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Gene Cloning, Nucleotide Sequence, and Expression of a Cephalosporin-C Deacetylase from *Bacillus subtilis*

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The gene encoding a cephalosporin-C deacetylase (CAH) from *Bacillus subtilis* SHS 0133 was cloned and sequenced. The nucleotide sequence contained an open reading frame encoding a polypeptide consisting of 318 amino acids, the molecular weight of which was in good agreement with the value obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The deduced amino acid sequence contained the common sequence Gly-X-Ser-X-Gly found in many esterases, lipases, and serine proteases. This indicates that CAH is a serine enzyme. A possible promoter sequence which is very similar to the consensus sequences of −35 and −10 regions recognized by *B. subtilis* RNA polymerase utilizing sigma factor H was found in the 5′-flanking region of the CAH structural gene. Two repeated A+T-rich blocks consisting of 24 bp were also found in the upstream region of the initiation codon. We constructed a series of expression plasmids by inserting the CAH gene into *Escherichia coli* ATG vectors. The degree of CAH gene expression depended on promoters and vector plasmids, which have different replication origins. The expressed CAH protein was an active form in the soluble fraction obtained after cell disruption. The highest expression level was accomplished with an expression plasmid, pCAH400, which has the *trp* promoter and the replication origin derived from pAT153. In the fermentation using a 30-liter jar fermentor, the transformant *E. coli* JM103(pCAH400) produced 440 U of CAH per ml of culture during a 24-h incubation. This value corresponded to 2.1 g of CAH protein in 1 liter of culture broth.

Cephalosporin-C deacetylase (CAH; systematic name, cephalosporin-C acetylhydrolase) is an esterase that catalyzes the deacetylation of cephalosporins such as cephalosporin C and 7-aminocephalosporanic acid. The resulting products, deacetyl cephalosporins, are used as starting materials for various semisynthetic cephalosporin antibiotics, for example, S-1108 (38) and cefuroxime (27). The enzyme activities have been found in citrus peels (16), some strains of *Bacillus subtilis* (1, 2, 18), and other microorganisms (7, 9, 14). Recently, we found that a strain (SHS 0133) of *B. subtilis* isolated from a soil sample produced a novel CAH. Previously (36), we reported the purification, characterization, and partial amino acid sequences of the enzyme from *B. subtilis* SHS 0133 and proved that the CAH is composed of eight identical subunits with a molecular mass of 35 kDa. This CAH differs from known CAHs of other *B. subtilis* strains in the following respects: molecular weight, subunit structure, substrate specificity, and isoelectric point (1, 2, 18). The CAH has favorable features for industrial production of deacetyl cephalosporins because of its high k_{cat} values to various cephalosporins and negligible product inhibition. Therefore, we intended to breed a strain which produces large amounts of the enzyme by means of genetic engineering with *Escherichia coli.*

Herein, we describe the cloning and nucleotide sequencing of the CAH gene from *B. subtilis* SHS 0133 and its expression with high efficiency in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage vector. *B. subtilis* SHS 0133 (FERM BP-2755), the producer of CAH, was isolated from a soil sample (36). *E. coli* HB101 (5) was used as a host strain for gene cloning and expression. *E. coli* JM103 (23) was used for gene expression.

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Plasmid pUC13 (Ap^r) was used as a cloning vector. Plasmids pKK233-2 (Ap^r; purchased from Pharmacia-LKB, Uppsala, Sweden) (3), pTrp · pAT · ATG (Tc^r) (supplied by H. Teraoka, Research Laboratories, Shionogi & Co., Ltd.), and pAT153 (Ap^r Tc^r; purchased from Toyobo, Osaka, Japan) were used for gene expression. Phage vector M13mp11 (purchased from Takara Shuzo, Kyoto, Japan) was used to prepare single-stranded DNA for nucleotide sequencing and preparation of a structural gene.

Culture conditions. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar (30). If necessary, ampicillin (40 μg/ml) or tetracycline (20 μg/ml) was added to the medium. For the CAH production at a flask level (50 ml of medium per 500-ml flask), the cells were grown for 24 h with constant shaking (170 rpm) at 37°C in modified M9 medium, which contained (per liter) 20 g of casein hydrolysates (Difco, Detroit, Mich.), 10 g of yeast extract (Difco), M9 basal salts (6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 2 mmol of $MgSO_4$, and 0.1 mmol of $CaCl_2$), and antibiotic (ampicillin or tetracycline). The practical production of CAH with a 30-liter jar fermentor was carried out as follows. The seed culture was performed in LB broth containing tetracycline (100 ml in a 500-ml flask) at 28°C for 16 h on a rotary shaker at 200 rpm. The entire contents of the seed flask was added to 20 liters of production medium, which contained (per liter) 40 g of glycerol, 20 g of casein hydrolysates, 3 g of yeast extract, M9 basal salts, and tetracycline, and incubated at 37°C for 24 h. The pH of the broth was maintained at 7.0 with 28% NH_4OH and 43% H_3PO_4. Agitation was maintained at 500 rpm, and air was sparged at a rate of 0.75 vol/vol/min at a pressure of 0 kg/cm^2 (gauge). Bacterial growth was monitored by measuring the A_{650}.

Synthesis of oligonucleotide probes and hybridization. The oligonucleotide mixture was synthesized as hybridization probes (34) with an automated DNA synthesizer, ZEON-GENET A-II (Nihon Zeon, Japan). The probes were labeled radioactively with [γ-^{32}P]ATP by the procedure of Wallace et al. (40). Hybridization was performed as described by Southern (33).

DNA manipulations. Chromosomal DNA from *B. subtilis* SHS 0133 was prepared by the method of Harris-Warrick et al. (13). Plasmid DNA was isolated by the alkaline-sodium dodecyl sulfate (alkaline-SDS) method of Birnboim and Doly (4). Cleavage with restriction enzymes, dephosphorylation with bacterial alkaline phosphatase, ligation with T4 DNA ligase, end filling by Klenow fragment (large fragment of *E. coli* DNA polymerase I), and S1 nuclease treatment were carried out with enzymes from Takara Shuzo Co., Ltd., and Toyobo Co., Ltd., under the conditions recommended by the manufacturers. The agarose and polyacrylamide gel electrophoreses were performed as described by Sambrook et al. (30). DNA fragments were electroeluted from the gels and recovered. Transformation of *E. coli* with recombinant molecules was performed by the competent cell method described by Hanahan (12).

Cloning of a CAH gene. Chromosomal DNA from *B. subtilis* SHS 0133 was digested with *Dra*I and subjected to 0.8% agarose gel electrophoresis. DNA fragments of 2 to 4 kb were recovered from the gel and ligated to *Sma*I-digested and dephosphorylated pUC13. The ligated DNA was introduced into *E. coli*

HB101 cells. Transformants were selected on LB agar containing ampicillin and used as the genomic library. The ampicillin-resistant transformants on the master plates were transferred to the nitrocellulose membrane and subjected to colony hybridization as described by Grunstein and Hogness (11). Positive clones were selected on the master plates and isolated.

Determination of nucleotide sequence. The nucleotide sequence was determined by the dideoxy chain termination method (31) with an M13 sequencing kit (Takara Shuzo) and universal M13 primers as well as synthesized internal primers. The nucleotide sequence was determined from both strands.

Preparation of a structural gene of CAH. Preparation of a structural gene of CAH with an oligonucleotide primer was performed as follows. The fragment containing the open reading frame of CAH was inserted into phage vector M13mp11 to generate recombinant plasmid M13-CAH. By introducing the plasmid M13-CAH into *E. coli* JM103, single-stranded DNA was obtained by the procedures of Messing (22). An oligonucleotide having a base sequence (18-mer, CAACTATTCGATCTGCCG) corresponding to Gln-2 to Pro-7 of CAH was synthesized as a primer (designated CAH-P1). The structural gene moiety of CAH was prepared by the method of Goeddel et al. (10). The primer CAH-P1 and single-stranded DNA of phage M13-CAH were mixed and annealed by incubation at 60°C for 20 min and then left standing at room temperature. To the mixture, dATP, dCTP, dGTP, and dTTP as well as Klenow fragment were added, and the primer extension was carried out at 37°C for 2 h. The remaining parts of single-stranded template DNA were digested with S1 nuclease and then blunt ended with Klenow fragment. The resulting double-stranded DNA was digested with an appropriate restriction enzyme, whose site lies approximately 0.3 kb downstream from the termination codon, to obtain the CAH fragment containing the structural gene and 3′ noncoding region.

Assay for recombinant CAH activity. The expression levels of recombinant CAH by *E. coli* transformants harboring various plasmids were compared by measuring the activities of the expressed enzymes with a crude extract. The cells collected from the culture broth were suspended in the same volume of 0.1 M phosphate buffer (pH 7.0) and disrupted with an ultrasonicator. The supernatant obtained by centrifugation was used as a sample solution. CAH activity was assayed as described previously (36). One unit of enzyme activity was defined as the amount producing 1 μmol of *p*-nitrophenol from *p*-nitrophenyl acetate in 1 min at 30°C.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (19). Proteins were stained with Coomassie brilliant blue. The subunit molecular mass of the protein was determined with the following standard proteins (molecular mass is shown in parentheses): phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrolase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL, GenBank, and DDBJ data banks under accession number D10935.

RESULTS

Cloning of the CAH gene. Previously (36), we determined the partial amino acid sequences of various *Achromobacter* proteinase I-digested fragments derived from purified CAH. The following amino acid sequence was selected to design the oligonucleotide mixture to be used as hybridization probes: Glu-Met-Val-Asn-Trp-Ala. The 17-mer mixed-oligonucleotide probes [3′ CT(C/T)TACCANTT(A/G)ACCCG 5′ (N represents A/C/G/T)] representing all possible sequences of antisense strand were synthesized. The 5′-termini of the oligonucleotides were labeled radioactively and used in Southern hybridization with restriction enzyme-digested fragments of *B. subtilis* SHS 0133 chromosomal DNA. The probes were found to hybridize to the 2- to 3-kb *Dra*I fragment, ca. 4-kb *Hin*dIII fragment, and ca. 8-kb *Pvu*II fragment. The chromosomal DNA was digested with *Dra*I, and fragments ranging from 2 to 4 kb were ligated with *Sma*I-digested and dephosphorylated pUC13. The genomic library of *B. subtilis* SHS 0133 in *E. coli* HB101 was screened by colony hybridization with the same probes, and two positive clones were selected. The recombinant plasmids obtained were designated pCAH01 and pCAH02. A preliminary analysis of the plasmids indicated that two and three *Dra*I fragments were contained in pCAH01 and pCAH02, respectively, and one of these *Dra*I fragments was found to hybridize to the mixed probes. Therefore, the desired *Dra*I fragment was subcloned into the *Sma*I site of pUC13 and designated pCAH03. Restriction enzyme mapping of the plasmid pCAH03 carrying the 2.5-kb *Dra*I fragment from *B. subtilis* chromosomal DNA was performed (Fig. 1). Southern analysis showed that the mixed probes hybridized to the 0.24-kb *Hin*dIII-*Eco*RI fragment of the insert.

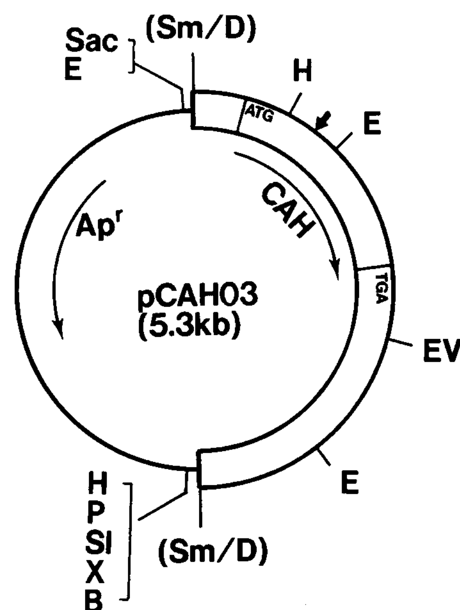

FIG. 1. Restriction map of recombinant plasmid pCAH03. The open bar indicates chromosomal DNA of *B. subtilis* SHS 0133. The solid line is vector plasmid pUC13. *Eco*RI, *Sac*I, *Sma*I, *Dra*I, *Hin*dIII, *Eco*RV, *Pst*I, *Sal*I, *Xba*I, and *Bam*HI cleavage sites are indicated by E, Sac, Sm, D, H, EV, P, Sl, X, and B, respectively. The thick arrow indicates the hybridization site of the mixed probes used. The thin arrows represent the transcriptional directions of the CAH gene and the ampicillin resistance (Ap^r) gene.

Nucleotide sequence determination. The approximately 2.0-kb region between *Dra*I and *Eco*RI was sequenced. Computer analysis of the sequence identified one major open reading frame (nucleotides 187 to 1140), with the capacity to encode a polypeptide of 318 amino acid residues (Fig. 2). The deduced amino acid sequence contained the previously determined sequences from five *Achromobacter* proteinase I-digested fragments of the CAH. The calculated molecular weight of the polypeptide is 35,571, which was in good agreement with the value of 35 kDa determined for the purified enzyme by SDS-PAGE (36). The deduced amino acid composition was essentially identical to that of the purified enzyme (36). These findings confirmed that the cloned sequence was encoding the CAH from *B. subtilis* SHS 0133. No other significant homologies were found when either the nucleotide sequence or the predicted amino acid sequence was compared with the EMBL and GenBank nucleotide or SWISSPROT protein sequences.

A purine-rich sequence, AAAGGGG, that probably serves as a ribosome-binding site (20, 24) was found 12 nucleotides upstream from the initiation codon ATG (nucleotides 187 to 189). A pair of sequences, CAGGC (nucleotides 32 to 36) and GAAAGTCT (nucleotides 56 to 63), preceding the CAH structural gene was very similar to the consensus sequences of the −35 and −10 regions for the σ^H form of *B. subtilis* RNA polymerase (28, 37). As shown in Fig. 2, four tandem repeats of an A-rich box (AAACAAA) existed between the putative promoter and initiation codon. Overlapping the two A-rich boxes, two repeated sequences with a common sequence (AAAT--TTA-AAAACAAAG--GAA) were found immediately in front of the initiation codon. These sequences matched

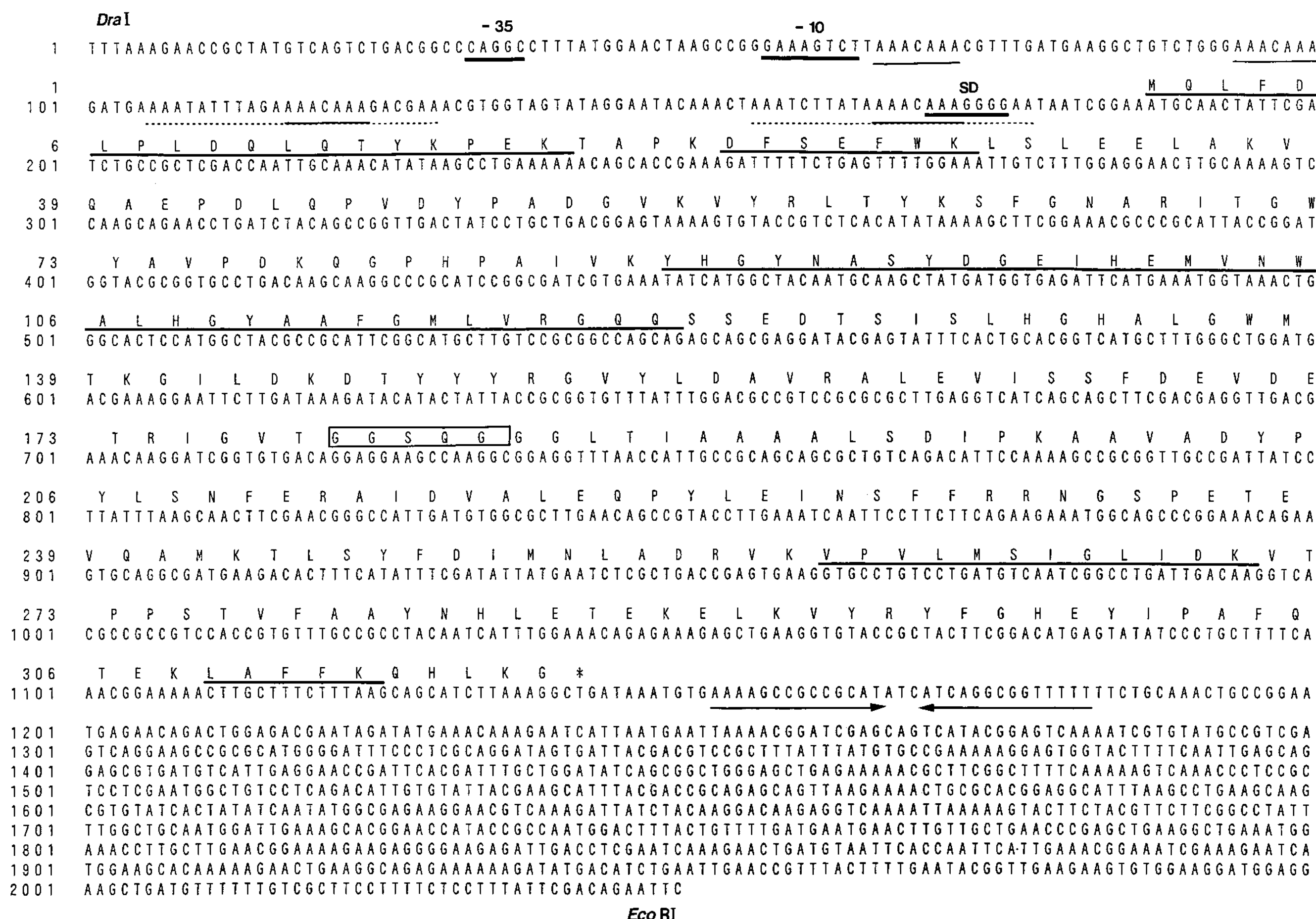

FIG. 2. Nucleotide sequence of the 2.0-kb *Dra*I-*Eco*RI fragment containing the *B. subtilis* SHS 0133 CAH structural gene. The deduced amino acid sequence is indicated above the nucleotide sequence in a single-letter code. Amino acids are numbered starting with the initiation methionine (M) as 1. The underlined amino acid sequences represent the previously determined sequences of purified CAH (36). The amino acid sequence from position 179 to 183, G-X-S-X-G, is boxed. The termination codon is designated by an asterisk. The probable Shine-Dalgarno (SD) sequence and the putative promoter (−35 and −10 regions) are shown by thick lines. The probable transcription terminator is indicated by arrows (→ and ←). The tandem repeats of the A-rich box and the repeated sequences constituting the A+T-rich block are indicated by thin and dashed lines, respectively.

in 19 of 24 bp of the common sequence. In the downstream region from termination codon TGA, an extended region of dyad symmetry was observed. This region is thought to be a rho-independent transcriptional termination site because a thymine-rich region exists just behind the stem-loop structure.

Construction of expression plasmids. The structural gene moiety of CAH in the recombinant plasmid pCAH03 was prepared as described in Materials and Methods. The CAH structural gene fragment which starts at CAA, coding the second amino acid (Gln), and extends to the 3′ noncoding region was obtained. To construct expression plasmids, two types of ATG vectors (26) were utilized (Fig. 3). One of the vectors was pKK233-2, which has the hybrid promoter *trc*, and the other was the pTrp · pAT · ATG vector, which has the *trp* promoter. Both vectors have unique *Nco*I sites between the Shine-Dalgarno sequence and the ATG initiation codon. Digestion with *Nco*I followed by filling in with Klenow fragment exposes the initiation codon. The CAH structural gene fragment with a *Pst*I site in the 3′ noncoding region was inserted between *Nco*I-Klenow and *Pst*I sites of pKK233-2, and the expression plasmid pCAH21 was constructed. In the same manner, the expression plasmid pCAH400 was constructed by ligating the CAH structural gene fragment digested with *Xba*I in the 3′ noncoding region with the pTrp · pAT · ATG vector cleaved with *Nco*I-Klenow fragment and *Xba*I.

Plasmid pCAH21 has a replication origin from pBR322 and an ampicillin resistance marker (Ap^r). To increase the copy number of the plasmid and to change the resistance marker, plasmid pCAH21 was improved. The *Bam*HI-*Sca*I fragment harboring the *trc* promoter, the CAH gene, and the 5S *rrnB* T1T2 terminator from pCAH21 was ligated to the *Eco*RI-*Dra*I fragment harboring the replication origin (*ori*) and tetracycline resistance marker (Tc^r) from pAT153, and new expression plasmids, pCAH211 and pCAH212, were constructed. In both plasmids, the transcriptions of the CAH gene and the tetracycline resistance marker are in opposite directions.

Expression of the CAH gene in *E. coli*. The expression plasmids mentioned above were introduced into *E. coli* JM103 and HB101 by transformation. Various transformants carrying each plasmid were cultured in 50 ml of the expression medium at 37°C for 24 h. The expressed CAH did not form an inclusion body but existed in an active form in the cells. The expression levels were compared by measuring the CAH activities in the soluble fractions obtained from sonicated cells. Table 1 shows the enzyme activities expressed by the various *E. coli* transformants. The CAH activities of *E. coli* JM103 carrying pCAH21,

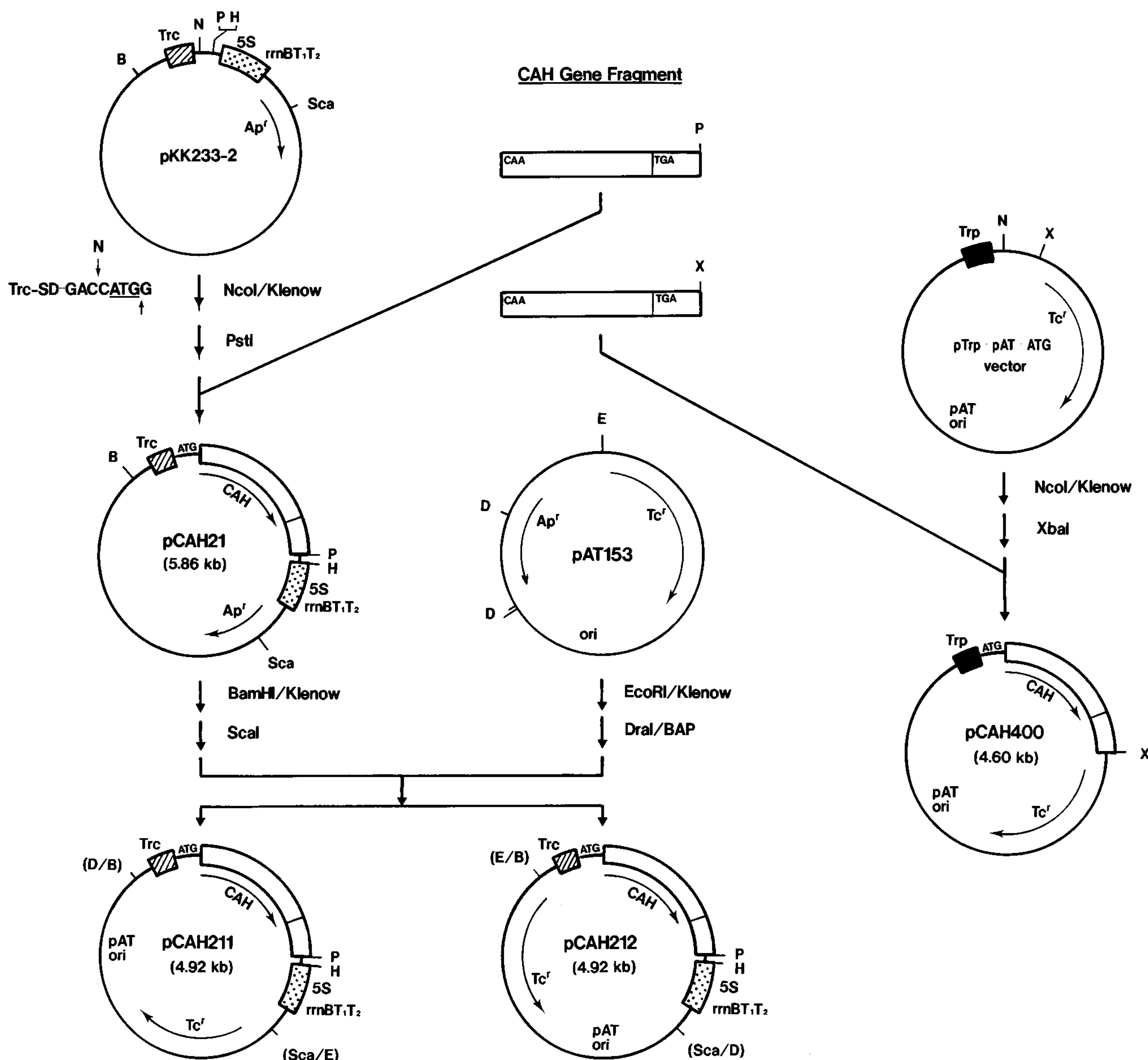

FIG. 3. Construction of various expression plasmids for CAH in *E. coli*. Symbols: open bar, CAH structural gene and 3′ noncoding region from *B. subtilis* SHS 0133; solid line, vector plasmid DNA; hatched bar, *trc* promoter; solid bar, *trp* promoter; stippled bar, 5S *rrnB* T_1T_2 terminator. *Nco*I and *Sca*I cleavage sites are indicated by N and Sca, respectively. Other restriction site abbreviations are the same as those described in the legend to Fig. 1. Abbreviations: Klenow, Klenow fragment; BAP, bacterial alkaline phosphatase.

pCAH211, and pCAH212 were about 1, 11, and 10 U/ml of culture, respectively. No induction effect of isopropyl-β-D-thiogalactopyranoside (IPTG) on the plasmids with the *trc* promoter was observed. The expression level of *E. coli* JM103 harboring pCAH400 was 115 U/ml of culture. These facts indicate that the CAH gene is expressed more effectively under the control of the *trp* promoter rather than the *trc* promoter. *B. subtilis* SHS 0133 showed productivity of 0.2 U/ml of culture under the same culture conditions. The production of CAH protein from *E. coli* JM103 harboring pCAH400 was also analyzed by SDS-PAGE of the soluble fraction from cell lysates (Fig. 4). An increase in the intensity of the protein band having a molecular mass of 35 kDa, corresponding to the CAH subunit, was observed over a time course of 21 h. After 18 to 21 h of cultivation, the expression of the protein was estimated to be almost 50% of the total proteins in the soluble fraction.

Figure 5 shows the typical pattern of large-scale CAH production. *E. coli* JM103 carrying pCAH400 was cultured in a 30-liter jar fermentor at 37°C in 20 liters of production medium containing 4% of glycerol as a carbon source. CAH production was associated with cell growth. The activity of the expressed enzyme increased during cultivation and reached a maximum value, 440 U/ml of culture, after 24 h of incubation.

DISCUSSION

Several research groups have reported on the substrate specificity of CAHs from citrus peels and *B. subtilis* and other

TABLE 1. Comparison of CAH expression levels of various recombinant strains

Host strain	Plasmid	Addition of IPTG[a]	Growth (A_{650})	CAH activity[b] (U/ml)
E. coli JM103	pCAH21	−	6.69	0.8
		+	6.96	0.9
	pCAH211	−	7.65	9.0
		+	5.31	12.1
	pCAH212	−	6.39	8.6
		+	6.00	10.4
	pCAH400	−	4.62	115.2
E. coli HB101	pCAH400	−	6.27	117.2
B. subtilis SHS 0133		−	10.2	0.2

[a] If necessary, IPTG was added at 1 mM at 4 h of incubation.
[b] CAH activity was measured in the soluble fraction obtained after 24 h of culture.

microorganisms (1, 9, 16, 18); however, these enzymes have not been characterized at the gene level. In this report, we describe the cloning and expression of a CAH gene from *B. subtilis* SHS 0133. Determination of the nucleotide sequence showed that the CAH structural gene was composed of 954 bp and coded for the subunit of CAH constituting the octamer structure. The primary structure of the CAH subunit was elucidated from the deduced amino acid sequence. The sequence from positions 179 to 183, Gly-Gly-Ser-Gln-Gly, corresponded to the common sequence Gly-X-Ser-X-Gly (6, 17, 29, 32) found in many esterases, lipases, and serine proteases. Ser-181 in the sequence is thought to be an active-site residue because CAH activity was strongly inhibited by the serine affinity labeling agent diisopropylfluorophosphate (36).

From the nucleotide sequence shown in Fig. 2, we presumed the locations of the promoter region and the ribosome-binding site. In the upstream region of the CAH structural gene, four tandem repeats of an A-rich box and two repeated sequences of a large A+T-rich block were also detected. The existence of a sequence resembling the A-rich box has been reported on other promoters, e.g., *sdh* (21) and *spo0A* (8) of *B. subtilis* and *nprM* (41) and *nprT* (35) of *Bacillus stearothermophilus*. These boxes are known to be a common region recognized by some activating factors. Overlapping the A-rich box, two large A+T-rich blocks were situated between the promoter-like region and the initiation codon. Similar repeated sequences were found in the upstream regions of the *Bacillus circulans* xylanase gene (42) and some powerful *E. coli* promoters such as *trp* (25) and λ phage p_L promoters (15). Since these sequences are shown to be necessary for maximal transcriptional activity of their own promoters, the A+T-rich blocks in the 5′ flanking region of the CAH gene may have a similar function.

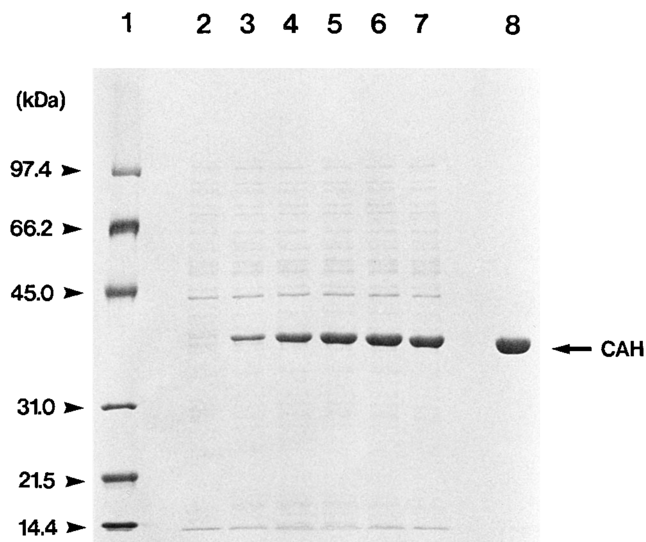

FIG. 4. SDS-PAGE analysis of the soluble fraction from cell lysates of *E. coli* harboring pCAH400. The soluble fractions were prepared from cells harvested at various culture times and used as analysis samples. Lanes: 1, molecular weight markers; 2 to 7, soluble fractions from *E. coli* JM103(pCAH400) harvested at 6, 9, 12, 15, 18, and 21 h, respectively; 8, purified CAH. The numbers on the left indicate the molecular masses of the protein standards. The position of CAH is indicated by an arrow.

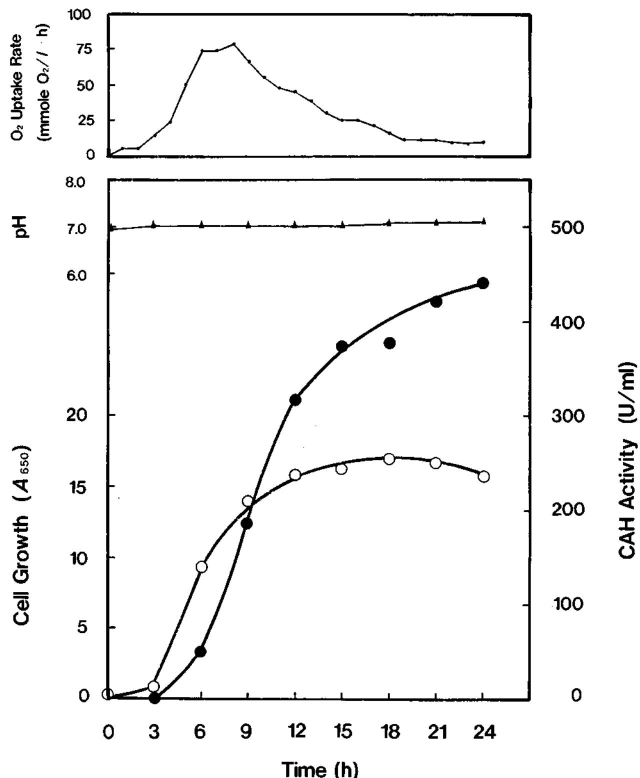

FIG. 5. Time course of the CAH production of *E. coli* harboring pCAH400 in a 30-liter jar fermentor. Symbols: ○, cell growth (A_{650}); ●, CAH activity in the soluble fraction from cell lysates (units per milliliter of culture); ▲, pH of the broth. The panel at the top of the figure indicates the O_2 uptake rate (millimoles of O_2 per liter of culture per hour).

The cloned CAH gene was easily expressed as an active form in *E. coli* by use of various recombinant plasmids. The fact that recombinant *E. coli* harboring pCAH211 or pCAH212 expressed 10 U of CAH per ml of culture, which was 10 times higher than that of *E. coli* carrying pCAH21 (Table 1), indicates that the expression level depends on the plasmid vector used. Although the plasmid pCAH21 was derived from pBR322, plasmids pCAH211 and pCAH212 were constructed on the basis of pAT153, a high-copy-number variant of pBR322 (39). The plasmid pCAH400 was created to express CAH under the control of the *trp* promoter from the pTrp · pAT · ATG vector having the same replication origin as pAT153. In contrast to pCAH211 or pCAH212 harboring the *trc* promoter, the CAH expression level of pCAH400 reached over 100 U/ml of culture. These findings indicate that the CAH gene expression was markedly influenced by promoters used for plasmid construction.

To develop a practical method of CAH production, we optimized the culture conditions by using a 30-liter jar fermentor. As shown in Fig. 5, *E. coli* JM103(pCAH400) produced 440 U of CAH per ml of culture during 24 h of incubation. This value corresponds to 2.1 g of CAH protein in 1 liter of culture broth because the specific activity of the purified recombinant CAH is 210 U/mg (data not shown).

Another notable feature of CAH expression is that the CAH protein was produced in a soluble state and did not form an inclusion body frequently found in the *E. coli* expression system. As a result, the expressed CAH was easily recovered as an enzymatically active octamer structure from the soluble fraction obtained after cell disruption. Thus, the expression system using recombinant *E. coli* provides an industrial method for CAH production.

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