15 h with constant shaking (180 rpm) at 36.5°C in LB medium. The practical production of CAH with a 5-l jar fermenter was carried out as follows. The seed culture was performed in LB broth (250 ml in a 2-l flask) containing ampicillin, at 36.5°C for 12 h on a rotary shaker at 180 rpm. The entire contents of the seed flask were added to 3 l of a production medium, which contained 1.5% glycerol, 2% yeast extract, 2% corn steep liquor, 0.05% $MgSO_4 \cdot 7H_2O$, 0.6% KH_2PO_4, 0.9% K_2HPO_4, and 50 mg/ml ampicillin. The pH of the broth was maintained at 6.8 and incubation was at 28°C for 16 h. The agitation was maintained at 400 rpm, and the air was sparged at a rate of 1 vol/vol/min at a pressure of 0 kg/cm^2. The bacterial growth was monitored by measuring the O.D. at 600 nm.

DNA Manipulations

The chromosomal DNA from *Bacillus* sp. KCCM-10143 was prepared by the method of Harris-Warrick *et al.* [9]. The plasmid was isolated using the alkaline sodium dodecyl sulfate method of Birnboim and Doly [3]. Cleavage with the restriction enzymes, dephosphorylation with bacterial alkaline phosphatase, and ligation with T4 DNA ligase, were carried out with enzymes from Boehringer Mannheim Co. Ltd., according to the conditions recommended by the manufacturer. The agarose and polyacylamide gel electrophoreses were performed as described by Sambrook *et. al.* [15]. The DNA fragments were eluted from the agarose gel by a Gene Clean kit, which was purchased from Bio101. Co. Ltd. The transformation of *E. coli* with recombinant molecules was performed using the electroporation method as described by BIO-RAD, Inc [6].

Construction of Genomic Library and Cloning of Gene

The chromosomal DNA from *Bacillus* sp. KCCM-10143 was partially digested with *Sau*3AI, and the DNA fragments of 2 to 4 kb were recovered using the sucrose gradient ultracentrifugation method. The fragments were ligated to *Bam*HI-digested and dephosphorylated pUC18. The ligated DNA was then introduced into the *E. coli* JM109 cells. The transformants were selected on an LB agar containing ampicillin and used as the genomic library. The ampicillin-resistant transformants on the master plate were transferred to a screening LB plate of naphthylacetate hydrolysis selection, which contained 0.02% naphthylacetate in ethylene glycol monomethyl ester and 0.012% fast blue RR salt in a Tris-malate buffer (pH 7.6). Positive red-brown colonies were selected on the master plates and isolated. Positive colonies were then cultured in an LB

medium and the recombinant CAH activity was measured for deacetyl 7-ACA production. The clone with the CAH activity was cultured and the plasmid with the 2.7 kb insert fragment was isolated.

DNA Sequencing

The restriction map from the 2.7 kb insert was analyzed and the 1.6 kb DNA fragment containing the CAH activity was resubcloned. Plasmid pSPF1.6 is a recombinant plasmid consisting of the 1.6 kb CAH gene from *Bacillus* sp. KCCM10143. The 1.6 kb DNA fragment of pSPF1.6 contained several *Sau*3AI restriction sites and was partially digested by *Sau*3AI. DNA fragments of 0.6 to 1.2 kb were subcloned and the plasmids carrying 0.6, 0.8, 1.0, 1.2, and 1.3 kb fragments were then reisolated. The nucleotide sequences were determined using the dideoxy chain termination method with a Cy5TM Auto ReadTM sequencing kit (Pharmacia Co. Ltd.), and read by the ALF express automatic sequencer (Pharmacia Co, Ltd.). The nucleotide sequences were determined using both strands and overlapped in sequence of the reading fragments.

Construction of Expression Plasmid

Based on pSPF1.6, the structural gene of CAH was prepared by the polymerase chain reaction (PCR) method. The region containing the open reading frame of CAH was determined by the molecular weight of the purified enzyme, and N-terminal amino acid was sequenced based on DNA fragment analysis. Olgonucleotides with a base sequence (21 mer: 5-TGA GCC ATG GCG AAT CAC ATT-3) corresponding to Ala-1 to Ile-4 of the N-terminal region and a complementary base sequence (22 mer: 5-TCA CAA GCT TCA CCC TTC TTT G-3) corresponding to Ile-213 to Gly-216 of the C-terminal region were synthesized as the primer. A PCR amplified DNA fragment including the *Nco*I and *Hin*dIII restriction sites was inserted into the pTrc99A vector, which was predigested by *Nco*I and *Hin*dIII restriction enzymes. The resulting expression plasmid was named pDST654 (KCCM-10140).

Enzyme Assay

The expression level of the recombinant CAH by the *E. coli* transformant-harboring plasmid (pDST654) was measured using the crude extract as the expressed enzymes. The cells collected from the culture broth were suspended in the same volume of a 10 mM Tris-HCl (pH 7.8) buffer and 1 mM EDTA, and disrupted with an ultrasonicator. The supernatant was obtained by centrifugation (12,000 ×g × 10 min). The enzyme activity was assayed in a 50 mM Tris-HCl buffer (pH 7.8), containing 10 mM 7-aminocephalosporanic acid (7-ACA) as the substrate. The enzyme reaction was performed at 30°C and stopped by adding a 0.2 M sodium acetate buffer (pH 4.0). One unit of enzyme activity was defined as the amount of 1 M deacetyl-7-aminocephalosporanic acid (DACA) produced per min at 30°C.

The protein concentrations were measured according to the method of Bradford [4] (Bio-Rad laboratories) using bovine serum albumin as the standards.

N-terminal Amino Acid Sequencing

The amino-terminal residues of the purified enzyme were extracted using a precise protein sequencing system (Applied Biosystems) and serviced by the Korea Basic Science Institute (Seoul branch). The samples were analyzed using 10 base amino-terminal amino acid sequencing.

Nucleotide Sequence Accession Number

The nucleotide sequence reported in this paper has been submitted to the GenBank database under the accession number of AF184175.

RESULTS

Enzyme Purification

Table 1 summarizes the result of a typical purification from 41 of *Bacillus* sp. KCCM-10143. The enzyme was purified with a specific activity of 31.0 U/mg protein and a yield of 6.4%. The purity of the enzyme eluted from the final column step was assessed by SDS-PAGE (Fig. 3).

Molecular Property

The molecular weight of the enzyme was estimated to be about 48 kDa by the Sephacryl S-300 gel filtration. SDS-PAGE gave a molecular weight of 25 kDa (Fig. 3), indicating that the CAH protein was a dimeric enzyme composed of identical subunits.

Table 1. Purification of the Cephalosporin-C deacetylase.

Step	Protein (mg)	Activity (unit)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	5825.4	27,000	4.63	100	1.0
Salting-out	3940.6	18,270	4.64	67.7	1.0
Ultrafiltration	2437.7	13,117	5.38	48.6	1.2
DEAE-sepharose	277.1	7,691	27.75	28.5	6.0
Gel-filtration	55.6	1,725	31.03	6.4	6.7

Table 2. Properties of the Cephalosporin-C deacetylase.

Properties	*Bacillus* sp. KCCM10143	*Bacillus subtilis* SHS0133
Molecular Weight	48 kDa	280 kDa
Structure	Dimer	Octamer
K_m(for 7-ACA*1)	18.8 mM	7.3 M
K_m(for CPC*2)	14.6 mM	24.3 mM
K_m(for MIFACA*3)	17.2 mM	-

*1 7-ACA: 7-aminocephalosporanic acid *2 CPC: Cephalosporin-C. *3. MIFACA: 2-methoxyimino-2-furylacetic cephalosporanic acid

Enzyme Kinetics

The CAH showed broad substrate specificity and followed Michaelis-Menten kinetics with various substrates. Table 2 shows the K_m values for the substrates. From double-reciprocal plots, the K_m values for cephalosporin-C, 7-ACA, and MIFACA were estimated to be 14.6 mM, 18.8 mM, and 17.2 mM, respectively.

Cloning of the CAH Gene

The genomic library of *Bacillus* sp. KCCM10143 in *E. coli* was screened by naphthylacetate hydrolysis positive selection. Among 1,800 colonies examined, five positive clones were detected. The five selected clones were assayed by an enzyme reaction between 7-ACA and one of the selected colonies which contained a 2.7 kb DNA fragment. Restriction enzyme mapping of the plasmid pUCF2.7 carrying the 2.7 kb fragment from the *Bacillus* sp. KCCM10143 chromosomal DNA was performed (Fig. 1). After removing the *Sph*I fragment, the remaining 1.6 kb fragment was resubcloned as the selected recombinant plasmid pSPF1.6. The transformant with pSPF1.6 exhibited an enzyme activity that was about five times higher than that of *Bacillus* sp. KCCM10143.

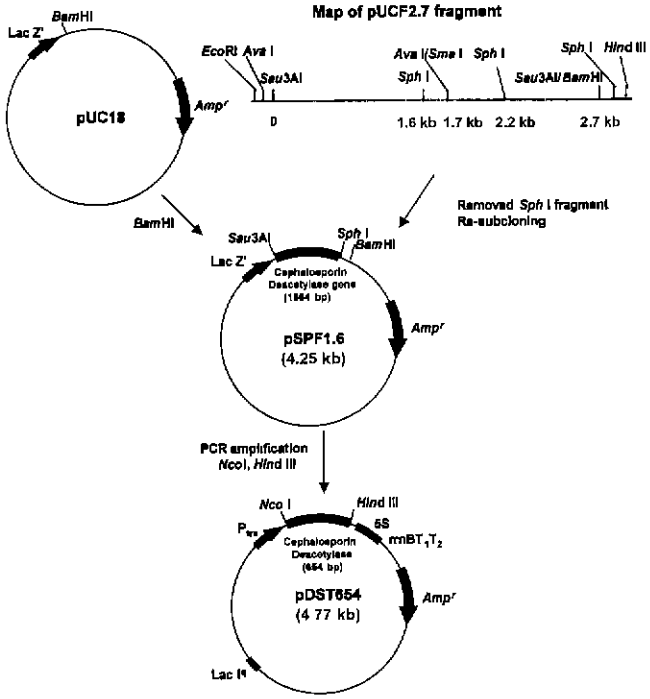

Fig. 1. Restriction map of the CAH gene and construction of the expression plasmid.

Plasmid pUCF2.7 is a recombinant plasmid consisting of pUC18 and the 2.7 kb CAH gene of *Bacillus* sp. KCCM10143. Plasmid pSF1.6 was constructed by ligation with the 1.6 kb *Sph*I fragment including the CAH gene from pUCF2.7 and pUC18, and the 1.6 kb fragment encoded the enzyme activity against 7-ACA.

Nucleotide Sequencing

The approximate 1.6 kb region was sequenced and the sequencing strategies are shown in Fig. 1. A computer analysis of the sequence identified one major open reading frame (ORF, nucleotides 580 to 1233), with the capacity to encode a polypeptide of 217 amino acid residues (Fig. 2). The calculated molecular weight of the polypeptide was 24,000, which was in good agreement with the value of 25 kDa determined by SDS-PAGE for the purified enzyme (Fig. 3).

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ATCGAGCTCGGTACCGGGGATCATGACTGGGACGGAACGGTCGGCAGGCCGAAGATTGAA                60
AAGTGGGATGCTGAAAACCGCTGTCTGAAGACGATTTTTCAGCCGGACGGCGTGCTGTCC               120
AACAACGGCACAAAAGGAAATCCTGTTCTTCAGGCCAACCTGTTCGGAGACTGGCCGGAA               180
GAAGTGATATGGAGAACGGAAGACAGCAGCGCACTCCGCATCTATACAACGACACATCTC               240
ACCCGCCATCGCTTTTACACGCTTATGCACGATCCGGTTTACAGGCTCGGCATCGCCTGG               300
CAGAATACCGCCTACAACCAGCCGCCGCACACGGGCTTTTATCTCGGAACGGGAATGAAA               360
AAACCACCGAAGCCCGCCCTGTACATAGCGGGCAGCAAAGCGGAGGCGCCGCTATAGGAG               420
GAACTAAGGGATAAGAAGCAAAGGCTGAACAGTACGACAAGATGACTTATCAAAGCCAGG               480
ACGACTAATGTTCAGGCGCTGAAATCACCGGTTTTTTCAAGGGAAGAGCTGTCCATGATT               540
GATGCCATATGAAACGAAAAAAACGAGGGBGAATGAGAA ATG GCG AAT CAC ATT TAT CTT         600
                          SD        1    Ala Asn His Ile Tyr Leu             6

GCT GGC GAT TCA ACT GTT CAA ACG TAT GGA GAC AGC ACA AAT CAA GGG GGG         651
Ala Gly Asp Ser Thr Val Gln Thr Tyr Gly Asp Ser Thr Asn Gln Gly Gly          23

TGG GGG CAG TTT CTC GGC TCG CAT CTG CCG GAG CAT ATT CAA GTG ATT AAC         702
Trp Gly Gln Phe Leu Gly Ser His Leu Pro Glu His Ile Gln Val Ile Asn          40

AGA GCA ATC GGG GGA AGA AGC TCG AAA ACA TTT GTG GAA GAG GGC AGG CTT         753
Arg Ala Ile Gly Gly Arg Ser Ser Lys Thr Phe Val Glu Glu Gly Arg Leu          57

CAG GCG ATT CTT GAT GTG ATT GAG CCG GAT GAC TGG CTG TTT GTG CAG ATG         804
Gln Ala Ile Leu Asp Val Ile Glu Pro Asp Asp Trp Leu Phe Val Gln Met          74

GGC CAT AAT GAC GCG TCG AAA AAT AAG CCG GAG CGC TAC ACC GAG CCC TAT         855
Gly His Asn Asp Ala Ser Lys Asn Lys Pro Glu Arg Tyr Thr Glu Pro Tyr          91

ACC ACC TAT AAA CAA TAT TTA AAG CAG TAT ATC GCA GGC GCG CGG GAA AAA         906
Thr Thr Tyr Lys Gln Tyr Leu Lys Gln Tyr Ile Ala Gly Ala Arg Glu Lys         108

GGC GCC CAT CCG CTT CTC ATT ACC CCT GTA GCA CGC TTT CAT TAT GAA AAT         957
Gly Ala His Pro Leu Leu Ile Thr Pro Val Ala Arg Phe His Tyr Glu Asn         125

GAC ATG TTT TTG AAC GAC TTT CCT GAC TAT TGC ATT GCC ATG AAG CAG ACG        1008
Asp Met Phe Leu Asn Asp Phe Pro Asp Tyr Cys Ile Ala Met Lys Gln Thr         142

GCT GCT GAG GAG AAT GTC CAG CTC ATT GAT CTG ATG GAG AAA AGT CTT GCT        1059
Ala Ala Glu Glu Asn Val Gln Leu Ile Asp Leu Met Glu Lys Ser Leu Ala         159

TTC TTT ACC GAG AAG GGC GAG GAA AAA GTG TAC ACC TAT TTT ATG GTG TCA        1110
Phe Phe Thr Glu Lys Gly Glu Glu Lys Val Tyr Thr Tyr Phe Met Val Ser         176

GAA GGA ATT AAC GAT TAC ACG CAC TTT ACA AAA AAA GGC GCA AAT GAA ATG        1161
Glu Gly Ile Asn Asp Tyr Thr His Phe Thr Lys Lys Gly Ala Asn Glu Met         193

GCG AAA CTT GTG GCA AAA GGC ATA AAG GAA CTC GGC CTG CCA TTG ACA GAA        1212
Ala Lys Leu Val Ala lys Gly Ile Lys Glu Leu Gly Leu Pro Leu Thr Glu         210

TCG ATC ATC AAA GAA GGG TGA   AAAATGTGAGGAGAAAACTGTATCACGGCG              1263
Ser Ile Ile Lys Glu Gly ***                                               216

CTTGTTATTATCCCGAATTATGGGATGAAGAGACGATTCAGCAGGACATTGACATCATGC              1323
GTGAAGTTGGCGTGAATGTTGTGCGGATCGGTGAATTTGCCTGGTCTGTCATGGAACCGG              1383
AAGAAGGAAAAATTGACGTCAGTTTTTTCAAAGAGATCATCACCCGTCTGTATGATAA               1445
CGGCATCGAAACGATCATGTGCACGCCGACGCCTACACCGCCGATTTGGCTGTCGCACGG              1505
CCGGCCGGAGCGTATGCATGTAAACGAAAAAAGAGAGGTCATGGGGCATGGCTCCCGTCAG            1562
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Fig. 2. Nucleotide and amino acid sequences of the CAH gene of strain *Bacillus* sp. KCCM10143.

The amino acid sequence corresponding to the ORF beginning with the first start ATG codon begins at nucleotide 580 bp. The stop codon is indicated by (*).

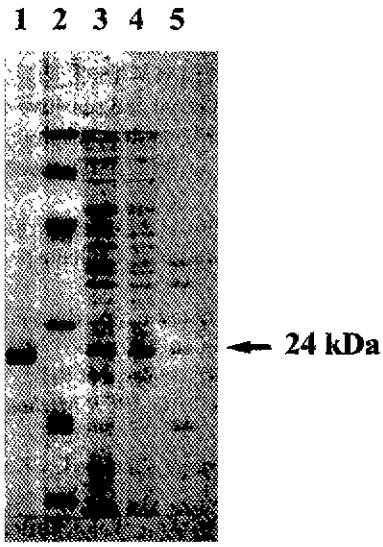

Fig. 3. SDS-PAGE of purification of the CAH enzyme and expression in *E. coli* JM 109 harboring pDST654 (KCCM10140).
Lane 1, Partial-purified enzyme; Lane 2, Standard markers (94 K, 67 K, 43 K, 30 K, 20 K, 14.4 K), Lane 3, Crude lysate: Lane 4, Soluble fraction; Lane 5, Insoluble fraction.

The N-terminal amino acid sequence of 10 base residues (Ala-Asn-His-Ile-Tyr-Leu-Ala-Gly-Asp-Ser) starting from the ORF was essentially identical with that of the purified enzyme, except for Met, which was subsequently deleted in the purified enzyme of *Bacillus* sp. KCCM10143. Accordingly, it was confirmed that the cloned sequence encoded the CAH from *Bacillus* sp. KCCM10143.

When the nucleotide sequence was compared with the NIH BLASTIN search, 92% homologies were found between the DNA sequence of the CAH and the *Bacillus subtilis* in the regions of 172923 to 173602, the complete sequence known since 1997 [18].

Expression of CAH Gene in *E. coli*

The structural gene moiety of CAH in the recombinant plasmid pDST654 was prepared as described in Materials and Methods [16]. The plasmid pDST654 included a *trc* promoter and unchanged ampicillin resistance marker (Am^r).

The expression plasmids were introduced into *E. coli* JM109 by transformation. The expressed enzyme formed a low level inclusion body, yet existed in an active form in the cells. The expression level was measured in a soluble fraction obtained from the sonicated cells in the culture broth. Table 3 shows the enzyme activities expressed by the transformant.

The induction effect of isopropyl-D-thiogalactopyanoside (IPTG) on the plasmids with the *trc* promoter was significant, however, the CAH gene was expressed autonomously in the culture medium, which was 70% of the level of the IPTG induction effect. The expression level

Table 3. Comparsion of expression levels of recombinant strains.

Host strain	Plasmid	Induction factor	Enzyme activity (units/ml broth)
E. coli JM109	pUC18	(-)	N.D
	pSPF1.6	(-)	0.47
	pDST654	(-)	2.80
	pDST654	0.5 mM IPTG	4.12
Bacillus sp. KCCM10143			0.11

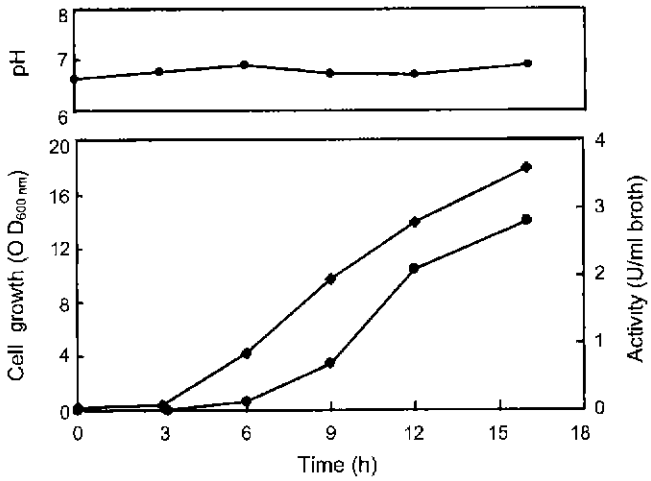

Fig. 4. Time course of CAH production of *E. coli* harboring pDST654 in a 5-l jar fermenter.
Symbol. ● Enzyme activity. ◆ Cell Growth.

of *E. coli* JM109 harboring pDST654 was 3.0–4.0 U/ml of the culture. Figure 4 shows the typical pattern of CAH production. *E. coli* JM109 harboring pDST654 (KCCM10140) was cultured in a 5-l jar fermenter at 28°C in 3 l of the production medium. The CAH production was associated with the cell growth. The activity of the expressed enzyme increased during cultivation and reached to a maximum value of 4.12 U/ml of culture after 16 h of incubation.

DISCUSSION

The CAH of *Bacillus* sp. KCCM10143 differs from those of *Bacillus subtilis* in the following respects: molecular weight, structure, and the substrate reactivity [13, 16, 19]. The results are shown in Table 2, which reveals that the enzyme shows similar reactivities to all of the three cephalosporin derivatives. In particular, this enzyme can deacetylate 2-methoxyimino-2-furyl-acetic cephalosporanic acid (MIFACA) with high reactivity, which are used as starting materials for semisynthetic cephalosporin antibiotics such as cefuroxime.

In the present study, the cloning and expression of the CAH gene from *Bacillus* sp. KCCM10143 were described. The determination of the nucleotide sequence showed that the structural gene was composed of 654 bp, and the CAH existed as a dimeric structure of identical subunits. The primary structure of the subunit was elucidated from the deduced amino acid sequence.

The nucleotide sequence is shown in Fig. 2. Since these sequences were shown to be necessary for the maximal transcriptional activity of their own promoters, the cloning plasmid of pSPF1.6 exhibited a high level of expression using its own promoter. The downstream region included an internal repeated sequence with six T residues. These regions were predicted to be the translational terminator regions.

In comparison of the gene sequence of CAH and the *Bacillus subtilis* complete genome regions, it was found that the protein-coding genes matched by 92%. The regions of a hypothetical protein in the *Bacillus subtilis* genome were different from those of the recombinant CAH, which mismatched four amino acid residues. From these results, it was predicted that the *Bacillus subtilis* strain contained a functionally similar enzyme.

The cloned gene was easily expressed in its active form in *E. coli* by the use of a recombinant plasmid. Recombinant *E. coli* harboring pDST654 was expressed at 4.12 U/ml of culture, which was 10 times higher than that of *E. coli* carrying pSPF1.6.

For the enzyme production, the culture conditions were optimized using a 5-l jar fermenter. As shown Fig. 4, *E. coli* JM109 (pDST654, KCCM10140) produced 2.80 U of enzyme per ml of culture during 16 h of incubation, and an inducer for gene expression was not necessary. This value corresponds to 50 g of cells in 1 l of culture broth and the specific activity was 0.25 U/mg protein.

Another notable feature of the gene expression in the present study was that the enzyme was produced in a soluble state and did not form an inclusion body which is frequently found in the *E. coli* expression system. As a result, the expressed enzyme was easily recovered as an active dimeric structure from the soluble fraction obtained after cell disruption. Accordingly, this expression system using recombinant *E. coli* could be readily applied for an industrial method for enzyme production.

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