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Cloning and Characterization of a Conjugated Bile Acid Hydrolase Gene from *Clostridium perfringens*

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The gene encoding a conjugated bile acid hydrolase (CBAH) from *Clostridium perfringens* 13 has been cloned and expressed in *Escherichia coli*, and its nucleotide sequence has been determined. Nucleotide and predicted amino acid sequence analyses indicated that the gene product is related to two previously characterized amidases, a CBAH from *Lactobacillus plantarum* (40% identity) and a penicillin V amidase from *Bacillus sphaericus* (34% identity). The product is apparently unrelated to a CBAH from *C. perfringens* for which N-terminal sequence information was determined. The gene product was purified from recombinant *E. coli* and used to raise antibody in rabbits. The presence of the protein in *C. perfringens* was then confirmed by immunoblot analysis. The protein was shown to have a native molecular weight of 147,000 and a subunit molecular weight of 36,100, indicating its probable existence as a tetramer. Disruption of the chromosomal *C. perfringens* CBAH gene with a chloramphenicol resistance cartridge resulted in a mutant strain which retained partial CBAH activity. Polyacrylamide gel electrophoresis followed by enzymatic activity staining and immunoblotting indicated that the mutant strain no longer expressed the cloned CBAH (CBAH-1) but did express at least one additional CBAH (CBAH-2). CBAH-2 was immunologically distinct from CBAH-1, and its mobility on native polyacrylamide gels was different from that of CBAH-1. Furthermore, comparisons of pH optima and substrate specificities of CBAH activities from recombinant *E. coli* and wild-type and mutant *C. perfringens* provided further evidence for the presence of multiple CBAH activities in *C. perfringens*.

Among the many compounds metabolized by the gastrointestinal microflora of mammals are the endogenously produced bile acids. These "biological detergents" are synthesized in the liver from cholesterol and are typically secreted in the form of amino acid conjugates, with an amide bond between the bile acid carboxyl group and the amino group of either glycine or taurine. Once in the intestine, the bile acids may be converted to a number of metabolites by the intestinal microflora. One of the more common reactions, the hydrolysis of the amide linkage, is catalyzed by a class of microbial enzymes referred to collectively as conjugated bile acid hydrolases (CBAH). These enzymes are produced by a number of different bacteria, including, but not limited to, members of the genera *Clostridium*, *Bacteroides*, *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* (1, 5, 9, 10, 12–14, 17, 20, 23–26, 33, 42, 43, 45). Because the efficient enterohepatic recirculation of bile acids is partially dependent on their recognition in the conjugated form by active transport sites in the terminal ileum (8, 36), bacterial CBAH may be viewed as being in competition with these active transport sites. Binding of a conjugated bile acid to an active transport site results in its return to the liver, while hydrolysis by bacterial CBAH results in production of a free bile acid which binds with a lower affinity to the transport sites (36, 40) and thus may pass into the large intestine or cecum, where further metabolism may occur. The most important metabolic transformation which these free bile acids may undergo is 7-dehydroxylation. This reaction results in the conversion of cholic acid or chenodeoxycholic acid to deoxycholic acid or lithocholic acid, respectively. These last two "secondary" bile acids are more hydrophobic than their parent bile acids and have been implicated in a variety of cytotoxic phenomena (3, 30, 34, 37). Because of the important role which CBAH plays in these subsequent reactions, we are interested in the enzymology and the regulation of CBAH activity.

A CBAH from *Clostridium perfringens* is available commercially and has been widely used for hydrolysis of conjugated of bile acids preliminary to chemical analysis. Although a CBAH from *C. perfringens* MCV 815 has been purified and characterized (11), little is known about the enzymology of the reaction or the role the enzyme plays in the growth of the organism in the gastrointestinal tract. The early studies of Nair and coworkers (27–29, 35) suggested that there were actually multiple forms of CBAH present in *C. perfringens* on the basis of observations of differential stabilities of activity against different conjugated bile acids substrates. We present in this work a confirmation of this earlier observation of multiple CBAHs in *C. perfringens* and provide the nucleotide sequence and derived amino acid sequence for one CBAH isozyme of *C. perfringens* 13.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *C. perfringens* 13 was obtained from Edmund Stellwag, Department of Biology, East Carolina University, and was grown on brain heart infusion medium (BHI) under an atmosphere of CO_2-H_2-N_2 (10:10:80) in a Coy anaerobic chamber at 37°C. *Escherichia coli* DH5αMCR and NM522 were obtained from GIBCO-BRL Life Technologies and were grown aerobically on Luria-Bertani medium on a rotary shaker at 37°C. pUC18 (*Bam*HI and bacterial alkaline phosphatase digested) and pKK233-2 were obtained from Pharmacia Biotech, Inc., and pHR106 (38) was obtained from Phillip Hylemon, Medical College of Virginia, Virginia Commonwealth University. The following compounds were added to growth media when appropriate at the indicated final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 10 μg/ml; and isopropyl-β-D-thiogalactoside (IPTG), 0.2 mM.

Recombinant DNA techniques. Plasmid DNA was isolated from *E. coli* and *C. perfringens* by the alkali lysis procedure (21). Templates for DNA sequencing were purified with Qiagen columns (QIAGEN, Inc., Chatsworth, Calif.). Chromosomal DNA was isolated from *C. perfringens* by the procedure of Marmur (22), with the inclusion of the lysozyme pretreatment. Restriction endonuclease

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digestions were carried out as recommended by the suppliers. Electrophoresis of DNA, ligation reactions, sucrose gradient centrifugation, and other nucleic acid manipulations were performed as described by Maniatis et al. (21).

Plasmid library preparation. Genomic DNA from *C. perfringens* 13 was partially digested and subjected to sucrose density gradient centrifugation as described by Maniatis et al. (21). Fractions containing DNA in the 7-kb size range were pooled, and an aliquot was ligated to *Bam*HI-digested bacterial alkaline phosphatase-treated pUC18 and transformed into *E. coli* DH5αMCR (Library Efficiency; GIBCO-BRL). Following a 1-h outgrowth period, the cells were spread onto plates containing the differential medium described by Christiaens et al. (6). This medium is a modified Luria-Bertani agar containing additional glucose as well as 0.5% sodium taurodeoxycholate and is buffered to pH 6.0. Action of CBAH in this environment causes hydrolysis of the soluble sodium taurodeoxycholate to deoxycholate, which is insoluble at the medium pH and forms a white precipitate. After 12 to 24 h, plates were observed for colonies with precipitated deoxycholate halos. These were picked and restreaked to purity.

DNA sequencing. DNA was sequenced by the dideoxynucleotide chain-terminating method (39) with alkali-denatured double-stranded plasmid templates, according to the procedure supplied with the Sequenase version 2.0 sequencing reagent kit (U.S. Biochemical). Labeling reactions were performed with [α-^{35}S]ATP (1325 Ci/mmol; New England Nuclear). Universal M13/pUC forward and reverse primers were used for sequencing those regions flanking vector sequences, while internal regions were sequenced with specific primers based on observed DNA sequences. All primers were synthesized by the DNA Synthesis Core Laboratory, School of Medicine, East Carolina University.

Analysis of sequence data. Nucleic acid and protein sequence analyses were performed with the GCG Sequence Analysis Software Package (Genetics Computer Group, Inc., Madison, Wis.). Sequence information was submitted to GenBank using AUTHORIN (Intelligenetics, Inc., Mountain View, Calif.).

Disruption of CBAH gene in *C. perfringens*. The clostridial chloramphenicol acetyltransferase (CAT) gene contained on plasmid pHR106 (38) was excised by digestion with *Eco*RI and *Hin*dIII. After the ends were blunted by treatment with Klenow fragment, the CAT gene was ligated to a derivative of the original CBAH-pUC18 recombinant plasmid. This derivative was generated by exonuclease III deletion of the upstream sequence to within 68 bp of the translational start site, followed by digestion with *Xba*I and *Sma*I. The resulting CBAH-CAT construct consisted of 679 bp of the N-terminal portion of the CBAH reading frame (approximately two-thirds of the total), followed by the CAT gene, followed by vector sequence. Since the pUC replicon is not active in *C. perfringens* 13, this construct was designed to disrupt the *C. perfringens* CBAH gene via chromosomal recombination. The plasmid was introduced into *C. perfringens* by electroporation. Cells were prepared by growing them anaerobically in BHI to the mid-logarithmic phase of growth. After the cells had been harvested by centrifugation at 6,000 × *g* for 15 min, they were washed twice in ice-cold 2.5 mM sodium phosphate–10% (vol/vol) polyethylene glycol (pH 6.5) and resuspended in 0.02× the original culture volume of this same buffer. Cell suspensions (120 μl) containing 1 to 5 μg of plasmid were electroporated at 5,000 V/cm with a Bio-Rad Gene Pulser with Pulse Controller (0.2-cm cuvettes, 1,000 V, 25 μF, infinite resistance setting). They were then immediately transferred to 5 ml of BHI and incubated anaerobically at 37°C for 3 h before aliquots were plated on BHI agar plates containing 10 μg of chloramphenicol per ml. Chloramphenicol-resistant colonies were restreaked to purity before large-scale liquid cultures were grown. These cultures were used for protein and enzyme analysis, as well as for DNA analysis to verify the presence of the CAT gene and altered CBAH gene by restriction enzyme digestion and Southern blot analysis.

Protein and enzyme assays. Protein was assayed by the dye-binding method of Bradford (4) with bovine serum albumin as the standard with commercially available reagents (Bio-Rad Laboratories). Conjugated bile acid hydrolase activity was assayed colorimetrically by measuring the amount of taurine liberated after a fixed period at 37°C. The standard assay was performed in 1.5-ml microcentrifuge tubes; the assay mixture contained, in a final volume of 0.5 ml, 25 μmol of sodium acetate (pH 5.5), 0.5 μmol of dithiothreitol, 0.5 μmol of EDTA, 1 μmol of sodium taurocholate, and enzyme. Reactions were initiated by the addition of the sodium taurocholate after a 5-min equilibration at 37°C. After 5 min, the reaction was stopped by addition of an equal volume of 20% (wt/vol) trichloroacetic acid. After centrifugation for 5 min to remove precipitated protein, 100 μl of the mixture was assayed for free amino groups by the ninhydrin reaction, using the procedure described by Lee and Takahashi (16). One unit of activity is defined as that amount which catalyzes the liberation of 1 μmol of taurine in 1 min under the conditions described above. To determine the effect of buffer pH on enzymatic activity while avoiding the possibility of buffer-specific reaction differences, a mixed-buffer system was used. A stock 2× buffer which contained 30 mM citric acid, 30 mM boric acid, and 30 mM monobasic potassium phosphate was prepared. The pH of the buffer was adjusted to various points from 2.5 to 9.0 in 0.5 pH unit increments with sodium hydroxide. Each of these buffer stocks was used in place of the sodium acetate component in the standard assay described above. The CBAH activity in chromatography fractions during purification was monitored by a precipitation assay. Assays were performed in round-bottom 96-well microtiter plates. Aliquots (1 to 20 μl) of fractions were added to individual wells containing 100 μl of reaction cocktail (50 mM sodium phosphate [pH 6.0], 10 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM sodium taurodeoxycholate). Samples containing CBAH activity generate free deoxycholate, which is insoluble at the reaction pH. This is easily visualized as a white precipitate after approximately 15 min in samples with high activity. The length of time required to form visible precipitate corresponds roughly to the level of activity in the fraction. By scoring plates for activity every 5 min, the relative activity present in different fractions can be estimated very accurately.

Preparation of *C. perfringens* and *E. coli* cell extracts and purification of CBAH from *E. coli*. All steps were performed at 4°C, except for column chromatographies, which were performed at 25°C. *E. coli* NM522 containing plasmid with the CBAH gene downstream from the *trp-lac* promoter (see Results) was grown from a 5% inoculum with overnight cultures in Luria-Bertani medium containing 100 μg of ampicillin per ml and 0.2 mM IPTG. Cells were harvested in the early stationary phase of growth (approximately 7 h after inoculation) by centrifugation and resuspended in 3 volumes of 20 mM sodium phosphate–10 mM 2-mercaptoethanol–1 mM EDTA (pH 7.0) (buffer A). The suspension was lysed by two passages through a French pressure cell (14,000 lb/in^2) and centrifuged at 17,000 × *g* for 45 min to remove debris and unbroken cells. The supernatant was adjusted to a protein concentration of approximately 20 mg/ml, brought to 50% saturation with solid ammonium sulfate, equilibrated for 30 min at 4°C, and then centrifuged at 14,000 × *g* for 30 min. The pellet was suspended in 0.5× the original volume in buffer A containing 0.5 M ammonium sulfate (pH 6.0) (buffer P). Following centrifugation at 5,000 × *g* for 15 min to remove insoluble material, the solution was applied to a phenyl-Sepharose CL-4B column (2.5 by 9.0 cm) equilibrated with buffer P. After the column was washed with buffer P until the effluent A_{280} returned to baseline, CBAH was eluted with a linear decreasing salt gradient (0.6 to 0 M ammonium sulfate in 20 mM sodium phosphate–10 mM 2-mercaptoethanol [pH 7.0]). Active fractions (as assayed by the 96-well plate precipitation method) were combined and applied to a Q Sepharose Fast Flow column equilibrated with 20 mM sodium phosphate–10 mM 2-mercaptoethanol–5% glycerol (pH 7.0) (buffer Q). After the column was washed with buffer Q until the effluent A_{280} had returned to baseline, CBAH was eluted with a 0 to 0.5 M NaCl gradient in buffer Q (200 ml total volume). Active fractions were combined, concentrated by ultrafiltration through an Amicon YM-10 membrane, and stored at −20°C. Extracts from *C. perfringens* 13 (wild type and mutant) were prepared by growing cells in BHI to either mid-logarithmic phase or late stationary phase. The harvested cell pellets were suspended in 3 volumes of buffer A and disrupted by two passages through a French pressure cell. Following centrifugation at 17,000 × *g* for 45 min, the supernatant was dialyzed overnight against buffer A and used without further purification for enzyme assays and polyacrylamide gel electrophoresis (PAGE) analyses.

Production of antibody. Antibodies to CBAH were produced in the laboratory of Mason Smith, School of Medicine, East Carolina University. CBAH purified from *E. coli* was used to raise antibody in a female New Zealand White rabbit. Following an initial injection in Freund's complete adjuvant, booster injections were given in Freund's incomplete adjuvant. Serum from test bleeds was assayed by Western blot (immunoblot) analysis to determine reactivity and specificity. Following final bleeding by cardiac puncture, serum was allowed to clot at room temperature for 2 h and centrifuged. The supernatant was stored overnight at 4°C and recentrifuged to remove additional precipitate. Immunoglobulins were then enriched by ammonium sulfate precipitation as described by Ausubel et al. (2). Nonspecific antibodies to *E. coli* proteins were removed by treatment of aliquots of the antibody solution with crude extracts prepared from *E. coli* NM522 coupled to Sepharose (Affi-Gel 15; Bio-Rad), as described by Maniatis et al. (21). Coupling of protein to the Affi-Gel was performed as specified by the manufacturer.

Protein electrophoresis and immunoblotting. Sodium dodecyl sulfate (SDS)-PAGE was carried out in 0.75-mm-thick gradient gels (7 to 20% total acrylamide [T], 2.7% cross-linker [C]), using the discontinuous buffer system of Laemmli (15). High- and low-molecular-weight protein standards (Bio-Rad Laboratories) or 10-kDa molecular mass markers (GIBCO-BRL) were used as size markers. Following electrophoresis, proteins were stained with 0.03% (wt/vol) Coomassie brilliant blue R-250 in 15% (vol/vol) methanol–10% (vol/vol) acetic acid. Native gel electrophoresis was carried out in 1.5-mm gradient gels (5 to 15% T, 2.7% C) with the Laemmli buffer system, omitting the SDS and including 5 mM sodium thioglycolate. Electrophoresis was conducted at 4°C at 150 V (constant voltage) until the bromphenol blue tracking dye reached the bottom of the gel. CBAH activity was detected by first washing the gel twice for 15 min each in 250 ml of 0.5 M sodium acetate–10 mM 2-mercaptoethanol–1 mM EDTA (pH 5.5) at 10°C and then incubating the gel at 37°C in 50 ml of 0.5 M sodium phosphate (pH 5.5)–10 mM 2-mercaptoethanol–10 mM sodium taurodeoxycholate. Within 1 h, CBAH activity in the gel resulted in the formation of a white precipitate of deoxycholic acid at the position of the enzyme. The gels were then photographed under tungsten light on a glossy black background with Polaroid Type 55 film. The negative was then rephotographed to produce the negative image shown in this work. Control gels were incubated with the reaction buffer lacking taurodeoxycholate to ensure that observed bands were not due to acid denaturation and precipitation of proteins. For Western analyses, proteins were electrotransferred to nitrocellulose membranes immediately following electrophoresis. The methanol-Tris-glycine transfer buffer of Towbin et al. (46) was used for both SDS-containing and native gels; however, native gels were incubated at 37°C for 30 min in Laemmli tank buffer containing 0.5% SDS prior to electrotransfer to facilitate the migration of proteins from the gel. Following incubation with the NM522 extract-absorbed antiserum to CBAH, membranes were incubated with

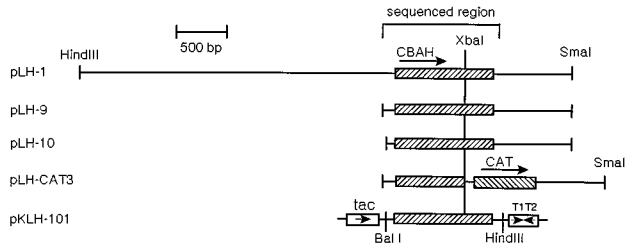

FIG. 1. Relationships between pLH-1 and derivatives. The originally cloned fragments and four derivatives referred to in the text are shown. All members of the pLH series are inserts in pUC-18, while pKLH-101 is derived from the expression vector pKK233-2. The arrows above the CBAH and CAT genes indicate the direction of transcription and translation. The arrow within the *tac* promoter box indicates the orientation for transcript initiation. The downstream terminator of pKK233-2 (T1T2) is indicated by the opposing arrows within the boxed region. The bracket at the top of the figure indicates the region for which the nucleotide sequence is reported in this work. This region was sequenced in both strands over its entirety.

horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. Detection was then performed with 4-chloro-1-naphthol reagent.

Determination of native molecular weight. CBAH purified from recombinant *E. coli* as described above was chromatographed on a Sephacryl S-200 column (2.5 by 95 cm) equilibrated with 20 mM sodium phosphate (pH 7.0), 100 mM sodium chloride, and 10 mM 2-mercaptoethanol. The peak fractions were identified by measuring enzymatic activity in the 96-well plate assay. The column was calibrated with cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), yeast alcohol dehydrogenase (150 kDa), and sweet potato β-amylase (200 kDa).

Nucleotide sequence accession number. The nucleotide sequence of the DNA fragment containing the CBAH gene has been submitted to GenBank with the accession number U20191.

RESULTS

Identification and subcloning of the CBAH gene. A genomic library of *C. perfringens* 13 was constructed by ligation of strain 13 DNA which had been partially digested with *Mbo*I-to-*Bam*HI-digested, phosphatase-treated pUC18. Following transformation into *E. coli* DH5αMCR and plating on differential medium (6), 1 colony from the approximately 3,000 total screened produced a halo of precipitate. This colony was streaked to purity, plasmid was isolated, and crude extracts were prepared and subjected to enzymatic analysis. Plasmid isolates were shown to contain a 5,000-bp insert in pUC18 and were designated pLH-1. Enzymatic analyses of crude extracts verified that the strain was catalyzing the hydrolysis of taurodeoxycholate. Restriction mapping and exonuclease III deletion analyses were performed to localize the CBAH gene on this fragment. The restriction map and relationship of various subclones to the parent fragment are depicted in Fig. 1. Deletions from the *Hin*dIII end of the multiple cloning region into the insert retained CBAH activity down to an insert size of approximately 1,900 bp. Two of these deletion plasmids, differing in length by 36 bp, were used for the majority of the sequencing, mutagenesis, and *E. coli* expression studies and are designated pLH-9 and pLH-10. Additional deletions extending further into the insert were used for DNA sequencing purposes.

Nucleotide and amino acid sequence analysis. The region of pLH-9 proximal to the *Hin*dIII side of the pUC18 multiple cloning region, as well as more extensive exonuclease III-deleted inserts, were subjected to chain-terminating nucleotide sequence analysis of both strands (Fig. 2). The resulting 1,087 bp of sequence information revealed a 990-bp open reading frame (bases 69 to 1058), with a coding potential for a 329-residue polypeptide (37,185 Da). The base composition of the open reading frame (27.5% G+C) is consistent with a clostridial origin. A potential Shine-Dalgarno sequence was found with a spacing of 11 bases to the putative start codon. A protein database search revealed that the hypothetical polypeptide (CPE13-A) is related to a CBAH from *Lactobacillus plantarum* (LPL; 39.8% identity) (6), as well as to the penicillin V amidase from *Bacillus sphaericus* (BSPPVA; 34.2% identity) (31) (Fig. 3). Also included in the sequence comparison are the sequences of N-terminal peptides determined from the CBAHs purified from *C. perfringens* MCV 815 (CPE815) (11) and from *Lactobacillus* sp. strain 100-100 (LSP100-A) (18). While the two *Lactobacillus* sequences had 13 identical amino acids over a stretch of 25 residues at the N terminus, there was little sequence homology between the CBAHs from the two *C. perfringens* strains (three matches over 13 residues, including the N-terminal methionine). Although no definitive conclusions should be drawn from comparisons within such a small region, this does suggest that there may be fundamental differences between the two *C. perfringens* enzymes. Also indicated in Fig. 3 are residues which are conserved throughout all three of the proteins whose complete predicted sequence is shown. Several highly conserved regions are notable. Protein database searches with these short peptide sequences provided no information pointing to additional relatives to this cluster.

Expression and purification of CBAH from *E. coli*. To confirm the existence of a protein corresponding to the cloned CBAH in the parent organism, we used immunoblot analyses. For this purpose, it was necessary to purify sufficient amounts of protein from recombinant *E. coli* for antibody preparation. Expression was maximized by subcloning the CBAH reading frame, along with the Shine-Dalgarno sequence, into a position downstream from the *trp-lac* promoter of the expression vector pKK223-3. This was facilitated by PCR amplification of the cloned fragment with primers engineered to contain a *Bal*I site (5′ end) and a *Hin*dIII site (3′ end) (Fig. 2). After digestion of the amplification reaction mixture with these two enzymes and gel purification, the fragment was ligated to pKK223-3 prepared by digestion with *Nco*I (promoter proximal) and filling in protruding ends with Klenow fragment, followed by digestion with *Hin*dIII (promoter distal). The resulting construct, pKLH-101, when transformed into *E. coli* NM522, produced high levels of CBAH activity in response to the presence of IPTG (0.2 mM) in the growth medium. The enzyme from *E. coli* NM522(pKLH-101) cultures induced with IPTG was purified by a combination of ammonium sulfate fractionation, hydrophobic interaction chromatography (Phenyl Sepharose CL-4B), and anion-exchange chromatography (Q Sepharose Fast Flow). The results of a typical purification from 7 g (wet weight) of cells (2 liters of culture) are summarized in Table 1. Approximately 200 to 300 μg of this material was used to raise rabbit antibody. The remainder was used for physical and biochemical characterization.

Native and subunit size of CBAH. Chromatography of purified CBAH-1 on a calibrated Sephacryl S-200 column provided a native molecular weight estimation of 147,000 (data not shown). SDS-PAGE analysis of purified CBAH-1 indicated a single polypeptide with molecular weight of 36,100. This correlates well with the calculated molecular weight based on analysis of the open reading frame (i.e., 37,185). Together, these data suggest that the enzyme exists in its native state as a tetramer.

Insertional mutagenesis of CBAH gene in *C. perfringens* 13. The gene in *C. perfringens* corresponding to the cloned CBAH gene was disrupted by insertional mutagenesis with a suicide vector (pLH-CAT3) based on pUC8 and containing an N-terminal portion of the CBAH gene upstream from the clos-

FIG. 2. Nucleotide sequence of CBAH gene. The sequence represents the 5′-proximal region of pLH-9. The 5′ end of pLH-10 is at residue 37 (▼). The deduced amino acid sequence is shown below the nucleotide sequence. A putative ribosome-binding region is underlined. The two oligonucleotide sequences used to amplify the CBAH gene for overexpression in *E. coli* are shown at the 5′ and 3′ ends, along with the position of the *Bal*I and *Hin*dIII sites contained within them. The sequence of pUC18 which adjoins the 5′ end of the CBAH gene in pLH-10 is shown between the CBAH gene and the 5′ amplification primer. The *Xba*I cleavage site at residue 748 represents the 3′ end of the CBAH derivative used for mutagenesis, after the introduction of the CAT gene between this position and the *Hin*dIII site of pUC18.

tridial CAT gene of pHR106 (Fig. 1). Thus, following electroporation of pLH-CAT3 into *C. perfringens* 13, the presence of chloramphenicol-resistant colonies is indicative of recombination of the plasmid into the chromosome. This would most probably be directed by the homology between the chromosomal CBAH gene and the fragment of the same gene present on the plasmid. Southern blot analysis of DNA isolated from several chloramphenicol-resistant colonies with a CBAH gene-specific probe revealed a band shift relative to the wild-type DNA, indicating that the recombinational event had taken place within the CBAH gene (data not shown). One of these mutants was chosen for enzymatic analyses and was designated *C. perfringens* 13-3. It was grown in the presence of chloramphenicol at all times because of the potential instability of this single-crossover type of insertional mutation.

CBAH expression in bacterial extracts. CBAH assays (ninhydrin method) were performed on extracts prepared from *E. coli* NM522, DH5αMCR(pLH-1), and NM522(pKLH-101) and *C. perfringens* 13 and 13-3. The results are shown in Table 2. Strain NM522(pKLH-101) (IPTG induced) expressed CBAH at levels approximately 25-fold higher than did DH5αMCR(pLH-1) and 180-fold higher than did *C. perfringens* 13. The putative insertional mutant, strain 13-3, continued to express CBAH activity at 86% of wild-type levels. This result suggested either that the CBAH-1 gene had not been inactivated completely or that other CBAH enzymes were present, with our enzyme, hereafter designated CBAH-1, making up only a small fraction (14%) of the total CBAH activity.

SDS-PAGE fractionation followed by Western blot analysis of crude extracts prepared from wild-type strain 13, mutant

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          1                                                  50
CPE815      MCrtklvi Tigas
CPE13-A   ..mCtglale tkdglhlFgR nmDieysfnq sIifiPRnFk c.vnksnkke
LPL       ..mCtaityq synn..yFgR nfDyeisyne mVtitPRkYp l.vfr.kven
LSP100-A    gTsIvY. SsNNhhYFGR NlDlqISfg
BSPPVA    mlgCsslsir ttddkslFaR tmDftmepds kViivPRnYg irllekenvv
CONSENSUS ---C------ -------F-R --D------- -$---PR-$- ----------

          51                                                 100
CPE13-A   lttkYAvlGm gtifddyPtF aDgmNEKGLg cAgLnFpvYv sYskediegk
LPL       ldhhYAiiGi tadvesyPlY yDamNEKGLc iAgLnFagYa dYkkyd.adk
BSPPVA    innsYAfvGm gstditsPvL yDgvNEKGLm gAmLyYatFa tYadepkkgt
CONSENSUS ----YA--G- -------P-$ -D--NEKGL- -A-L-$--$- -Y--------

          101                                                150
CPE13-A   tnIpvynfll wvLanfssVe EVkealknan ivdipiseni pnttLHWmis
LPL       vnItpfelip wlLgqfssVr EVkknigkln lvninfseql plspLHWlva
BSPPVA    tgInpvyvis qvLgncvtVd DViekltsyt llneaniilg fappLHYtft
CONSENSUS --I------- --L-----V- $V-------- ---------- ----LH$---

          151                                                200
CPE13-A   DitgkSIVVE qtKeklnVfd nnIGVLTNsP tFDWhvaNLn qYvglryNqv
LPL       D.kqeSIVIE svKeglkIyd npVGVLTNnP nFDYqlfNLn nYrals.Nst
BSPPVA    DasgeSIVIE pdKtgitIhr ktIGVMTNsP gYEWhqtNLr aYigvtpNpp
CONSENSUS D----SIV$E --K----$-- --$GV$TN-P -$$$---NL- -Y-----N--

          201                                                250
CPE13-A   pefkLgDq.s LtaLgqGtGl vGLPGDFtpa sRFiRvAFlr damikndkDs
LPL       pqnsFsEkvd LdsYsrGmGg lGLPGDLssm sRFvRaAFtk lnslpmqtEs
BSPPVA    qdimMgD.ld LtpFgqGaGg lGLPGDFtps aRFlRvAYwk kytekaknEt
CONSENSUS ----$-$--- L--$--G-G- -GLPGD$--- -RF-R-A$-- --------$-

          251                                                300
CPE13-A   idlieFFHIL nnVamvrGst rtvEeKsDlT qYtScmclek giYYYntYEN
LPL       gsvsqFFHIL gsVeqqkGlc evtDgKyEyT iYsSccdmdk gvYYYrtYDN
BSPPVA    egvtnLFHIL ssVnipkGvv ltnEgKtDyT iYtSamcaqs knYYFklYDN
CONSENSUS -----$FHIL --V----G-- ---$-K-$-T -Y-S------ --YY$--Y$N

          301                                  339
CPE13-A   nqInaIdMnk EnLdgnEikt Ykynktlsin hvN*.....
LPL       sqInsVnLnh EhLdttElis Yplrseaqyy avN*.....
BSPPVA    srIsaVsLma EnLnsqDlit Fewdrkqdik qlNqvnvms*
CONSENSUS --I--$-$-- E-L---$--- $--------- --N------
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FIG. 3. Amino acid sequence comparisons of the CBAH-PVA group. The entire amino acid sequences (based on nucleotide sequence data) for the CBAH from *C. perfringens* (CPE13-A; this work), the CBAH from *L. plantarum* (LPL) (6), and the penicillin V amidase from *Bacillus sphaericus* (BSPPVA) (32) are shown, along with the N-terminal sequences (italicized) of the CBAH from *C. perfringens* MCV 815 (CPE815) (11) and the α polypeptide of the CBAH-A from *Lactobacillus* sp. strain 100-100 (LSP100-A) (19). For the three entire sequences, conserved amino acids are shown in capital letters. Shown below them is the consensus sequence, with those residues identical in all three proteins indicated in capital letters and those positions with conserved but nonidentical residues indicated by the $. For the two N-terminal peptides, the capital letters designate positions where there is identity with the protein from the related organism (i.e., CPE815 versus CPE13-A and LSP100-A versus LPL).

strain 13-3, and *E. coli* NM522 with and without pKLH-101 indicated that CBAH-1 (37-kDa polypeptide) was present only in wild-type strain 13 and in *E. coli* NM522 carrying the CBAH-1 gene on the expression vector (Fig. 4A and B). No immunoreactive bands corresponding to the position of CBAH-1 were observed in *C. perfringens* 13-3 or *E. coli* NM522 lacking the CBAH gene, nor were new bands at different positions observed. The intensity of the CBAH-1 band in *C. perfringens* 13 extracts did not change in log-phase versus stationary-phase cells, indicating that the protein was produced constitutively throughout the growth cycle.

Native gel electrophoresis followed by CBAH activity staining clearly demonstrates the presence of an additional CBAH (CBAH-2) in wild-type strain 13 and mutant strain 13-3 (Fig. 4C). Western blot analyses of parallel gels show that CBAH-2 is not immuno-cross-reactive with CBAH-1 (Fig. 4D). It should be noted that the activities of CBAH-1 and CBAH-2 displayed differential stabilities to the activity staining procedure. Thus, the band intensity observed in Fig. 4C does not necessarily reflect the amount of enzyme present and should be interpreted only qualitatively.

TABLE 1. Typical purification of cloned CBAH from *E. coli*

Purification step	Total amt of protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	251	96	0.38	1	100
0 to 50% ammonium sulfate	142	86	0.61	1.6	90
Phenyl-Sepharose CL-4B	23	50	2.17	5.7	52
Q Sepharose Fast Flow	0.9	29	32.22	84.8	30

[a] One unit is that amount of enzyme releasing 1 μmol of taurine per min under the assay conditions described in Materials and Methods.

TABLE 2. Properties of CBAH activity from various extracts

Source	Sp act[a]	Relative activity[b]	T/G ratio[c]	pH optimum
C. perfringens 13	2.12×10^{-3}	1.00	0.52	4.5
C. perfringens 13-3	1.82×10^{-3}	0.86	0.32	4.5
E. coli NM522	ND[d]	0		
E. coli DH5αMCR(pLH-1)	1.45×10^{-2}	6.84	NT[e]	NT
E. coli NM522(pKLH-101)	3.80×10^{-1}	179	1.33	5.5
Cholylglycine hydrolase (Sigma Chemical Co.)	NT		0.38	4.5

[a] Micromoles of taurine formed per minute per milligram of protein.
[b] Specific activity relative to that of *C. perfringens* 13.
[c] T/G ratio, activity on taurocholate/activity on glycocholate.
[d] ND, no activity detected.
[e] NT, not tested.

Substrate specificity of CBAHs. The dissimilarity of CBAH-1 and CBAH-2 was apparent after substrate specificity analyses (Table 2). This property is demonstrated best by analyzing the T/G ratio, defined as the ratio of the hydrolysis rate for taurocholic acid to that for glycocholic acid. CBAH-1 assays performed with crude protein extracts from recombinant *E. coli* hydrolyzed taurocholate at a higher rate than glycocholate (T/G ratio, 1.33), while extracts from wild-type strain 13, mutant strain 13-3, and the commercially available enzyme preparation gave T/G ratios of 0.57, 0.32, and 0.38, respectively. The higher T/G activity ratio of extracts containing the cloned gene product relative to strain 13 extracts illustrates its difference from the major CBAH activity of strain 13. The lower ratio in the mutant strain 13-3 versus the wild type is consistent with the loss of a CBAH activity with preference for taurine-conjugated bile acids (CBAH-1) from a mixture of CBAHs with overall preference for glycine conjugates.

pH optima for CBAH activities. Differences among the various extracts were also apparent with regard to pH-activity profiles, and these are summarized in Table 2. The optimum for activities of CBAH from extracts of strains 13 and 13-3, as well as commercial cholylglycine hydrolase (Sigma), was pH 4.5. However, the activity expressed in extracts from *E. coli* NM522(pKLH-101) had an optimum at pH 5.5.

DISCUSSION

The heterogeneity of CBAH among different genera of bacteria became apparent once the characterization of several of these enzymes from different sources had been reported (11, 14, 28, 44). The major differences which have been observed are in substrate specificity and molecular weight. The enzyme from *Bacteroides vulgatus* was shown to preferentially hydrolyze taurine-conjugated bile acids (14), while those from two *Lactobacillus* species (6, 18), *C. perfringens* (11, 28), and *Bacteroides fragilis* (44) were generally more active on glycine conjugates, with the degree of difference in hydrolysis rates varying with the source organism as well as the bile acid moiety. In addition, earlier studies by Mitvedt and Norman (25) with bacterial cultures pointed to a diversity of enzymes. Some

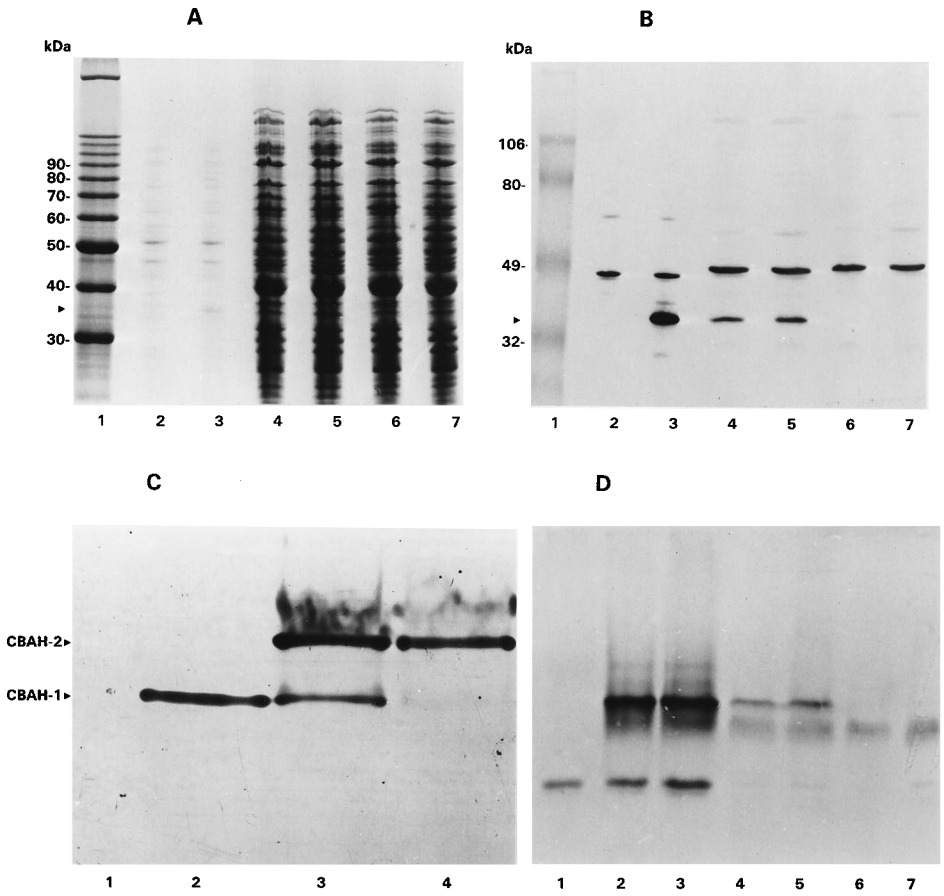

FIG. 4. SDS-PAGE and native PAGE analyses of CBAHs. (A) Coomassie blue stain of proteins separated on SDS-PAGE gels (0.75 mm; total acrylamide [T], 10%; cross-linker [C], 2.7%). Lanes: 1, 10-kDa marker; 2, NM522 (1 μg); 3, NM522(pKLH-101) (1 μg); 4, strain 13, log phase (50 μg); 5, strain 13, stationary phase (50 μg); 6, strain 13-3, log phase (50 μg); 7, strain 13-3, stationary phase (50 μg). (B) Western blot analysis of a gel identical to that in panel A, except that lane 1 contained prestained markers. (C) Negative image of CBAH activity stain of proteins separated on nondenaturing polyacrylamide gels (1.5 mm; T, 7.5%; C, 2.7%). Lanes: 1, NM522 (40 μg); 2, NM522(pKLH-101) (40 μg); 3, strain 13 (400 μg); 4, strain 13-3 (400 μg). (D) Western blot analysis of proteins separated as in panel C, except that the gel was 0.75 mm thick. Lanes: 1, NM522 (4 μg); 2 and 3, NM522(pKLH-101) (lane 2, 2 μg; lane 3, 4 μg); 4 and 5, strain 13 (lane 4, 50 μg; lane 5, 100 μg); 6 and 7, strain 13-3 (lane 6, 50 μg; lane 7, 100 μg).

organisms were capable of hydrolyzing both glycine and taurine conjugates, while others preferred one or the other of the two classes. These observations served as an indication that there were a variety of CBAH enzymes in nature with distinct substrate-hydrolyzing capabilities. It also was known, from work by Nair and coworkers (27, 29, 35) and more recently by Lundeen and Savage (19), that, even within a single strain of bacteria, there was a heterogeneity with respect to CBAH activities. *C. perfringens* ATCC 19574 was shown to produce several enzymes with distinct but overlapping CBAH activities against a variety of conjugated bile acid substrates. These enzymes also manifested differences with respect to one another in thermal stability (35). Despite the commercial use of extracts from *C. perfringens* for their CBAH activity, no concerted attempt has been made to purify and compare the individual components of this apparent mixture of activities. In the case of *Lactobacillus* sp. strain 100-100, at least four distinct CBAHs have been observed, each composed of different combinations of two separate polypeptides, α and β, in trimeric configuration (19).

We have been interested in elucidating the role that this group of enzymes plays in the gastrointestinal ecosystem and had focused on *C. perfringens* 13 as the model organism. This choice was based primarily on its utility for genetic studies because of the relative ease with which it could be transformed by electroporation (41). After being unsuccessful in attempts to identify a CBAH gene from *C. perfringens* genomic libraries with a DNA probe based on the N-terminal sequence of a CBAH purified by Gopal-Srivastava and Hylemon (11), we resorted to the indicator media method (7) used by Christiaens et al. (6). We were promptly successful in cloning a gene which encodes a protein product with CBAH activity. This gene and its product, upon nucleotide sequence analysis and product characterization, exhibited very little sequence or structural similarity to the enzyme purified from *C. perfringens* MCV 815 (11). However, the product is related at the sequence level to the previously described CBAH from *L. plantarum* (6), as well as to the penicillin V amidase from *Bacillus sphaericus* (31, 32). All three of these enzymes catalyze amide bond hydrolysis, all have optimal activity at an acidic pH, and all are of similar size (ca. 37-kDa subunits with probably tetrameric native composition). Protein and nucleic acid database searches with these entire sequences or their highly conserved regions have not revealed other obvious members of this family. Other than inhibitor data suggesting the importance of a sulfhydryl group to catalytic activity (11), nothing is known of the enzymatic mechanism for any of these enzymes. Thus, it is difficult to even speculate on the location of active-site residues.

The lack of homology of the CBAH gene from strain 13 with that previously purified from *C. perfringens* MCV 815 led us to examine strain 13 for additional CBAH activities. Using the enzymatic activity assay after fractionation of extracts by PAGE, we have shown that there is at least one additional CBAH activity in crude extracts of strain 13. The failure of this second activity to cross-react with antibody raised against CBAH-1, along with the presence of this second activity in mutant strains with a disrupted CBAH-1 gene, indicates that this second enzyme is not a heteromultimer containing CBAH-1 subunits, as was the case with the multiple forms of CBAH observed in *Lactobacillus* sp. strain 100-100 (19). The observation that the CBAH from *C. perfringens* MCV 815 had a subunit size of 56 kDa (11), along with its N-terminal sequence dissimilarity, leads to the possibility that it is derived from a different protein family from the penicillin amidase-CBAH cluster. We are currently purifying and characterizing the second CBAH (CBAH-2) from the strain 13 knockout mutant to determine its relationship to CBAH-1.

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