ABSTRACT

Rick Matthew Jordan. CLONING AND CHARACTERIZATION OF A CONJUGATED BILE ACID HYDROLASE GENE FROM *ENTEROCOCCUS FAECALIS*. (Under the direction of Dr. James Coleman) Department of Microbiology and Immunology, July, 2001.

Sequence analysis of a partial genome sequence of *Enterococcus faecalis* indicated the presence of an open reading frame (ORF) with homology to members of the conjugated bile acid hydrolase/ penicillin V amidase family. In order to define the function of this ORF, a genomic DNA fragment was cloned containing this sequence from *Enterococcus faecalis*, and its nucleotide sequence was confirmed. The DNA fragment was amplified using primers based on the flanking sequence, ligated into pUC19, and transformed into *E. coli*. Colonies containing inserts were streaked to purity and assayed for conjugated bile acid hydrolase activity. Plasmid DNA was subjected to automated DNA sequencing to confirm the genomic sequencing data. Following purification of the enzyme expressed in *E. coli*, substrate specificity assays demonstrated that the enzyme is a conjugated bile acid hydrolase. Activity was higher on glycine conjugates than on taurine conjugates. No hydrolytic activity toward penicillin V was observed. The enzyme displayed optimal bile acid hydrolysis at a pH of 5.0. Therefore, results confirm that this gene encodes a conjugated bile acid hydrolase, rather than a penicillin V amidase.

CLONING AND CHARACTERIZATION OF A CONJUGATED BILE ACID HYDROLASE GENE FROM *ENTEROCOCCUS FAECALIS*

A Thesis

Presented to

The Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science in Molecular Biology and Biotechnology

by

Rick Matthew Jordan

July 2001

CLONING AND CHARACTERIZATION OF A CONJUGATED BILE ACID HYDROLASE GENE FROM *ENTEROCOCCUS FAECALIS*

by

Rick Matthew Jordan

APPROVED BY:

DIRECTOR OF THESIS _____
James P. Coleman, Ph.D.

COMMITTEE MEMBER _____
Mary A. Farwell, Ph.D.

COMMITTEE MEMBER _____
Gerhard W. Kalmus, Ph.D.

COMMITTEE MEMBER _____
Everett C. Pesci, Ph.D.

CHAIR OF THE DEPARTMENT OF BIOLOGY _____
Ronald J. Newton, Ph.D.

DEAN OF THE GRADUATE SCHOOL _____
Thomas L. Feldbush, Ph.D.

DEDICATION

To my wife Courtney, for being there each and every time I have ever needed you, for putting your life and dreams on hold for the past three years, for believing in me when I didn't believe in myself, and for making me a better person than I actually am.

ACKNOWLEDGMENTS

I would like to thank Dr. James Coleman for taking me under his wing, when it seemed that no one else would, for sharing his knowledge, and for the invaluable direction that was absolutely necessary to fulfill this requirement.

A debt of gratitude is owed to my parents for their financial and emotional support through all of my years. Everything that I have, everything that I have done, and everything that I am, I owe to you.

I would also like to thank the Department of Microbiology and Immunology for the entire financial support of this project.

To Dr. Gerhard Kalmus, thank you for giving me the chance to pursue this degree, without that opportunity, none of this would have mattered. Also thank you for participating in the completion of this degree.

Thanks to Lynn Hudson and Robin Alligood for brightening up my days, for all of their help, and for answering the barrage of questions that I threw at them daily. To Dr. Jeffrey Smith for providing us with plasmids, bacterial strains, and for his help with the sequence analysis, and to Dr. Mary Farwell and Dr. Everett Pesci for their participation in the completion of this degree.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

A	absorbance
APS	ammonium persulfate
ATCC	American Type Culture Collection
bp	base pair
cm	centimeter
CTAB	Cetyltriethyl ammonium bromide
Da	Dalton
°C	degree Celsius
dCTP	deoxycytosine triphosphate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
g, *g*	gram, gravity
IPTG	isopropyl-β-D-thio-galactoside
Kb	kilobase pairs
kDa	kilodalton
K_m	Michaelis constant
L	liter
LB	Luria-Bertani broth
µFd	microfarad
µg	microgram
µl	microliter
µmol	micromole
mg	milligram
ml	milliliter
mM	millimolar
M	molar
nm	nanometer
nmol	nanomole
OD	optical density
^{32}P	phosphorous isotope
PCR	polymerase chain reaction
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TH broth	Todd Hewitt broth
TLC	thin-layer chromatography

TEMED	N, N, N', N' Tetramethyethylenediamine
Tris	Trizma-base
U	µmol/min/mg
V, v	volume, velocity
V_{max}	maximum velocity

INTRODUCTION

Bile acid transformation

One of the major functions of the liver is to secrete a solution known as bile. Bile serves two important functions: it plays an important role in fat digestion and absorption, and it also serves as a means for excretion of several important waste products from the blood. The most abundant substances secreted in the bile are the bile acids, accounting for about half of the total solutes. The precursor of the bile acids is cholesterol, which is either supplied by the diet or synthesized in the liver during fat metabolism (Fig. 1). Cholesterol is then converted to either cholic acid or chenodeoxycholic acid. In the liver these acids are linked, via an amide bond, with either glycine or taurine to form conjugated bile acids. The carbon-nitrogen bond present is unique in its ability to withstand cleavage by proteolytic enzymes, and requires drastic alkaline conditions for its chemical hydroysis (Midtvedt, 1974).

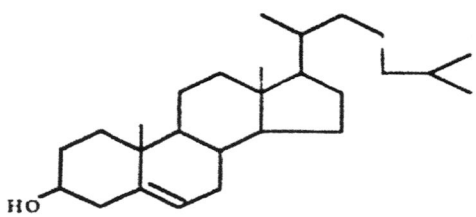


Figure 1. Cholesterol

Bile acids have two major functions: they help emulsify large fat particles and aid in the hydrolysis, transport, and absorption of the digested fat to and through the intestinal membrane. Active transport systems in the liver and the intestine limit the bile acids to these two organs. Most of the conjugated bile acids are not absorbed until they reach the last part of the small intestine, the ileum. Bile acids are rapidly transferred into the blood and returned to the liver where they are removed and recycled (Javitt, 1997).

Once in the intestine, the bile acids may be converted to a number of metabolites by the intestinal microflora. Hydrolysis of the amide linkage is catalyzed by a class of microbial enzymes known as conjugated bile acid hydrolases (CBAH)(Fig. 2). Recirculation of bile acids is partially dependent on their recognition in the conjugated form by active transport sites in the ileum, thus binding of a conjugated bile acid to an active transport site results in its return to the liver, while hydrolysis by CBAH results in a free bile acid with lower affinity to these receptors (Playoust and Isselbacher, 1964; Schiff et al., 1972), and which may pass into the large intestine, for further metabolism.

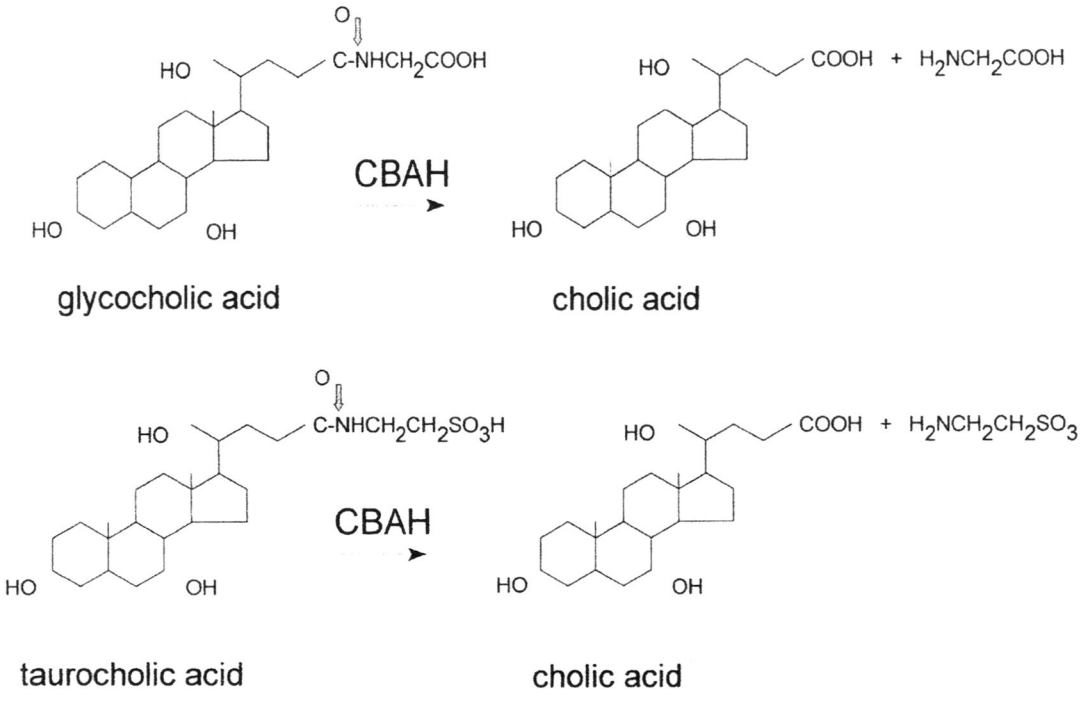


Figure 2. Deconjugation of glycocholic and taurocholic acid to cholic acid

The large intestine contains bacterial populations that modify the steroid ring. The most important metabolic transformation which these free bile acids undergo focuses on the 7α-hydroxyl group. Removal of this group results in the formation of deoxycholic acid from cholic acid and lithocholic acid from chenodeoxycholic acid (Fig. 3). Deoxycholic acid is reabsorbed from the intestines and returned to the liver for processing to conjugated bile acids. Lithocholic acid is very insoluble in water and binds to fecal residue so most is excreted from the body (Javitt, 1997).

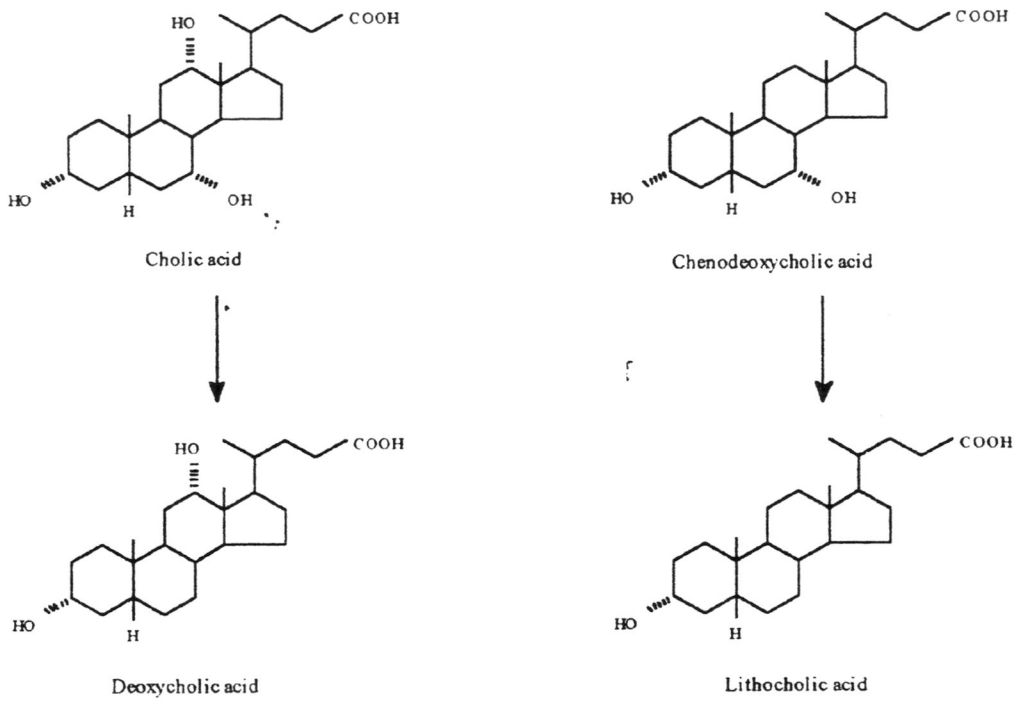


Figure 3. 7α-Dehydroxylation

Conjugated Bile Acid Hydrolase

Bile acid hydrolysis is a common reaction in the intestinal tract of animals (Christiaens et al., 1992). The enzyme, conjugated bile acid hydrolase (CBAH), catalyzes the hydrolysis of the amide bond that links bile acids to glycine or taurine (Hylemon, 1985). CBAH activity has been detected in members of the genera *Bacteroides, Bifidobacterium, Clostridium, Enterococcus, Fusobacterium, Lactobacillus, Peptostreptococcus, Streptoccus* (Hylemon, 1985) and *Veillonellae* (Aries and Hill, 1970). Deconjugation activity has been purified from *Bacteroides fragilis* (Stellwag and Hylemon, 1976), *Bacteroides vulgatus* (Kawamoto et al., 1989), *Bifidobacterium longum*

(Tanaka et al., 2000; Grill et al., 1995), *Clostridium perfringens* (Nair et al., 1967; Gopal-Srivastava and Hylemon, 1988; Coleman and Hudson, 1995), *Lactobacillus acidophilus* (Corzo and Gilliland, 1999), *Lactobacillus johnsonii* (Lundeen and Savage, 1990), and *Lactobacillus plantarum* (Christiaens et al., 1992).

There is considerable variation in occurrence of this enzymatic activity among species and strains (Hylemon, 1985). Hylemon reported differences in kinetic properties, substrate concentrations, and substrate itself between *B. fragilis* and *C. perfringens*. Differences in optimal pH, optimal temperature, and enzyme stability also exist. Coleman reported that the enzyme obtained from a *C. perfringens* is more related, structurally and at the sequence level, to the CBAH from *L. plantarum*, and also to the penicillin V amidase from *Bacillus sphaericus*, than it is to a CBAH purified from another strain of *C. perfringens* (Coleman and Hudson,1995). Christiaens reported that the CBAH from *L. plantarum* shares extensive similarity with penicillin V amidase of *Bacillus sphaericus*, whereas no similarity between the amino acid sequence of the CBAH enzymes of *Bacteroides vulgatus* and *Clostridium perfringens* is found (Christiaens et al., 1992).

Despite the many differences present, some similarities also exist between enzymes. Generally, the optimum pH for CBAH activity is in the 4.5-6.0 range, and the optimum temperature is 30-45° C. The enzymes from *C. perfringens* (Coleman and Hudson, 1995) and *L. plantarum* (Christiaens et al., 1992) were of similar size and both appear to exist as tetramers.

The ecological significance of bile acid hydrolysis, particularly the advantage

this activity offers the CBAH producing bacterium, is not fully understood (Christiaens et al., 1992). It is believed that bile acids have cytotoxic and bacteriostatic properties. Sung et al. offered the opinion that the bacteriostatic activity of bile acids is a function of the hydrophobicity of the molecules (Sung et al., 1993). They showed evidence to support the idea that free bile acids, which are generally more hydrophobic, have more significant inhibition on the growth of bacteria when compared with the hydrophilic (conjugated) bile acids. Therefore, it is possible that the deconjugation activity, possessed by certain bacteria, can be used to control their own population or the entire intestinal bacterial population.

Enterococcus faecalis

Enterococci (formerly group D streptococci) are gram-positive bacteria, and are among the leading causes of nosocomial infections in the United States, with the majority of clinical isolates being classified as *Enterococcus faecalis* (Xu et al., 1998). Over the past several decades, enterococci have developed resistance to almost all antimicrobial agents used in hospitals, posing a problem for treatment (Xu et al., 1997).

The *E. faecalis* genome is being sequenced by The Institute of Genomic Research (TIGR), and the organism has been intensely researched for its antibiotic resistance, its gene transfer qualities, and its stress tolerance ability. The CBAH activity has been identified in *E. faecalis*, but an enzyme has yet to be isolated. Our study will shed light on the function of CBAH in this bacteria, and may lead to a greater understanding of the role played by this bacterium in the gastrointestinal tract.

Penicillin V amidase

Pencillin V (phenoxymethylpenicillin) belongs to the group of small-spectrum β-lactam antibiotics. The chemical structure of the penicillins is characterized by a thiazolidine ring connected to a β-lactam ring, onto which a side chain is attached (Fig. 4). The most common mechanism of microbial resistance to β-lactam antibiotics is based on a group of enzymes that hydrolyze the β-lactam ring of penicllin, known as β-lactamases. This type of resistance spreads rapidly in bacterial populations by transfer of genetic information among populations and natural selection. Consequently, the massive use of antibiotics soon renders them ineffective (Valle et al., 1991).

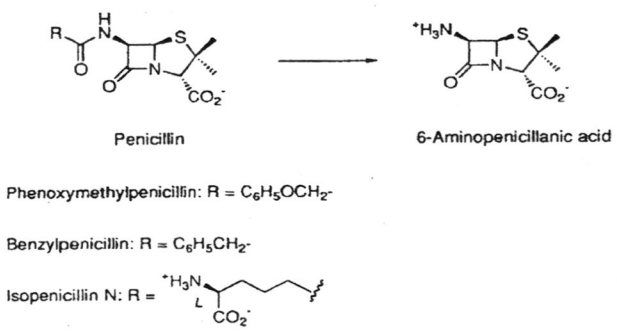


Fig. 1. Conversion of penicillin to 6-aminopenicillanic acid.

Figure 4. Conversion of penicillin

Penicillin V amidase catalyzes the hydrolysis of penicillin V to phenoxyacetic acid and 6-aminopenicillanic acid (Lowe et al., 1986). The resulting 6-amino penicillanic acid is an important industrial compound, since it is used as a precursor for many of the semisynthetic penicillins (Kerr, 1993). Penicillin V amidase activity has been detected in a wide range of bacteria and fungi, and has been isolated from *Bacillus sphaericus*

(Olsson et al., 1985; Pundle and SivaRaman, 1997), *Erwinia aroideae* (Vandamme and Voets, 1975), *Fusarium oxysporum* (Lowe et al., 1986), *Fusarium* sp. SKF (Sudhakaran and Shewale, 1995), *Penicillium chrysogenum* (Whiteman and Abraham, 1996), *Streptomyces chainia* (Chauhan et al., 1998), and *Streptomyces lavendulae* (Torres et al., 1999; Arroyo et al., 2000).

Penicillin V is highly active against Gram-positive cocci and is the drug of choice in the treatment of infections of the upper respiratory tract. Penicillin V is mainly excreted by the kidneys, but part of it is converted into the penicilloic acids. The presence of the amidase and its ability to break down penicllin V provides the bacteria with a resistance to this drug.

Ntn hyrolase family

Penicillin V amidase is a member of the Ntn (N-terminal nucleophile) hydrolase family, characterized by processing from an inactive precursor to reveal a catalytic nucleophillic residue at the new N-terminus whose nucleophilicity is enhanced by its own amino group. The simple presence of an N-terminal serine, threonine, or cysteine, in an enzyme with a nucleophillic mechanism may be sufficient to place it in the Ntn hydrolase family. Thus, since conjugated bile acid hydrolases (CBAH) have extensive sequence similarity to PVA (Christaens et al., 1992), and typically have a N-terminal methionine followed directly by cysteine, these enzymes can be assigned to the Ntn hydrolase family (Suresh et al., 1999).

In the PVA structure, cysteine was observed as the N-terminal residue, whereas the gene sequence predicts an N-terminal sequence of Met-Leu-Gly-Cys (Olsson and Uhlen, 1986). This shows that three amino acids are processed from the precursor N-terminus to unmask a nucleophile with a free α-amino group (Suresh et al., 1999).

Based on sequence comparisons, conjugated bile acid hydrolases are also members of the Ntn hydrolase family and several are believed to exist as tetramers. The major difference between Penicillin V amidases and conjugated bile acid hydrolases is in substrate specificity. Both of these enzyme classes catalyze amide bond hydrolysis, both have optimal activity at an acidic pH, and both are of similar size (37-kDa subunits with probably tetrameric native composition). Other than inhibitor data suggesting the importance of a sulfhydryl group to catalytic activity, nothing is known of the enzymatic mechanism for the conjugated bile acid hydrolases.

Statement of purpose

Conjugated bile acid hydrolysis activity has been shown to exist in many strains of *E. faecalis* (Aries and Hill, 1969; Kobashi et al., 1978). Kobashi et al., as well as Aries and Hill, found activity in 75% and 93% of the strains tested, respectively. Both showed that the CBAH enzyme in *E. faecalis* exhibits greater affinity for glycine conjugates over taurine conjugates. However, only crude extracts were tested, and no sequence data was presented. The in-progress sequencing of the *E. faecalis* genome by The Institute of Genomic Research has provided a data set to examine for the presence of the putative CBAH gene. The finding of a DNA sequence with homology to the CBAH

gene from *C. perfringens* implied that this was the CBAH in *E. faecalis*. However, the possibility that this gene encoded a penicillin V amidase needed to be excluded. The work described here addresses this question by proving the function of the unknown gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, & growth conditions

A clinical isolate of *Enterococcus faecalis,* strain DK, was obtained from Pitt County Memorial Hospital, Greenville, NC, and grown on brain heart infusion medium (BHI), without shaking, under aerobic conditions at 37°C. *E. coli* strains DH10B and NM522 were obtained from Gibco-BRL Life Technologies and were grown aerobically at 37°C on Luria-Bertani medium on a rotary shaker. Other *Enterococcus* strains used were: *E. faecalis* ATCC 29212, obtained from Diane Norris, Department of Biology, East Carolina University; *E. faecalis* ATCC 33186, *E. faecium* ATCC 6057, obtained from Dr. Daniel Martin, Department of Microbiology, Brody School of Medicine, East Carolina University, and *E. hirae* ATCC 8043, obtained from the American Type Culture Collection (Rockville, MD). Cell growth was monitored by optical density measurements at 600 nm using a Model 250 Spectrophotometer (Gilford Instruments; Oberlin, OH). pUC19 was obtained from Gibco-BRL (Bethesda, MD). The following components were added to growth media when appropriate, at the indicated final concentrations: ampicillin 100μg/ml; isopropyl-β-D-thiogalactoside (IPTG) 0.6mM; and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) 50μg/ml (obtained from Gibco-BRL).

Materials

Sodium salts of taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), and penicillin V (phenoxymethyl penicillanic acid) were obtained from Sigma (St. Louis, MO). Restriction endonucleases, T4 DNA ligase, and molecular markers for gel electrophoresis were purchased from Gibco-BRL (Bethesda, MD).

Recombinant DNA techniques

Chromosomal DNA was isolated from *E. faecalis* by the CTAB/NaCl procedure as described by Ausubel et al.(1987), with the inclusion of the lysozyme pretreatment. Polymerase chain reaction was performed using the following primers: 5'-CCCCGGGTGTACTATTTTAGTGAC-3 and 5'-CCGAGCTCATT TCGAAAATAC TTAT-3' and the following heating cycles: 95°C for 5 minutes, 30 cycles of the following: 95°C for 45 seconds, 45°C for 45 seconds, 70°C for 2 minutes, and then 70° for 5 minutes, using an Eri-comp twin block Easy Cycler Series Thermocycler. Recombinant plasmid DNA was isolated from *E. coli* by alkaline lysis procedure (Maniatis et al., 1982). DNA was quantified using 260/280 nm measurements. Templates for sequencing were purified with Qiagen-tip 100 columns using the Midi protocol (Qiagen, Inc., Chatsworth, CA). Restriction endonuclease digestions were

carried out as recommended by the suppliers. Electrophoresis of DNA, ligation reactions, and other nucleic acid manipulations were performed as previously described (Maniatis et al., 1982). DNA was purified from agarose gels using the Elu-Quik Purification Kit (Schleicher & Schuell, Keene, NH).

Identification of positive clones

Electrocompetent cells were prepared using a modified version of the procedure of Tung and Chow (1995). 100 ml of LB was inoculated with 1 ml of an overnight culture of *E. coli* and incubated at 37°C with vigorous shaking. When the OD_{600} of the culture reached 0.6, cells were chilled in an ice-bath for 10 minutes and centrifuged at 4200 rpm at 4°C for 20 minutes using a Beckman GSA rotor. Cells were washed twice with 50 ml of ice-cold 10% glycerol, and resuspended to a final volume of 0.2 ml in ice-cold GYT (10% glycerol, 0.125% yeast extract and 0.25% tryptone). Electrocompetent cells (50 µl) were transformed in a 2 mm gap cuvette using a BioRad Gene Pulser at the following settings: 1800 volts, 25µFd, and 200 ohms. Cells were then incubated in 1 ml SOC broth (20 g/L tryptone, 5 g/L yeast extract, 0.6 g/L NaCl, 0.5 g/L KCl, 20 mM glucose, 20 mM magnesium) for 1 hour, concentrated, and plated on appropriate medium containing X-gal. Colonies with inserts were picked, their plasmids purified as before, and electrophoresed on a 0.8% agarose gel. Colonies were also picked onto differential medium plates described by Christiaens et al. (LB with 0.35% glucose, 0.5% sodium taurocholate, and 100 µg/ml ampicillin, adjusted to a pH of 6.0)(1992), to determine

hydrolase activity. After 12 to 24 hours of incubation, plates were observed for colonies with precipitated deoxycholate halos. These were picked and re-streaked to purity.

DNA sequencing

Automated DNA sequencing was performed at the Molecular Biology Resource Facility at the University of Tennessee, Knoxville, TN.

Sequence analyses

Nucleic acid and protein sequence analyses were performed using the Wisconsin Package V10.0-UNIX (Genetics Computer Group, Inc., Madison, WI). Sequence information was obtained from the The Institute of Genomic Research (TIGR) and GenBank databases.

Sequence accession numbers

The previously cloned nucleotide sequence of the DNA fragment containing the CBAH gene from *Clostridium perfringens* can be found by using the accession number U20191. This gene sequence was used to discover the gene of interest in *E. faecalis*. Other sequences used were: Bile acid hydrolase peptide sequences from *Lactobacillus acidophilus* (AF091248), *Lactobacillus johnsonii* peptides *A & B* (AF297873 & AF054971), *Lactobacillus plantarum* (S51638), *Bifidobacterium longum* (AF148138), *Lactobacillus gasseri* (AF305888; Russell and Klaenhammer, 2001), and penicillin V

amidase gene sequences from *Bacillus sphaericus* (M15660), *Paramecium bursaria* (NP048638), and *Staphylococcus aureus* (BAB41488).

Preparation of *E. coli* and *E. faecalis* cell extracts

E. coli was grown from a 5% inoculum with overnight cultures in LB containing ampicillin. IPTG was added when cells had grown to an optical density of 0.8 at 590 nm. Cells were harvested after growth for an additional 2.5 hours, and resuspended in three volumes of Buffer A (20 mM sodium phosphate, 10 mM β-mercaptoethanol, and 1 mM EDTA, pH 7.0). The suspension was lysed by either two passages through a French Pressure Cell at 12,000 psi, or by sonication at a 60% duty cycle for 5 minutes using a Branson Sonifer 450. Lysates were centrifuged at 12, 000 x *g* for 45 minutes to remove debris and unbroken cells. Protein concentrations were estimated by absorbance readings at 230/260 nm (Kolb and Bernlohr, 1977). The supernatant was dialyzed against Buffer A.

Thin-layer chromatography

Media containing 1 mM CA, DCA, GCA, TCA, GDCA, TDCA, or 2.5 mg/ml Bile salts #3 (BBL) in Todd Hewitt Broth (TH Broth) were inoculated with *E. coli* containing a plasmid or *E. faecalis* and grown at 37°C. 1 ml samples were taken at 0, 24, and 48 hours, acidified with HCl, and extracted twice with equal volumes of water-saturated butanol to extract bile acids. The combined butanol layers were dried at 50°C under a stream of nitrogen, and resuspended in 250 µl of 100% ethanol. 1.0 µg of

standards as well as 5 µl samples were spotted onto a 20 x 20 cm plate. The plate was subjected to preheating for 5 minutes under vacuum at 100°C (Eneroth, 1963). The plate was developed in a solvent system consisting of benzene/dioxane/acetic acid (60:30:6). After the solvent had migrated to the top of the plate, the plate was removed from the tank and, after air-drying, sprayed with 10% (weight/volume) phosphomolybdic acid in ethanol and heated at 100°C for 5-15 minutes. Bile acids turned blue against a yellow background.

Substrate specificity assays

The standard assay mixture contained, in a final volume of 1 ml, 25 µmol of sodium acetate (pH 5.5), 0.5 µmol of dithiothreitol, 0.5 µmol of EDTA, and crude extract or purified protein. After addition of enzyme sample, reaction mixes were allowed to equilibrate at 37°C. Enzyme assay reactions were initiated by the addition of 1-10 mM bile acid or 2-10 mM penicillin V. After a set amount of time, (usually 5 minutes) the reaction was stopped by the addition of an equal volume of ice cold 20% trichloroacetic acid. After incubation on ice for 5 minutes, the mixes were centrifuged for 5 minutes to remove precipitated protein. 100 µl of the supernatant was assayed for free amino groups by the ninhydrin reaction, using the procedure described by Lee and Takahashi (1966). Briefly, 100 µl of reaction mixture was added to 500 µl of 0.2 M citrate buffer (pH 5.0), and 400 µl H_2O, for a total reaction volume of 1 ml. To this mixture was added 500 µl of ninhydrin reagent (0.2 g ninhydrin, 0.03 g hydrindantin in 7.5 ml DMSO added to 2.5 ml of 0.4 M lithium acetate buffer, pH 5.2). The mixture was heated at 95°C for 25 minutes,

and then cooled on ice for 15 minutes. The sample was then diluted 1:5 with 50% ethanol, and the absorbance determined at 570 nm. One unit (U) of specific activity is defined as μmol of substrate hydrolyzed/minute.

pH optimum of enzymatic reaction

The assay conditions described above were used except that the sodium acetate buffer was replaced with a citrate-borate-phosphate system. A 2X buffer was prepared containing 30 mM each of citric acid, boric acid, and monobasic potassium phosphate. The pH of this buffer was adjusted to 0.5 unit increments between pH 3.0 and pH 9.5. Addition of 5 mM GCA initiated the 1 ml reaction after a 5-minute equilibration period at 37°C. Reaction mixes were incubated for 5 minutes at 37°C using either purified enzyme or crude extract. The reaction was stopped by addition of an equal volume of ice-cold 20% trichloroacetic acid. Aliquots were then assayed for free amino groups using the same ninhydrin assay described above.

Enzyme purification

Enzyme purification was performed by Lynn Hudson, Department of Microbiology and Immunology, East Carolina University. Throughout the purification, enzymatic activity was monitored using a 96-well plate assay, as described previously by Coleman and Hudson (1995). Samples to be assayed (<20 ml) were added to 100 ml of assay cocktail (0.5 TDCA, 50 mM sodium phosphate, 10 mM β-mercaptoethanol, pH

5.5) in wells of a 96-well microtiter plate. At all stages of purification, samples were removed for protein and enzyme assay and SDS-PAGE analysis.

Ammonium sulfate precipitation

E. coli strain NM522 containing the CBAH insert in pUC19 was grown, harvested, and lysed as described above. Ammonium sulfate (ICN, Costa Mesa, CA) was added to 40% saturation (22.6 g/100 ml) at a pH of 7.0, and the mixture was centrifuged at 12, 000 x *g* for 30 minutes at 4°C. The pellet was resuspended in 10 ml of Buffer A (20 mM sodium phosphate, 1 mM EDTA, 10 mM β-mercaptoethanol, pH 7.0). To the remainder of the supernatant was added ammonium sulfate to 70% saturation (18.7 g/100ml), and then centrifuged as before. The 40-70% ammonium sulfate pellet was resuspended in Buffer A (pH 6.0) and frozen at -20°. The majority of CBAH activity was observed in the 40-70% pellet. This pellet was resuspended in Buffer A and further purified.

Phenyl-Sepharose

Phenyl-Sepharose was obtained from Pharmacia (Piscataway, NJ). The column (2.5 x 9.0 cm) was equilibrated in Buffer 1 (20 mM sodium phosphate, 1 mM DTT, 0.5 M ammonium sulfate, pH 6.0). The column was washed with Buffer 1 until the effluent A_{280} returned to baseline, and then washed with the same buffer at pH 7.0 (Buffer 2) until the effluent A_{280} returned to baseline. CBAH activity was then eluted with a decreasing ammonium sulfate gradient (0.5 M to 0 M, linear) in Buffer 2. CBAH activity assays were performed and positive fractions were pooled. Pooled fractions were concentrated using an Amicon YM-10 membrane to a final volume of 20 ml.

Sephacryl SR-300

Sephacryl was obtained from Pharmacia. The gel filtration column (2.5 x 4 cm) was equilibrated with Buffer 3 (20 mM sodium phosphate, 150 mM NaCl, 0.5 mM DTT, pH 7.0). Samples were applied in 10 ml portions. The column was eluted with Buffer 3 until the effluent A_{280} had returned to baseline. Fractions were checked for CBAH activity using the microtiter plate assay. Positive fractions were pooled, concentrated by ultrafiltration as above, and frozen after addition of glycerol to a final concentration of 10%.

Q-sepharose

Q-sepharose was obtained from Pharmacia. The anion exchange column (2.5 x 4 cm) was equilibrated with 20 mM sodium phosphate, 1 mM DTT, and 5% glycerol (pH 7.5) (Buffer 4). After the column was washed with degassed Buffer 4 until the effluent A_{280} had returned to baseline, CBAH was eluted with a linear 0 to 0.5 M NaCl gradient in Buffer 4 (200 ml total volume). Active fractions were combined, concentrated as before, and stored in Buffer 4 containing 10% glycerol at -20°C.

SDS-PAGE

Samples were precipitated as follows: to 1-30 mg of protein, deionized water was added to 1 ml, followed by 100 µl of 0.15% deoxycholic acid. After incubating at room temperature for 10 minutes, 100 µl of 72% trichloroacetic acid was added and the sample placed on ice for 10 minutes. The mixture was centrifuged for 10 minutes in a microcentrifuge at full speed, washed three times with 70% ethanol, dried, and

resuspended in SDS-sample buffer. The mixture was heated at 95°C for 5 minutes. Gel electrophoresis was performed using a BioRad Mini-Protean II Dual Slab Cell. A 12% polyacrylamide gel was loaded with sample and electrophoresis was performed at a constant voltage of 200 volts for 45 minutes. The gel was washed for 30 minutes (40% methanol, 10% acetic acid), and then stained with 15% methanol, 10% acetic acid, and 0.005% Coomassie Blue R-250. Standards were a mixture of Bio-Rad Low and High molecular weight markers.

Southern blot

Chromosomal DNA was obtained from 10 ml bacterial cultures by the CTAB/NaCl procedure obtained from Ausubel et al. (1987), was subjected to *Eco*RI endonuclease digestion at 37°C, and electrophoresis on 0.8% agarose gels. Following electrophoresis, the gel was agitated in 0.25 N HCl for 10 minutes, and rinsed with deionized water. DNA was denatured by soaking the gel in 0.4 N NaOH, 0.6 M NaCl for 30 minutes, followed by 1.5 M NaCl, 0.5 M Tris, pH 7.5 for 30 minutes, and finally 2X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 5 minutes. DNA was then transferred to a Gene Screen membrane (Dupont). Following overnight transfer, the membrane was rinsed briefly with 2X SSC and the DNA was fixed to the wet membrane using a Strata linker. Membranes were hybridized at 65°C with a probe consisting of the CBAH gene insert excised from the original pUC19 clone and labeled with α-^{32}P-dCTP using the RadPrime DNA labeling system (Life Technologies). Results were obtained by exposing X-ray film to the radioactively-labelled membrane.

Activity stain assay

An activity stain assay was performed, by electrophoresing purified protein or crude extract on a non-denaturing 7.5% acrylamide gel containing 5 mM thioglycollate. Crude extract (1 mg) from wild-type or purified protein (2 μg) were diluted in an equal volume of sample buffer (1 ml 1.5 M Tris, pH 6.8, 1ml glycerol, 5 mg bromphenol blue, in 10 ml final volume) and loaded onto the gel. Electrophoresis was performed at 70 volts until the tracking dye had migrated to the bottom of the gel. The gel was then soaked twice in 100 ml of 0.5 M sodium acetate, 1 mM EDTA, 10 mM β-mercaptoethanol, pH 6.5, for 15 minutes. Finally, the gel was then soaked in 25 ml of 0.5 M sodium phosphate, 5 mM TDCA, and 10 mM β-mercaptoethanol, and incubated at 37°C until a band of deoxycholic acid precipitate appeared.

RESULTS

Cloning of cbaH gene into *E. coli*

The nucleotide sequence of the proposed *E. faecalis* conjugated bile acid hydrolase gene was identified from the TIGR database by performing a BLAST search of *E.faecalis* strain V583 (ATCC 700802) using the *C. perfringens* cbaH nucleotide sequence. Chromosomal DNA was obtained from *E. faecalis* strain DK. Oligonucleotide primers containing *Sma*I and *Sst*I restriction sites were designed, and PCR was used to amplify the fragment. A 0.8% agarose gel confirmed the presence of this fragment (Fig. 5). The fragment, as well as pUC19 vector, was subjected to restriction endonuclease digestion using *Sma*I and *Sst*I, to allow for directional ligation. The ligation mix was transformed into *E. coli* strain DH10B by electroporation and the transformed cells were plated on LB plates containing 100 μg/ml ampicillin and X-gal. Recombinant colonies appeared white, while colonies containing the vector only appeared blue. Several white colonies were picked, and grown overnight for plasmid isolation. The plasmids were digested with *Eco*RI and *Hin*dIII, and electrophoresed on 0.8% agarose gel (Fig. 5). Several plasmids appeared to have inserted gene fragments. Sequence analysis data predicted that the insert containing the cbaH gene homolog would be approximately 975 base-pairs in size. As can be seen from Figure 5, an insert of similar size was obtained.

Recombinant colonies were picked from the original transformation plates, and CBAH activity was detected by using the plate assay described by Christiaens, et al.

(1992). The differential medium has a high glucose concentration, allowing an acidification below pH 5, and TDCA as a substrate for conjugated bile acid hydrolysis. Since taurine conjugates exhibit a pK_a that is less than 1.0 (Hofmann and Roda, 1984), only the deconjugated product (pK_a 5.0) precipitates at fermentative pH values. The halo formation of precipitated free bile acid permits the differentiation between hydrolase-positive and -negative clones. Colonies numbered 6 and 9 exhibited halos, and clone 6 was chosen for further analysis. For expression purposes, the recombinant plasmid containing the cbaH homolog was retransformed into *E. coli* strain NM522. This strain allows for IPTG controlled activation of transcription of the *lacZ* promoter. For future reference, the plasmid containing the cbaH homolog insert will be referred to as pJCG-6.

Nucleotide sequence of cloned fragment and flanking sequence

Figure 6 displays the nucleotide differences between *E. faecalis* strains V583(red) and DK(green), and the placement of the initial primers. Primers used for walking are underlined.

Thin-layer chromatography

A TLC of the *E. faecalis* wild-type strain shows hydrolysis of both taurine and glycine conjugates (Fig 7A). Figure 7B, offers proof that the cloned gene was that of a CBAH enzyme. *E. coli* strain NM522 without the gene was compared to NM522 containing the gene. Activity was achieved on all of the substrates tested (GCA, TCA,

GDCA, and TDCA), by cells, containing the CBAH insert, grown in the presence of bile acid.

Substrate specificity assays of NM522(pJCG-6) crude extracts

A ninhydrin assay was performed using increasing amounts of crude extract obtained from the *E. coli* strain NM522 containing pJCG-6, to determine enzyme specificity. As can be seen in Fig. 8, the CBAH enzyme has activity on both GCA and TCA, with a greater affinity for GCA, and minimal activity on penicillin V (Fig. 9).

Enzyme purification

Crude extract was obtained from *E. coli* strain NM522 as described in Materials and Methods, and subjected to ammonium sulfate precipitation, and tested for activity. The greatest activity was observed in the 40-70% pellet. The pellet was resuspended and applied to a phenyl-Sepharose hydrophobic interaction column. Active fractions were collected, pooled, and concentrated, and applied to a Sephacryl SR-300 gel filtration column. Positive fractions were again collected, pooled, and concentrated, and applied to a Q-Sepharose anion exchange column. Again, active fractions were collected, pooled, and concentrated to 94 μg/ml, and sample was subjected to SDS-PAGE. The expected molecular weight of the purified enzyme is 36, 932 Da, based on the predicted amino acid sequence. SDS-PAGE reveals a major band around 37, 000 Da (figure not shown).

Enzymatic properties of crude extracts and the purified CBAH enzyme

Figure 10A shows the effect of *E. coli* strain NM522(pJCG-6) crude extract concentration on the reaction rate. Once the protein had been purified, ninhydrin assays were performed to determine the optimal amount of enzyme that could be used to achieve an appropriate spectrophotometric signal. 0.75 μg of purified enzyme was chosen as an appropriate amount for future studies. Figure 10B is a plot of the effect of the purified enzyme concentration on the reaction rate.

For comparison purposes, *E. faecalis* crude extract was assayed to determine the optimal amount of crude extract to be used to achieve an appropriate spectophotometric signal. 2 mg of crude extract was chosen as an appropriate amount for future studies. Figure 10C is a plot of the effect of the *E. faecalis* crude extract concentration on the reaction rate.

These data allow the calculation of specific activities for the purified enzyme and crude extracts. The differences indicate a 15-fold purification from the NM522(pJCG-6) crude extract to the final purified stage. A 3280-fold difference in activity was achieved compared to the *E. faecalis* crude extract. The CBAH protein is estimated to be 0.03% of the total crude protein.

Determination of pH optimum

Ninhydrin assays were performed to determine the optimal pH for enzyme activity. The assay showed an optimal pH of 5.0, which is consistent with the findings of other conjugated bile acid hydrolases.

Determination of an optimal pH for *E. faecalis* crude extract was accomplished as before, using ninhydrin assays. The plot shows an optimal pH range from 4.5-5.5. A plot of the purified enzyme gives an optimal pH of 5.0. Figure 11 is a plot of the effect of pH on the purified CBAH and the crude extract reaction.

Substrate specificity determination

Determination of substrate specificity for the purified enzyme and *E. faecalis* crude extract was tested by ninhydrin assay. Comparison of the crude extract with the purified enzyme gives identical results, no activity on penicillin V, mild activity toward taurine conjugates, and highest activity toward glycine conjugates. Figure 12 is a plot of the substrate specificities of the purified enzyme and *E. faecalis* crude extract.

Determination of kinetic properties

Ninhydrin assays were used to produce Lineweaver-Burk double-reciprocal plots and substrate saturation curves of the purified enzyme with both glycine and taurine conjugates used as the substrate. The slope of the linear regression line was used to determine the K_m and V_{max} values. For all assays, 0.75 μg of purified enzyme was assayed against various concentrations of bile acids (1, 2, 5, and 10 mM) for 5 minutes at 37°C at a pH of 5.0.

The data points for taurine conjugated bile acids were non-linear, therefore, the K_m and V_{max} values can only be estimated using the previous method of determination.

Detection of CBAH activity in other *Enterococcus* strains

Another ninhydrin assay was performed to test the activity of other *E. faecalis* strains (DK, ATCC 29212, and ATCC 33186), and *E. faecium* ATCC 6057. Significant activity was observed in strain DK and *E. faecium* strain ATCC 6057, but not the other two strains (Fig. 19).

PCR reaction

The presence of the CBAH gene in various *Enterococcus* strains was determined by a PCR assay. PCR was performed as described in Materials and Methods, using the same primers that were used to obtain the original clone. Figure 20 shows a 1 Kb band wherever the CBAH gene is present. *E. faecalis* strain DK, *E. faecium* strain ATCC 6057, and *E. hirae* strain ATCC 8043 appear to have the gene present. *E. faecalis* strains ATCC 33186 and ATCC 29212 do not.

Southern blot

The presence of the CBAH gene in *Enterococcus* strains was also determined by Southern analysis. The DNA fragment to be used as a probe was obtained by excising the CBAH insert from the pUC19 plasmid by endonuclease digestion with *Eco*RI and *Hin*dIII. The fragment was then radioactively labelled with α-P^{32}dCTP and Klenow fragment. Chromosomal DNA was isolated from wild-type species as well as mutants using the CTAB procedure described in Materials and Methods.

Figure 21 is a photo of the X-ray film displaying the results of the Southern blot.

The presence of the CBAH gene is apparent in the *E. faecium* ATCC 6057, and *E. faecalis* DK.

Activity stain assay

An activity stain assay was performed to test the activity of the purified enzyme and the *E. faecalis* strain DK crude extract. Maintaining a rather low pH, will allow the TDCA to precipitate out once its conjugate was hydrolyzed, forming a white precipitous band. By looking at Figure 22, a white band was visualized in both lanes.

Sequence analysis data

The *E. faecalis* peptide sequence shows higher identity to other CBAH sequences, than to the penicillin V amidase sequences, which supports the other results obtained in this study. Sequence alignment was performed using a pileup program from SeqWeb for Figures 24 and 25. A gap creation penalty of 8, and gap extension penalty of 2 were used. Figure 23 is the proposed peptide sequence.

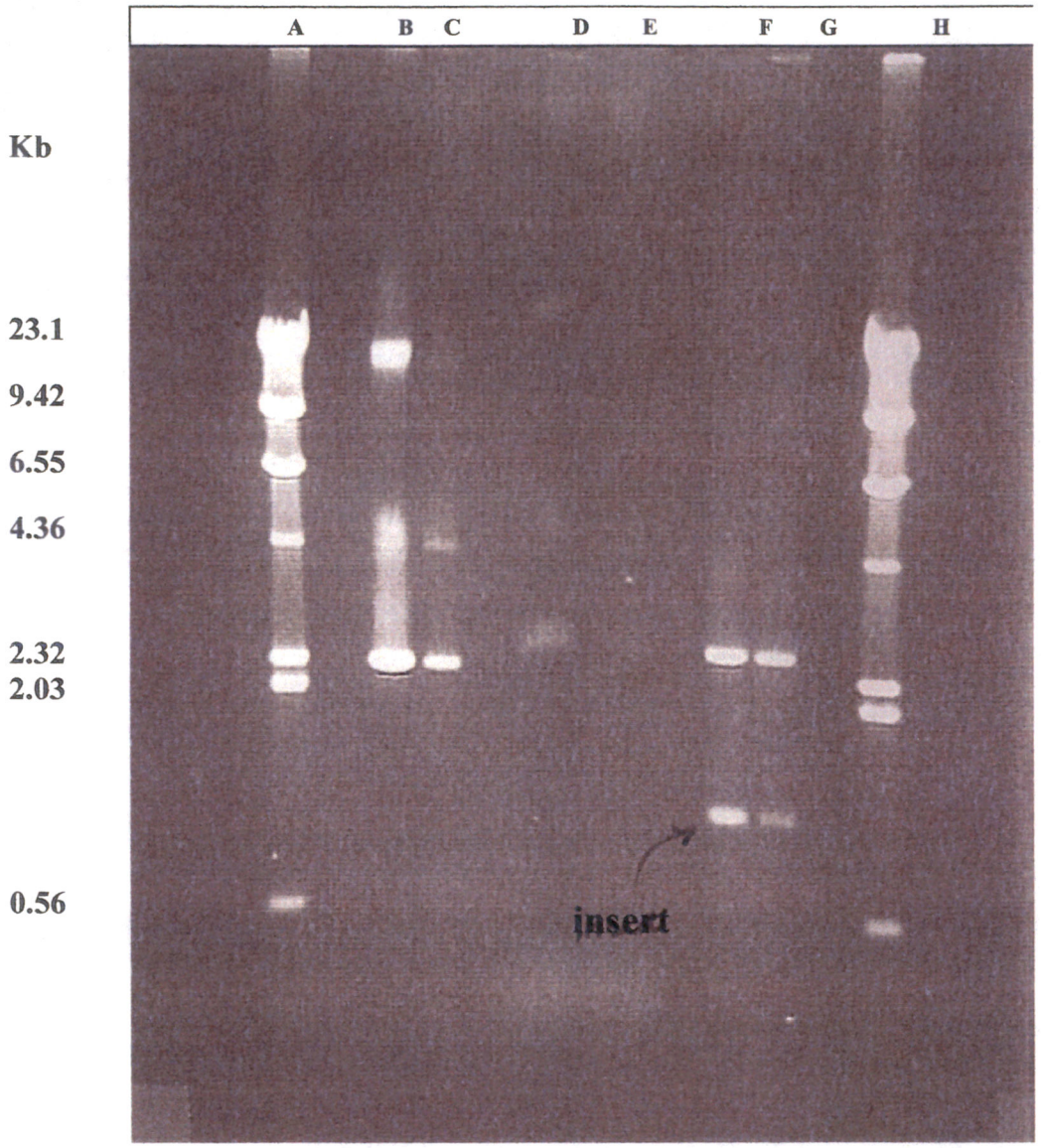
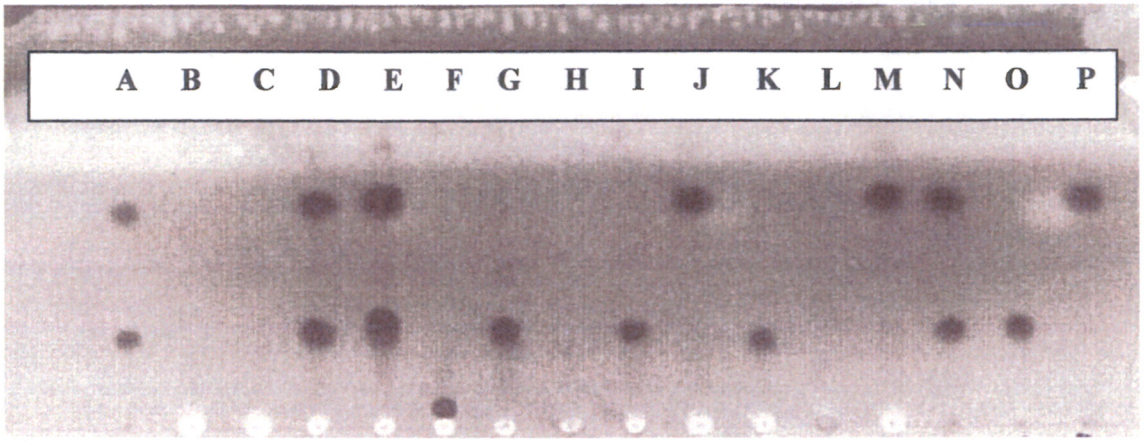
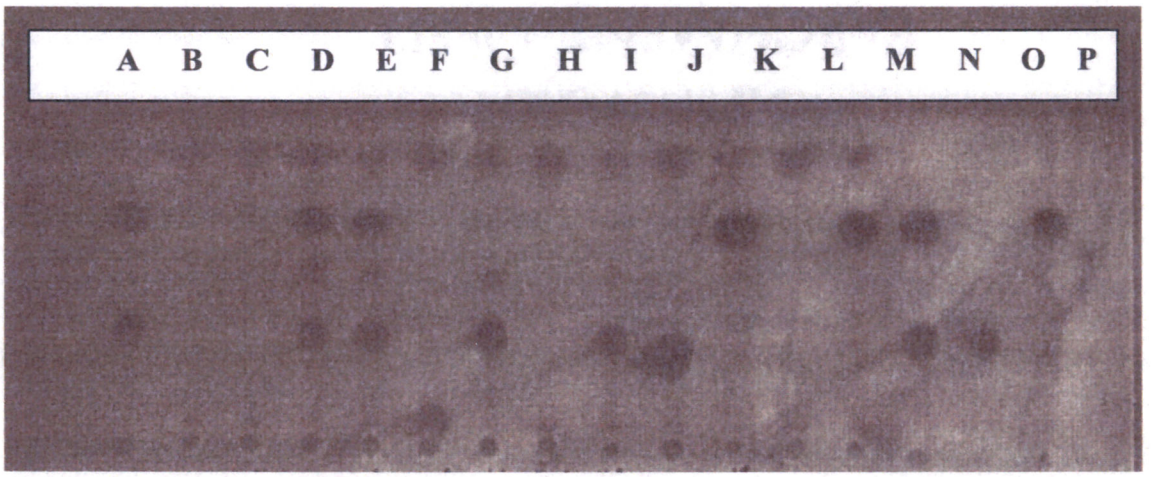


Figure 5. Gel electrophoresis of recombinant plasmids. Plasmids containing the *E. faecalis* CBAH gene were digested with *Eco*RI/*Hin*dIII. Plasmid DNA was purified, electrophoresed on a 0.8% agarose gel, and visualized after staining with ethidium bromide. Lanes: A, λ *Hin*dIII markers; B, uncut plasmid from clone 6; C, uncut plasmid from clone 9; F, clone 6 plasmid digested with *Eco*RI/*Hin*dIII; G, clone 9 plasmid digested with *Eco*RI/*Hin*dIII; H, λ *Hin*dIII markers.

Figure 6. Nucleotide sequence of cloned fragment and flanking sequence. Primer placement is depicted in blue and the coding region in red. The sequence shown was obtained from TIGR, and base-pair differences determined by automated DNA sequencing are shown in green. A Shine-Dalgarno sequence (shown in green) is located nine base-pairs upstream of the ATG initiation codon. The TAA stop codon can also be observed as the last three base-pairs of the coding sequence (red).



A.



B.

Figure 7. Thin-layer chromatographies exhibit bile acid hydrolysis.

A) Wild-type *E. faecalis* exhibiting bile acid hyrolysis of both glycine and taurine conjugates. Samples of *E. faecalis* were allowed to incubate in media containing a final concentration of 1 mM of conjugated bile acid. Samples were taken before inoculation (T_0) and after 24 hours of incubation (T_{24}). Lanes: A, CA and DCA standards; B-C, TH broth – T_0 and T_{24}; D-E, bile salts #3 – T_0 and T_{24}; F-G, GCA – T_0 and T_{24}; H-I, TCA – T_0 and T_{24}; J-K, GDCA – T_{24} and T_0; L-M, TDCA – T_0 and T_{24}; N, CA and DCA standards; O, CA standard; P, DCA standard;

B) NM522 with or without CBAH insert. Samples were taken at 24 hours post incubation. Lanes: A, CA and DCA standards; B-C, TH broth – (-)insert, (+)insert; D-E, bile salts #3 – (-)insert, (+)insert; F-G, GCA – (-)insert, (+)insert; H-I, TCA – (-)insert, (+)insert; J-K, GDCA – (-)insert, (+)insert; L-M, TDCA – (-)insert, (+)insert; N, CA and DCA standards; O, CA standard; P, DCA standard.

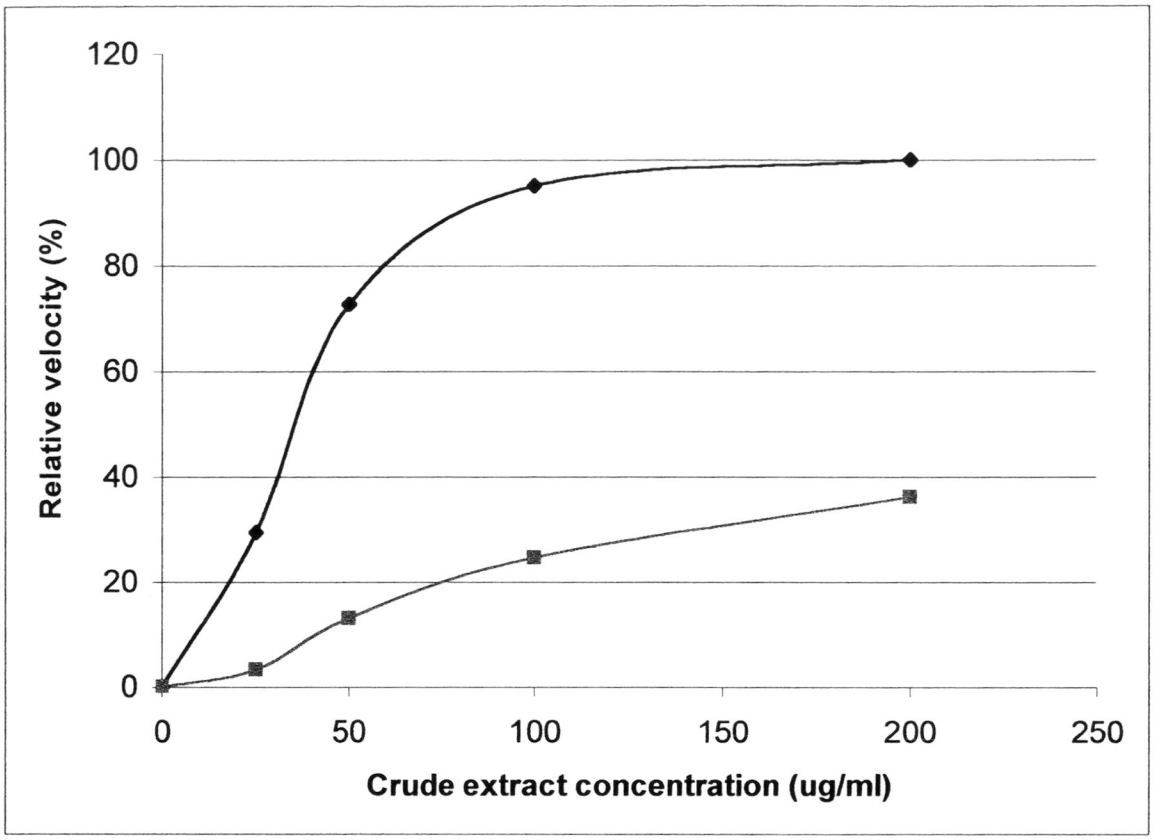
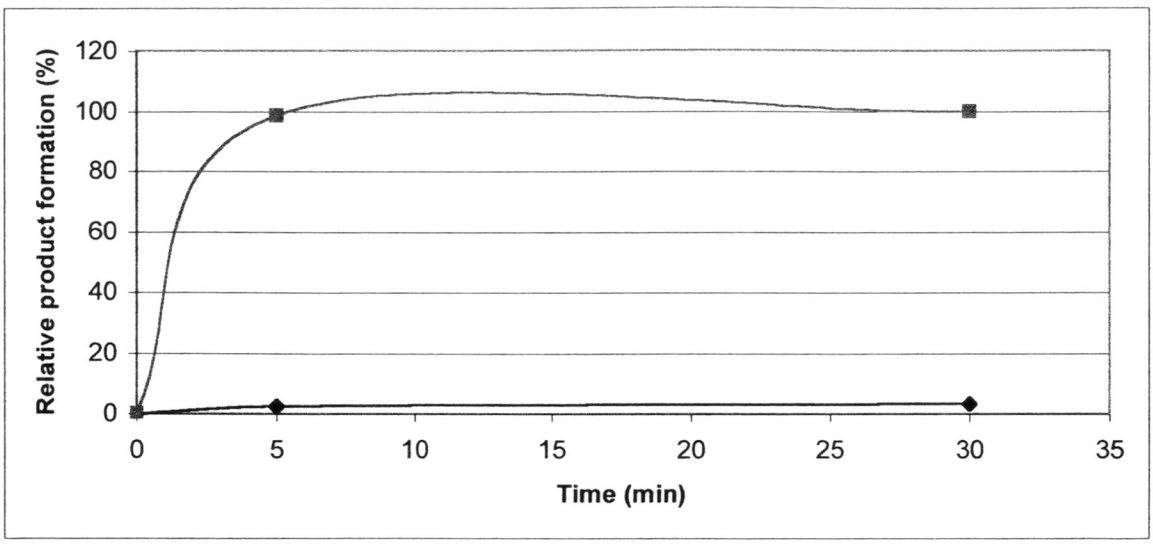
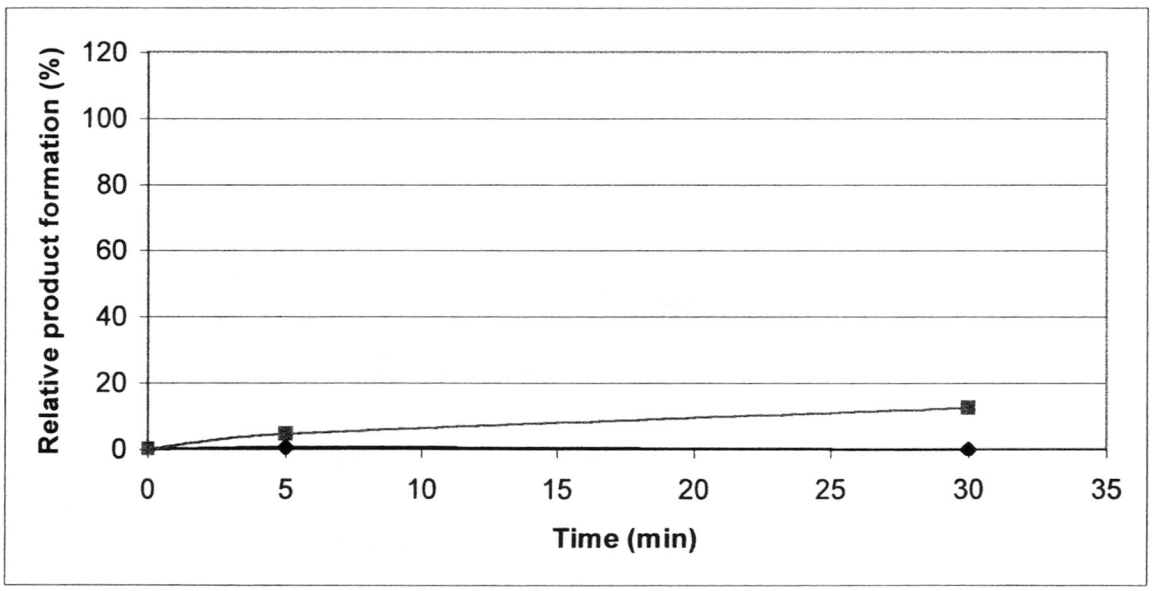


Figure 8. Hydrolysis of cholic acid conjugates. Various concentrations of crude extract (0, 25, 50, 100, and 200 μg) from *E. coli* strain NM522(pJCG-6) were assayed in a final volume of 1 ml, using 10 mM GCA and TCA. Samples were taken after a 5-minute incubation at 37°C. Diamonds represent GCA, and squares TCA.



A. GCA hydrolysis



B. Penicillin V hydrolysis

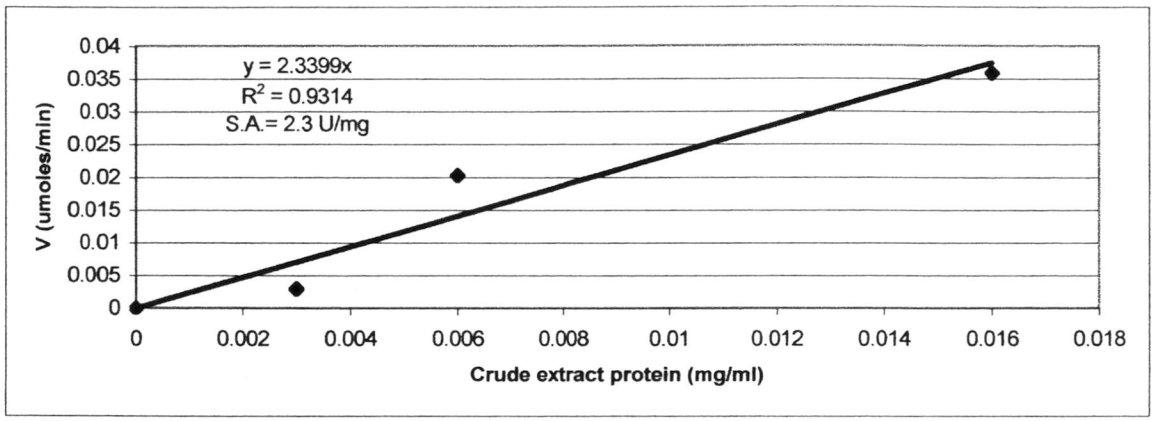
Figure 9. Hydrolysis of GCA and penicillin V. 500 μg of crude extract from *E. coli* strain NM522 was assayed in a final volume of 1 ml, with **A**)1 mM GCA and **B**) 2 mM penicillin V. Samples were taken at 0, 5, and 30 minutes during a 37°C incubation period. Diamonds represent *E. coli* strain NM522(pUC19), and squares represent NM522(pJCG-6).

Figure 10. Enzymatic properties of crude extracts and the purified CBAH enzyme.

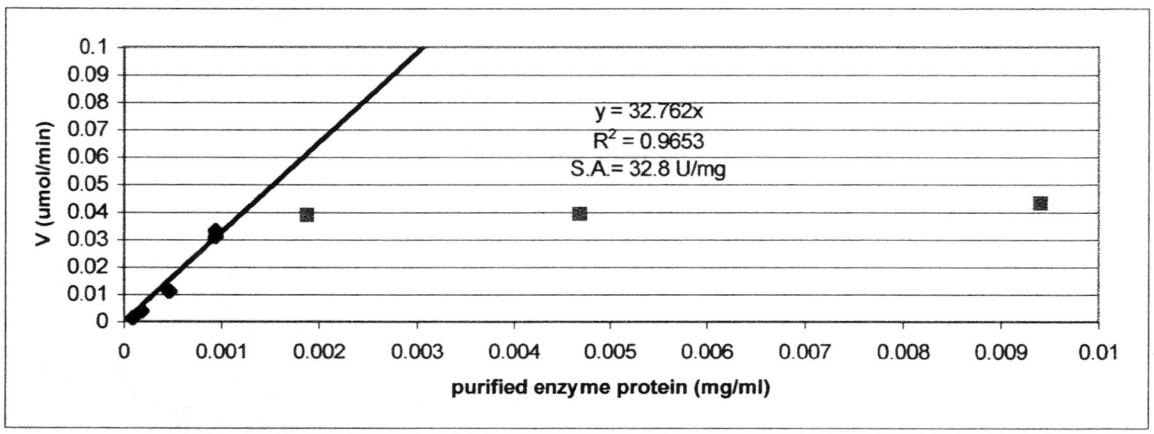
A) Effect of crude extract concentration on reaction rate. Various concentrations of NM522(pJCG-6) crude extract or purified enzyme were assayed in a final volume of 1 ml, using 5 mM GCA for 5 minutes at 37°C, at a pH of 5.0.

B) Effect of purified enzyme concentration on reaction rate. Various amounts of the purified CBAH enzyme were assayed in a final volume of 1 ml, using 5 mM GCA, for 5 minutes at 37°C at a pH of 5.5. The points not connected by regression line were in an enzyme excess region.

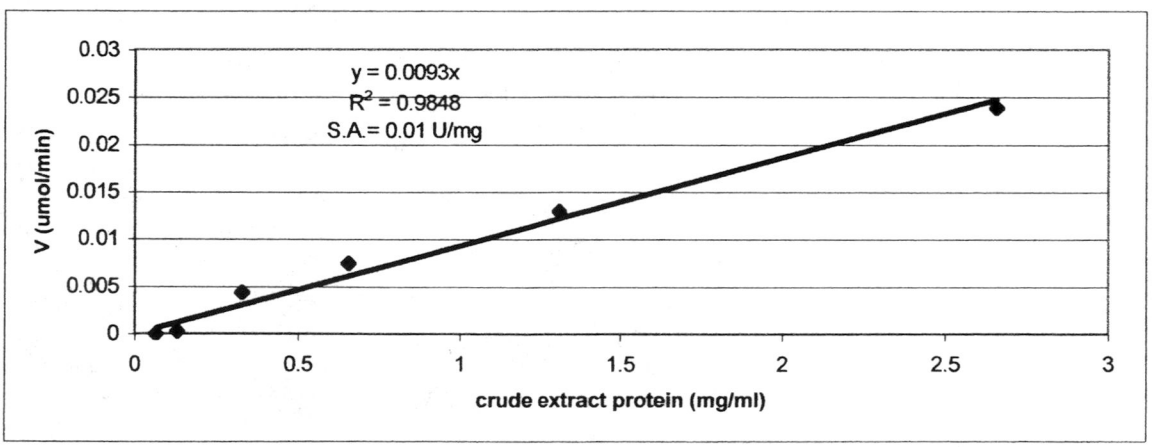
C) Effect of crude extract concentration on reaction rate. Various amounts of extract were assayed in a final volume of 1 ml, using 5 mM GCA for 5 minutes at 37°C, the pH was 5.5.



A. NM522/pJCG-6 crude extract activity



B. Purified CBAH activity



C. *E. faecalis* crude extract activity

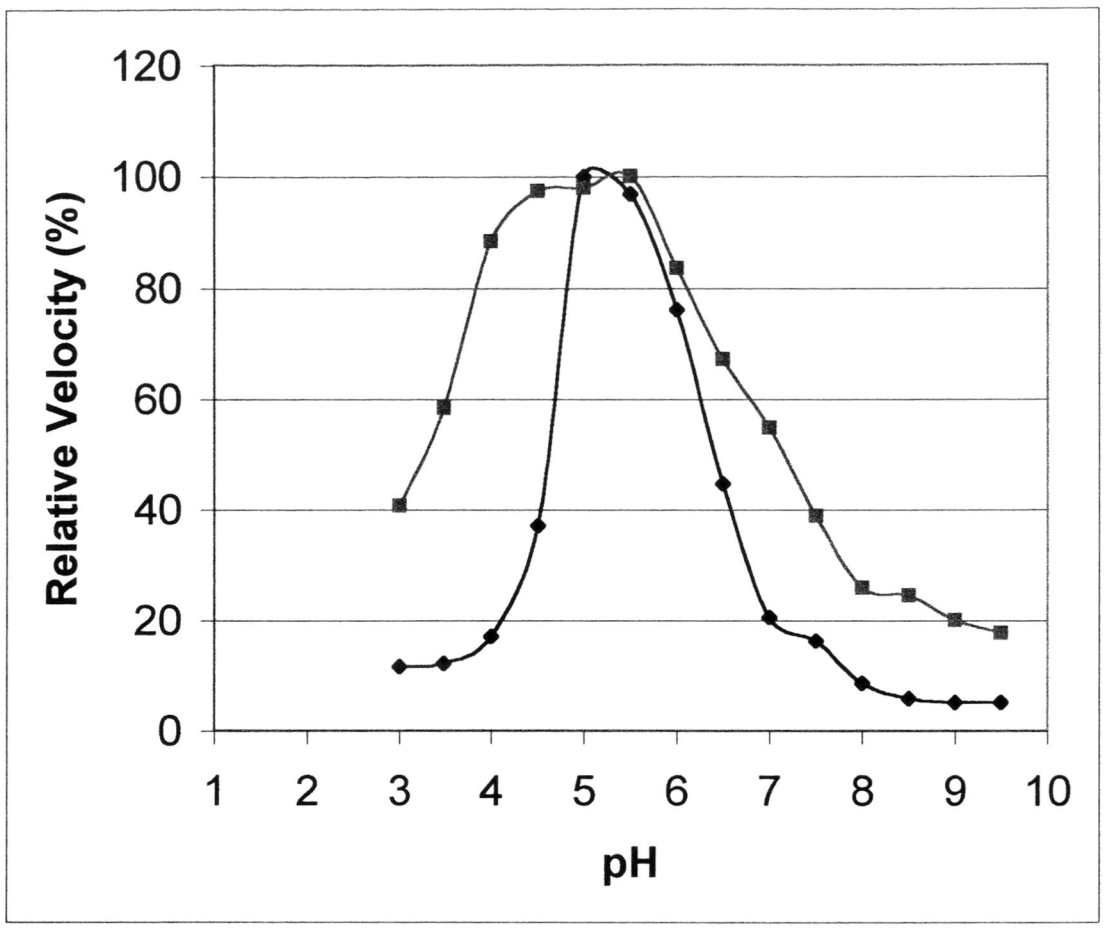


Figure 11. Effect of pH on the purified CBAH and *E. faecalis* crude extract reactions. 0.75 μg of purified CBAH enzyme and 2 mg of crude extract were assayed in a final volume of 1 ml, using 5 mM GCA at 37°C for 5 minutes. The pH of the reaction buffer was altered at 0.5 intervals. Squares represent crude extract, diamonds represent purified enzyme.

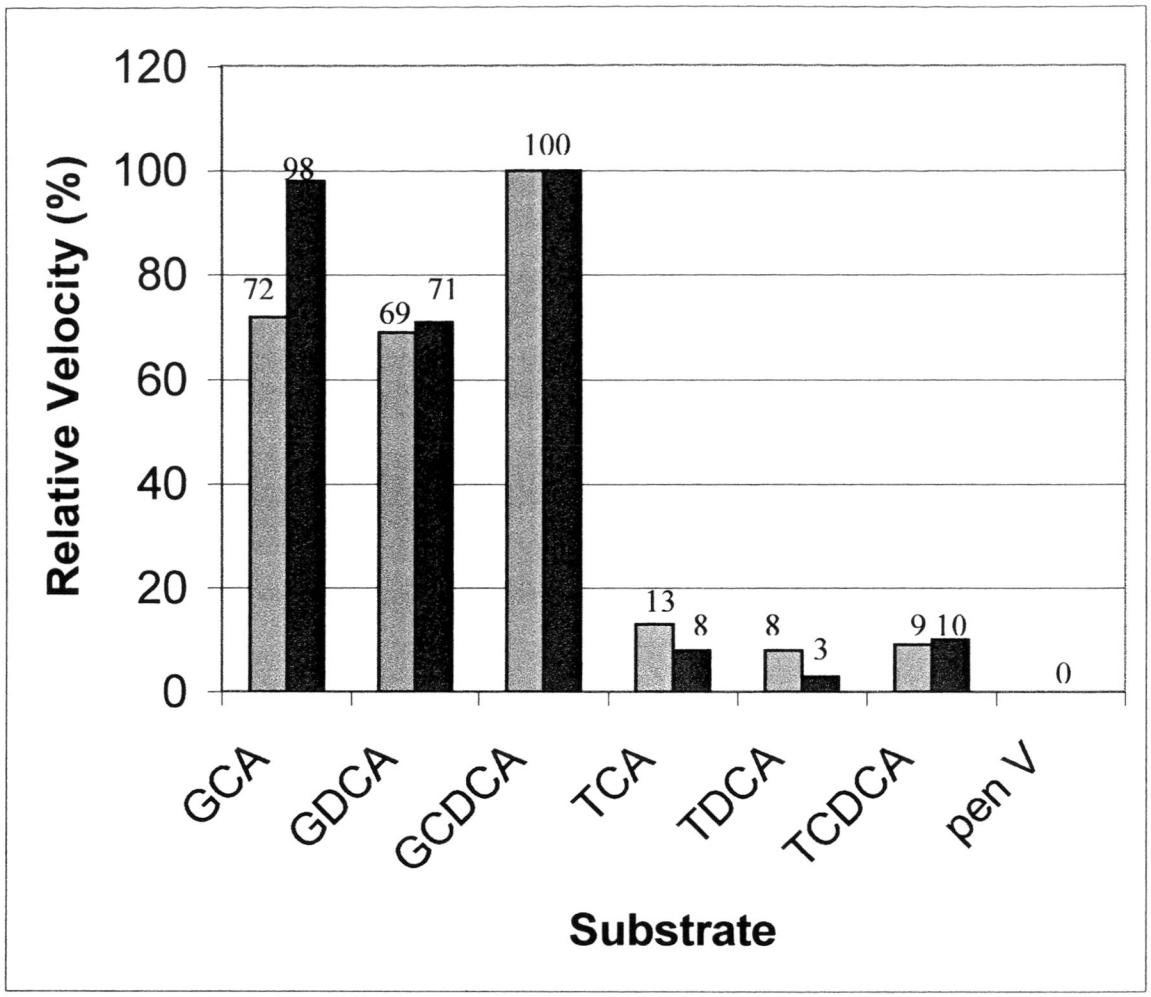


Figure 12. Substrate specificity of purified enzyme and *E. faecalis* crude extract. 0.75 μg of purified enzyme or 1 mg of crude extract was assayed in a final volume of 1 ml, using 5 mM of bile acid or penicillin V at 37°C for 5 minutes, pH 5.0. Light bars represent purified enzyme, dark bars represent *E. faecalis* crude extract.

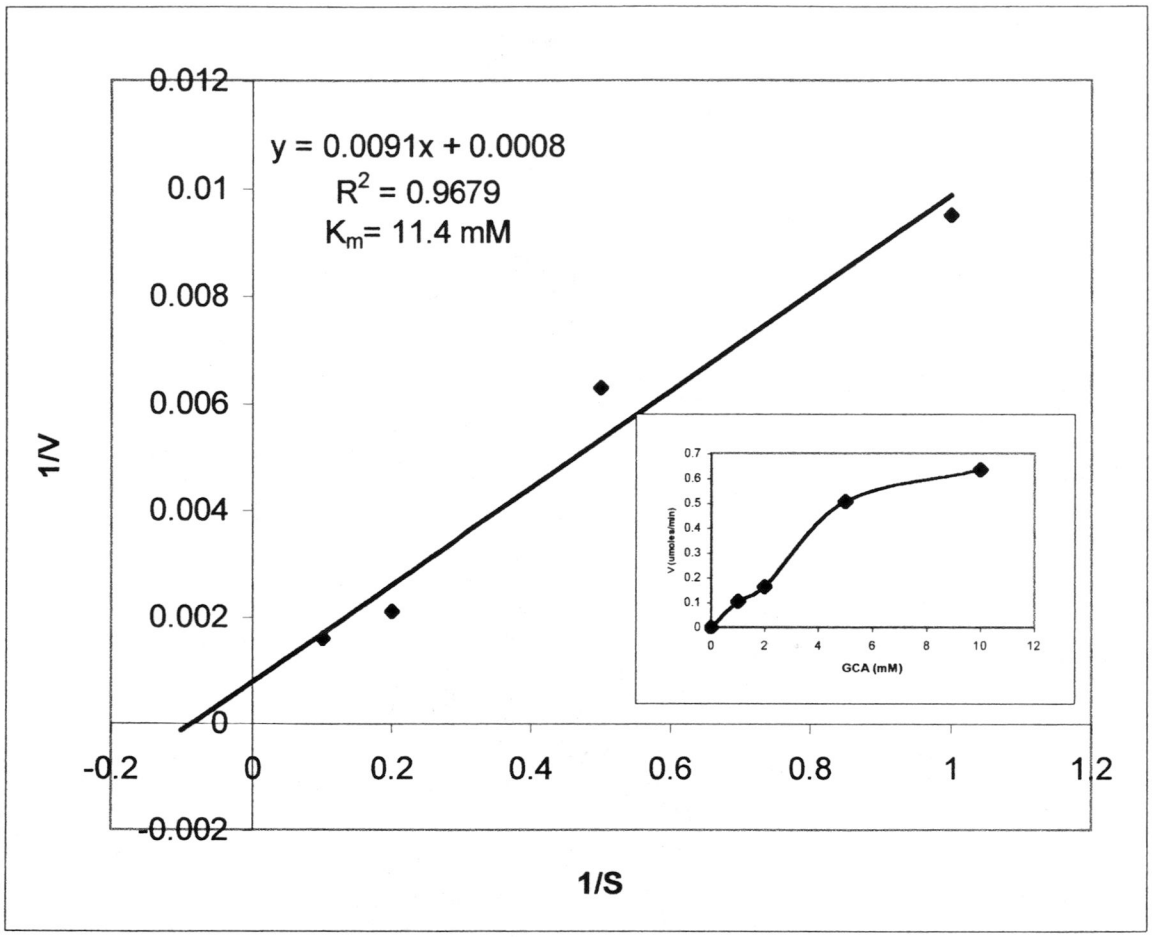


Figure 13. Lineweaver-Burk plot and substrate saturation curve for GCA.

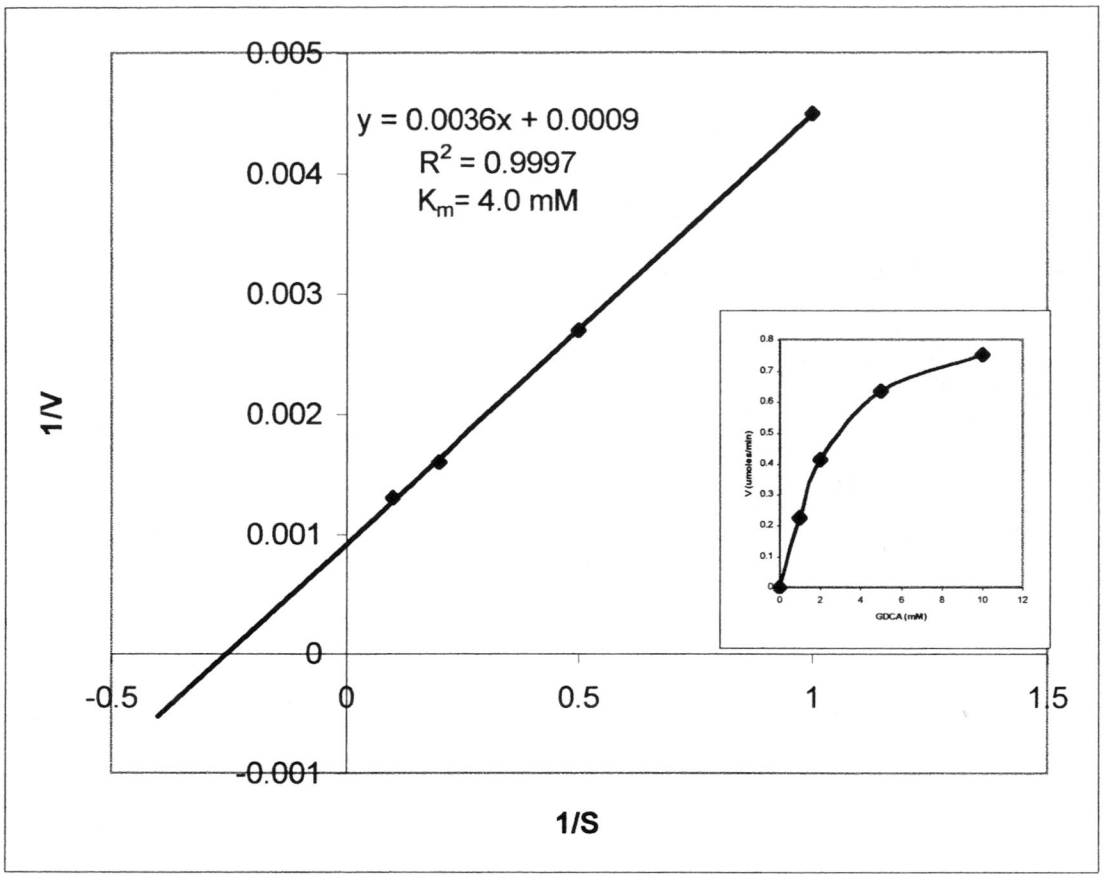


Figure 14. Lineweaver-Burk plot and substrate saturation curve for GDCA.

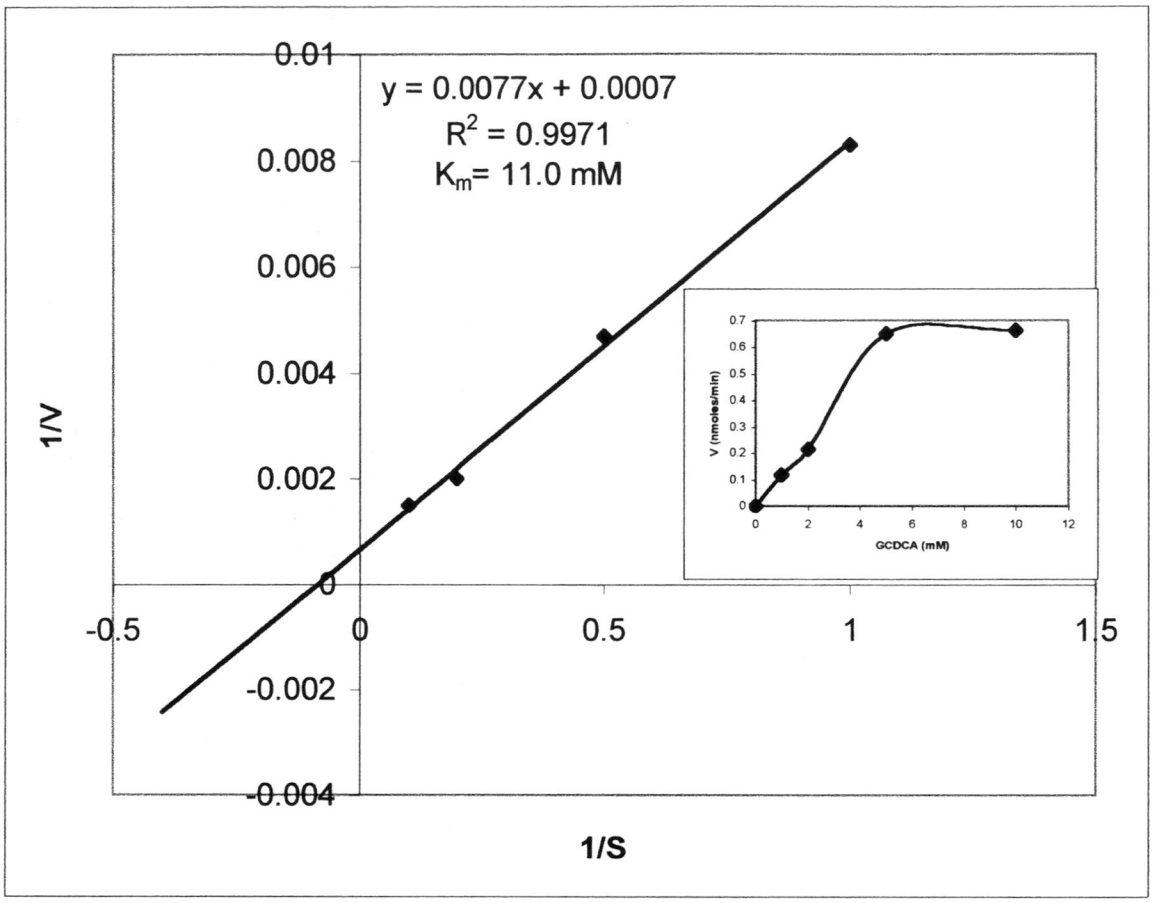


Figure 15. Lineweaver-Burk plot and substrate saturation curve for GCDCA.

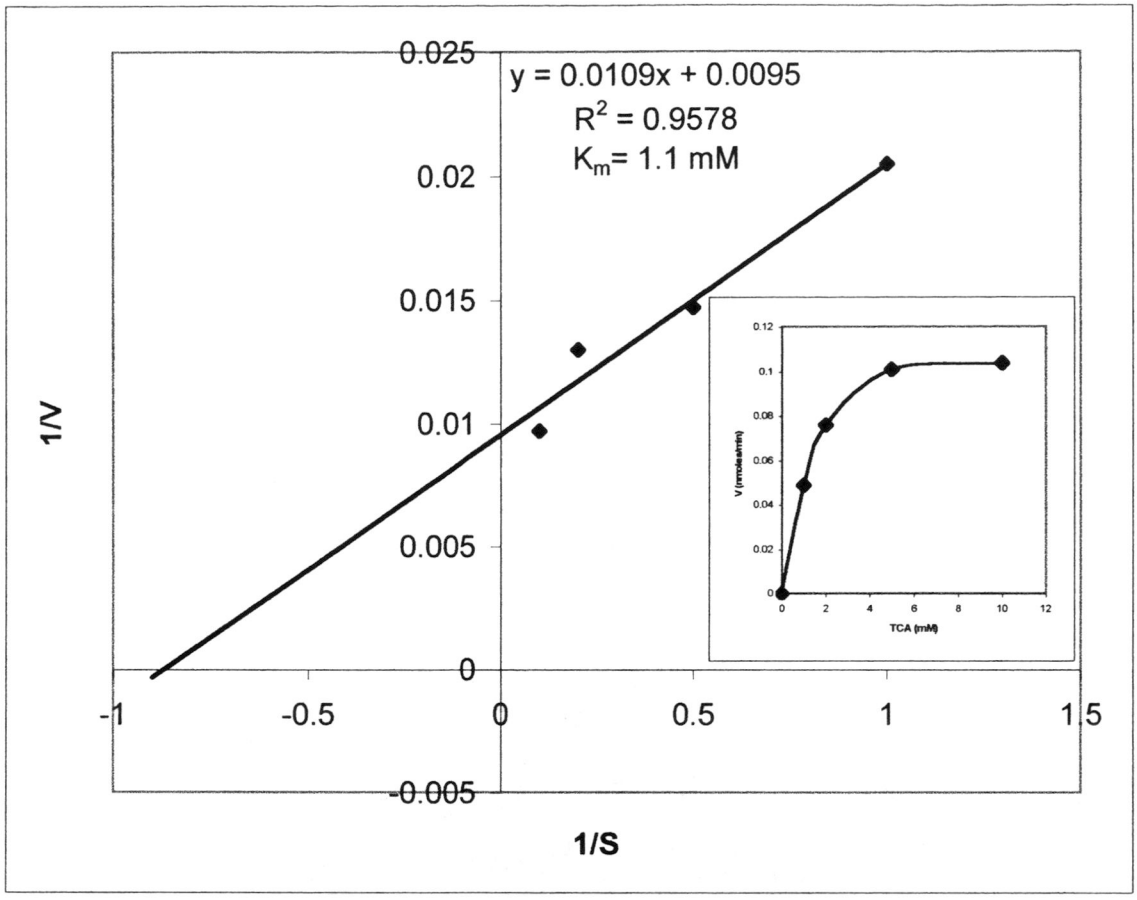


Figure 16. Lineweaver-Burk plot and substrate saturation curve for TCA.

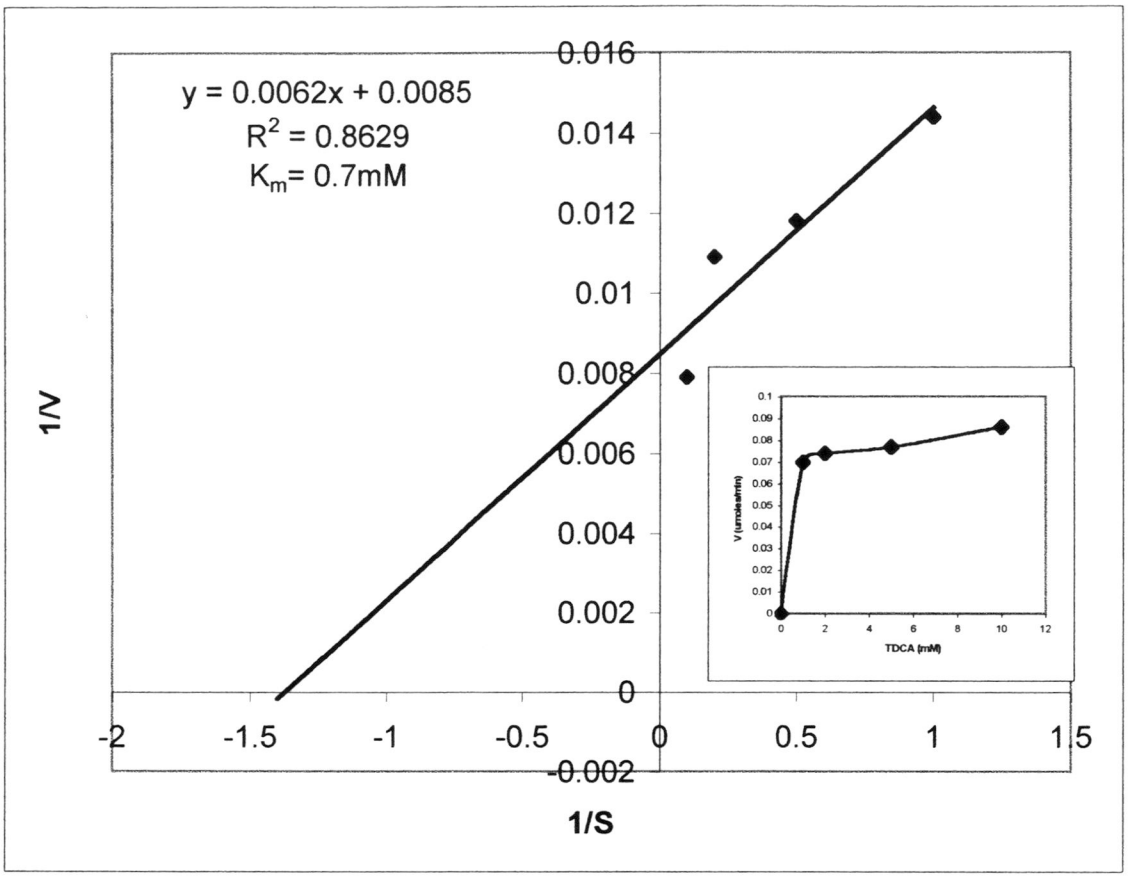


Figure 17. Lineweaver-Burk plot and substrate saturation curve for TDCA.

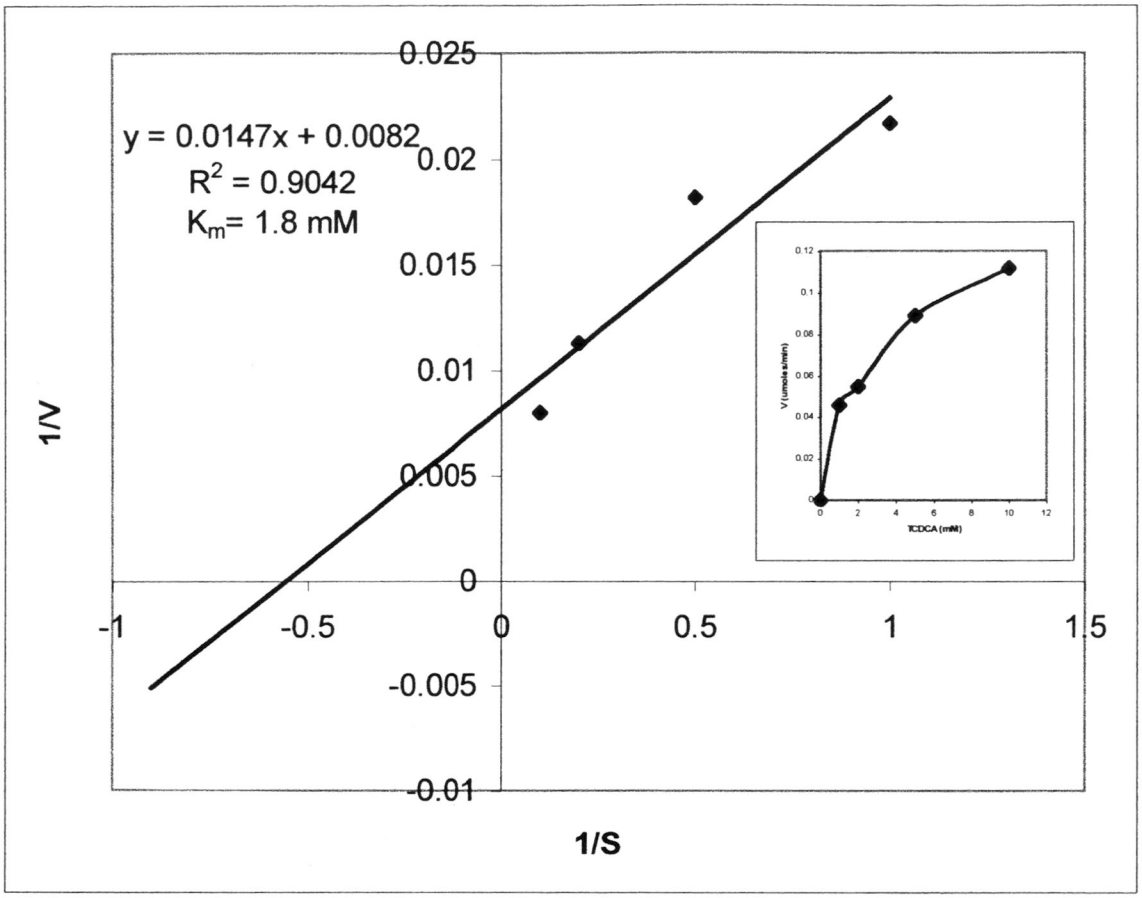


Figure 18. Lineweaver-Burk plot and substrate saturation curve for TCDCA.

TABLE 1. Summary of kinetic parameters

Substrate	K_m (mM)	Relative V_{max} (%)
GCA	11.4	87.5
GDCA	4.0	77.8
GCDCA	11.0	100
TCA	1.1*	7.4*
TDCA	0.7*	8.2*
TCDCA	1.8*	8.5*

* Obtained from non-linear double-reciprocal plots

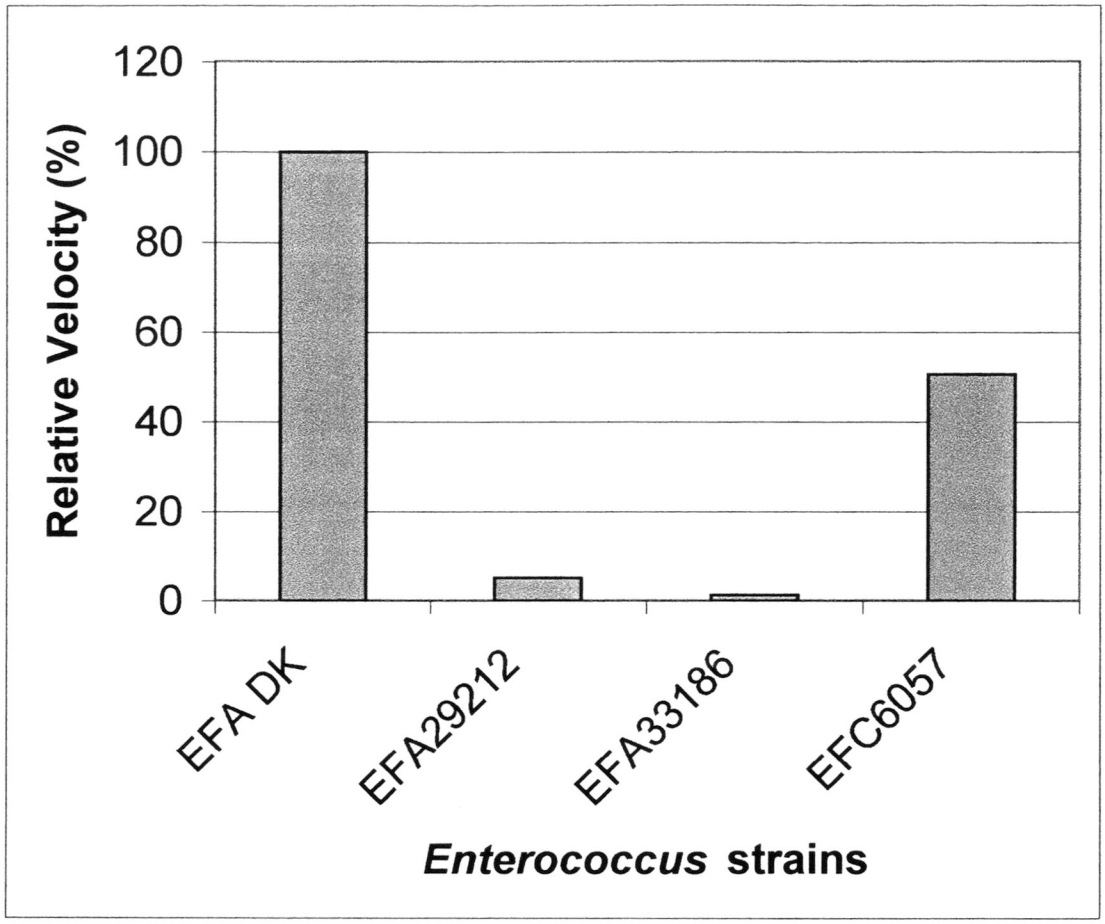


Figure 19. Ability of *Enterococcus* strains to hydrolyze GCA. 1 mg of crude extract was assayed in a final volume of 1 ml, using 5 mM GCA at 37°C for 30 minutes, pH 5.0.

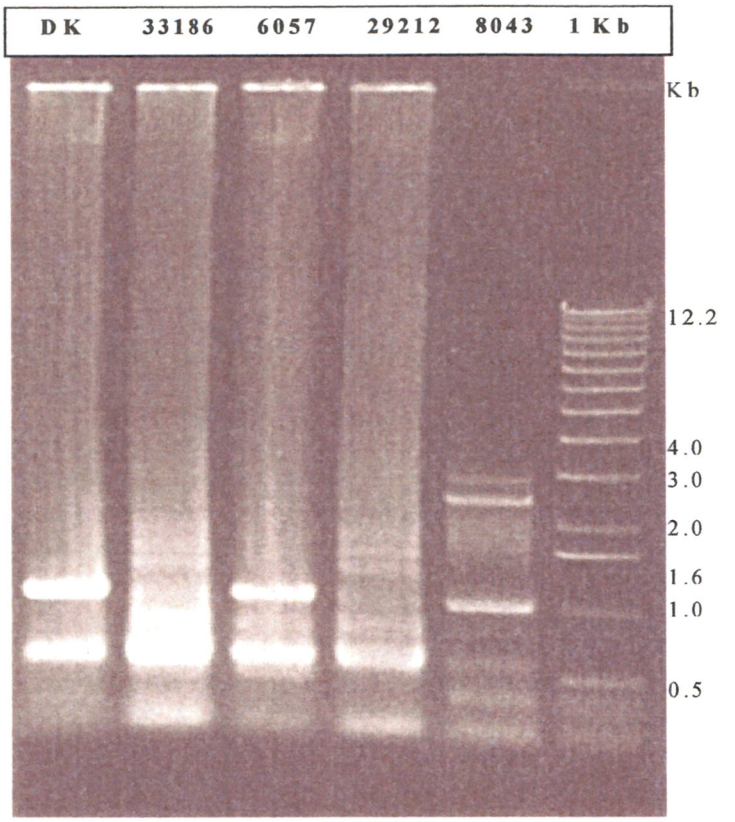


Figure 20. Gel electrophoresis of PCR fragments from *Enterococcus* strains. DNA exhibiting enough similarity to the *E. faecalis* CBAH gene will bind the primers, and exhibit a band approximately 1 Kb in size. Electrophoresis was performed at 70 volts for one hour. The gel was stained with 10 mg ethidium bromide.

Figure 21. Southern blot. The *E. faecalis* CBAH nucleotide sequence was used as a probe. Lanes: A, pJCG-6; B, 1 Kb ladder; C, *E. faecalis* strain ATCC 29212; D, *E. hirae* strain *ATCC* 8043; E, *E. faecium* strain ATCC 6057; F-G, *E. faecalis* strain DK; H, *E. faecalis* strain ATCC 33186. The CBAH gene is detected in *E. faecium* strain ATCC 6057, and *E. faecalis* strain DK.

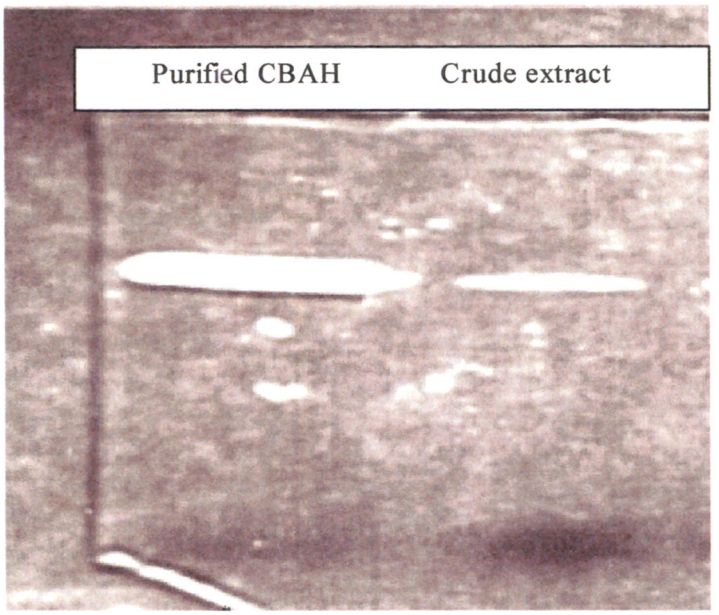


Figure 22. Activity stain assay of purified enzyme and crude extract. 1 mg of crude extract or 2 μg of purified enzyme was electrophoresed on a non-denaturing 7.5% acrylamide gel at 70 volts for one hour, and soaked in TDCA solution. The presence of a white band would represent side-chain hydrolysis of TDCA.

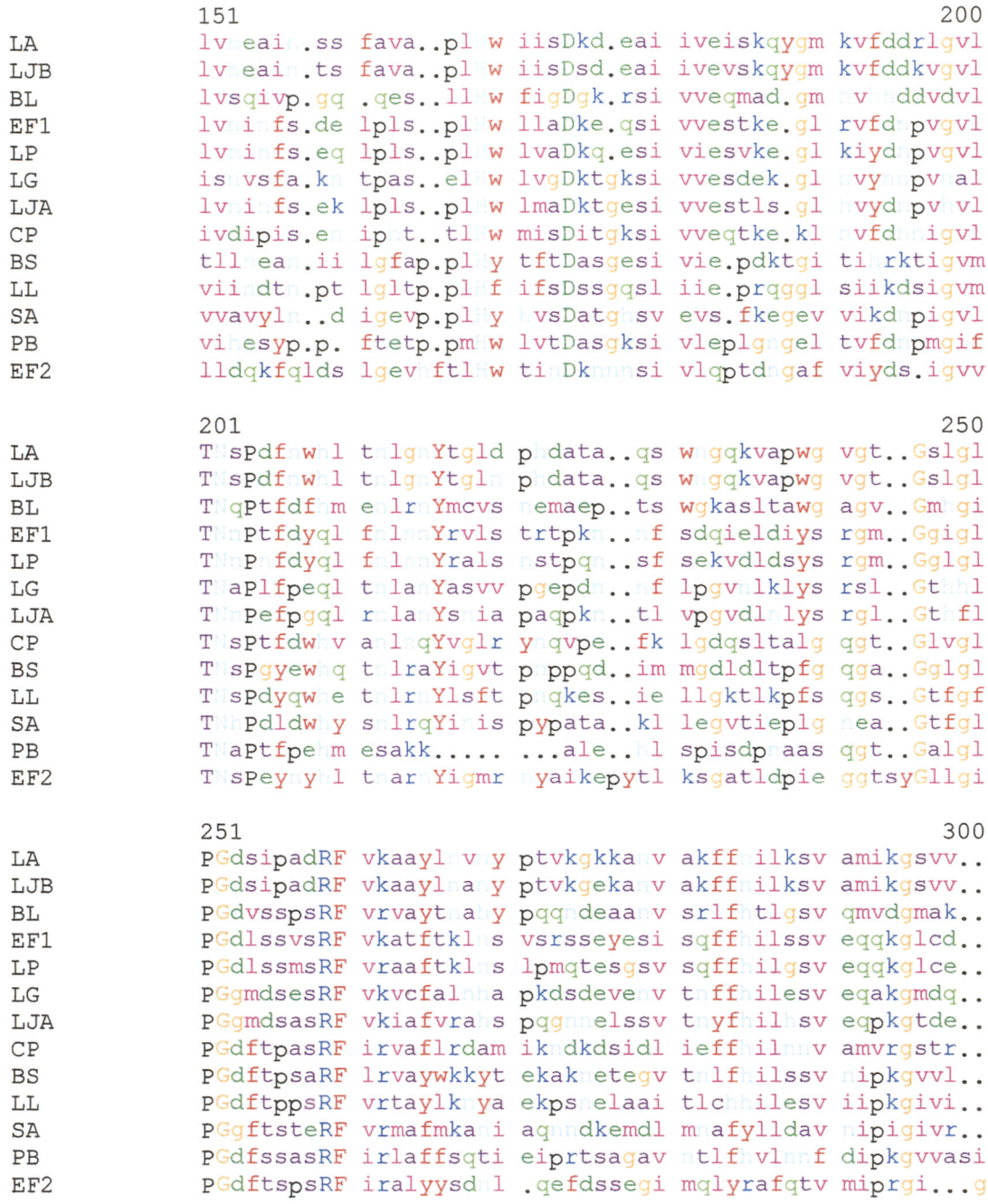
```
  1  mctaityvsk dhyfgrnfdy eisynevvti tprnykfsfr evgnldhhfa
 51  iigiaagiad yplyydaine kglgmaglnf sgyadykkie egkenvspfe
101  fipwvlgqcs tvdeakkllk nlnlvninfs delplsplhw lladkeqsiv
151  vestkeglrv fdnpvgvltn nptfdyqlfn lnnyrvlstr tpknnfsdqi
201  eldiysrgmg giglpgdlss vsrfvkatft klnsvsrsse yesisqffhi
251  lssveqqkgl cdvgdekyey tiyssccnle kgiyyyrtyd nsqitavdmn
301  kenlekdsli vypmvetqqi nyan*
```

Figure 23. Deduced peptide sequence of the *E. faecalis* strain V583 (obtained from TIGR). The TIGR sequence differs from the *E. faecalis* strain DK by two amino acids, a V to an A at pos. 28, and an E to a G at pos. 242.

```
            1                                                  50
LA            mCtglrft ddqgnlyfgR nldvgqd.yg egviitPrny plpykfldnt
LJB           mCtglrft ddqgnlyfgR nldvgqd.yg egviitPrny plpykfldnt
BL            mCtgvrfs ddegntyfgR nldwsfs.yg etilvtPrgy hydtvfgagg
EF1           mCtaityv skd..hyfgR nfdyeis.yn evvtitPrny kfsfr.evgn
LP            mCtaityq syn..nyfgR nfdyeis.yn emvtitPrky plvfr.kven
LG            mCtsilys pkd..hyfgR nldyeia.yg qkvvitPkny efeft.dlpa
LJA           mCtsivys snnh.hyfgR nldleis.fg ehpvitPrny efqyr.klpn
CP            mCtglale tkdglhlfgR nmdieys.fn qsiifiPrnf kcvnksnkke
BS          mlgCsslsir ttddkslfaR tmdftme.pd skviivPrny girllekenv
LL          migCssftle sqdnkhflsR tmdfqie.ma eqilfiPrnk eigfahsnee
SA            mCtgftiq tlnnqvllgR tmdydyp.ld gspavtPrny ..rwtsrtgt
PB            mCsglrii addgtvvvgR tle.....fg enil...... ..kfkkfvng
EF2           mCtgikii sktndifygR tmdftfdffg nedpiaPkip tliaqfpkgt

            51                                                 100
LA          t.tk...... ..kaviGmgi vvdgyps... ......yfDc fNEdGlgiag
LJB         t.tk...... ..kaviGmgi vvdgyps... ......yfDc yNEdGlgiag
BL          k.ak...... .pnaviGvgv vmadrpm... ......yfDc aNEhGlaiag
EF1         l.dh...... .hfaiiGiaa giadypl... ......yyDa iNEkGlgmag
LP          l.dh...... .hyaiiGita dvesypl... ......yyDa mNEkGlciag
LG          e.ks...... .hyamiGvaa vadntpl... ......ycDa iNEkGlgvag
LJA         k.ka...... .kyamvGmai vednypl... ......yfDa sNEeGlgiag
CP          l.tt...... .kyavlGmgt ifddypt... ......faDg mNEkGlgcag
BS          vinn...... .syafvGmgs tditspv... ......lyDg vNEkGlmgam
LL          tiet...... .syaclGmga meeghpv... ......lfDg iNEkGlmgat
SA          tgqt...... .qygfiGtg. tdmegfi... ......ygDg vNEhGvaist
PB          nir....... ......Gist pdgk...... ......llDg mNEhGlvifv
EF2         vlnsqlnpwt akyafmGlam sgtdqpandg ktvslaitDg iNEaGls.gd

            101                                                150
LA          lnfphfakfs dgpidgkinl asy...eiml wvtqnftkvs dvkealknvn
LJB         lnfphfakfs dgpidgkinl asy...eiml wvtqnfthvs evkealknvn
BL          lnfpgyasfv hepvegtenv atf...efpl wvarnfdsvd eveetlrnvt
EF1         lnfsgyadyk kie.egkenv spf...efip wvlgqcstvd eakkllknln
LP          lnfagyadyk kyd.adkvni tpf...elip wllgqfssvr evkkniqklh
LG          lsfagqgkyf pna.adkkni asf...efis yllatyetvd qvkesltnan
LJA         lnfdgpchyf pen.aeknnv tpf...elip yllsqcttva evkdalkdvs
CP          lnfpvyvsys kediegktni pvy...nfll wvlanfssve evkealknan
BS          lyyatfatya depkkgttgi npv...yvis qvlgncvtvd dviekl.tsy
LL          lyfpgyadys enikknqkgi spd...mvip tvltqasnle eiidlfdkkf
SA          qyfrgyssyg sthkadamni tqn...eivt wilgyttsie dmkqqasqih
PB          fyfknyakyg .cpsqtklni kpt...eval fllqkaknvk dvkaiaktln
EF2         iqylmessta paesladrgl tphiaeevla yilsnfesvd evkvafekig
```


151 200
LA lveain.ss fava..plww iisDkd.eai iveiskqygm kvfddrlgvl
LJB lveain.ts fava..plww iisDsd.eai ivevskqygm kvfddkvgvl
BL lvsqivp.gq .qes..llww figDgk.rsi vveqmad.gm nvnddvdvl
EF1 lvinfs.de lpls..plww llaDke.qsi vvestke.gl rvfdnpvgvl
LP lvinfs.eq lpls..plww lvaDkq.esi viesvke.gl kiydnpvgvl
LG isnvsfa.kn tpas..elww lvgDktgksi vvesdek.gl nvynnpvnal
LJA lvnifs.ek lpls..plww lmaDktgesi vvestls.gl nvydnpvnvl
CP ivdipis.en ipnt..tlww misDitgksi vveqtke.kl nvfdnnigvl
BS tllneam.ii lgfap.plyy tftDasgesi vie.pdktgi tinrktigvm
LL viindtn.pt lgltp.plff ifsDssgqsl iie.prqggl siikdsigvm
SA vvavylnn..d igevp.plyy nvsDatgnsv evs.fkegev vikdnpigvl
PB vinesyp.p. ftetp.pmww lvtDasgksi vleplgngel tvfdnpmgif
EF2 lldqkfqlds lgevnftlww tinDknnnsi vlqptdngaf viyds.igvv

201 250
TnsPdfnwll tnlgnYtgld pndata..qs wngqkvapwg vgt..Gslgl
LJB TnsPdfnwll tnlgnYtgln pndata..qs wngqkvapwg vgt..Gslgl
BL TnqPtfdfnm enlrnYmcvs nemaep..ts wgkasltawg agv..Gmngi
EF1 TnnPtfdyql fnlnnYrvls trtpkn..nf sdqieldiys rgm..Ggigl
LP TnnPnfdyql fnlnnYrals nstpqn..sf sekvdldsys rgm..Gglgl
LG TnaPlfpeql tnlanYasvv pgepdn..nf lpgvnlklys rsl..Gtnhl
LJA TnnPefpgql rnlanYsnia paqpkn..tl vpgvdlnlys rgl..Gtnfl
CP TnsPtfdwnv anlnqYvglr ynqvpe..fk lgdqsltalg qgt..Glvgl
BS TnsPgyewnq tnlraYigvt pnppqd..im mgdldltpfg qga..Gglgl
LL TnsPdyqwne tnlrnYlsft pnqkes..ie llgktlkpfs qgs..Gtfgf
SA TnnPdldwny snlrqYinis pypata..kl legvtieplg nea..Gtfgl
PB TnaPtfpelm esakk..... ...ale..nl spisdpnaas qgt..Galgl
EF2 TnsPeynyll tnarnYigmr nyaikepytl ksgatldpie ggtsyGllgi

251 300
PGdsipadRF vkaaylnvny ptvkgkkanv akffnilksv amikgsvv..
LJB PGdsipadRF vkaaylnany ptvkgekanv akffnilksv amikgsvv..
BL PGdvsspsRF vrvaytnahy pqqndeaanv srlfntlgsv qmvdgmak..
EF1 PGdlssvsRF vkatftklns vsrsseyesi sqffnilssv eqqkglcd..
LP PGdlssmsRF vraaftklns lpmqtesgsv sqffnilgsv eqqkglce..
LG PGgmdsesRF vkvcfalnha pkdsdevenv tnffnilesv eqakgmdq..
LJA PGgmdsasRF vkiafvrahs pqgnnelssv tnyfnilnsv eqpkgtde..
CP PGdftpasRF irvaflrdam ikndkdsidl ieffnilnnv amvrgstr..
BS PGdftpsaRF lrvaywkkyt ekaknetegv tnlfnilssv nipkgvvl..
LL PGdftppsRF vrtaylknya ekpsnelaai tlcnnilesv iipkgivi..
SA PGgftsteRF vrmafmkani aqnndkemdl mnafylldav nipigivr..
PB PGdfssasRF irlaffsqti eiprtsagav ntlfnvlnnf dipkgvvasi
EF2 PGdftspsRF iralyysdnl .qefdssegi mqlyrafqtv miprgi...g

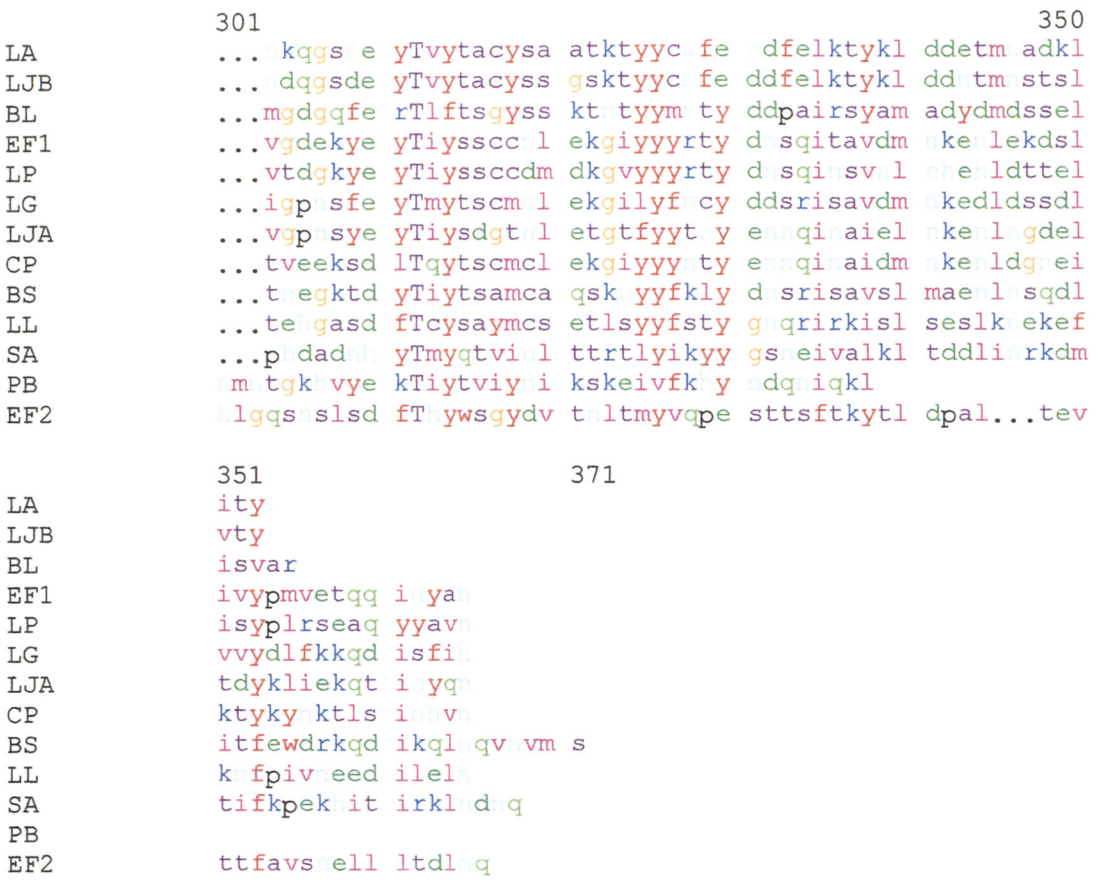


Figure 24. Sequence alignment of CBAH and penicillin V amidase peptide sequences. The *E. faecalis* CBAH exhibits most similarity to the CBAH from *L. plantarum* with 70% identity, and 82% homology. CBAH lanes: LA, *L. acidophilus*; LJB, *L. johnsonii* peptide B; BL, *B. longum*; EF1, *E. faecalis*; LP, *L. plantarum*; LG, *L. gasseri*; LJA, *L. johnsonii* peptide A; CP, *C. perfringens*; Penicillin V amidase lanes: BS, *B. sphaericus*; LL, *L. lactis*; SA, *S. aureus*; PB, *P. busaria*; EF2, *E. faecalis* gene of unknown function. The *E. faecalis* sequence shown is that of strain V583.

Phylogenetic dendrogram of known CBAH and penicillin V amidase peptide sequences

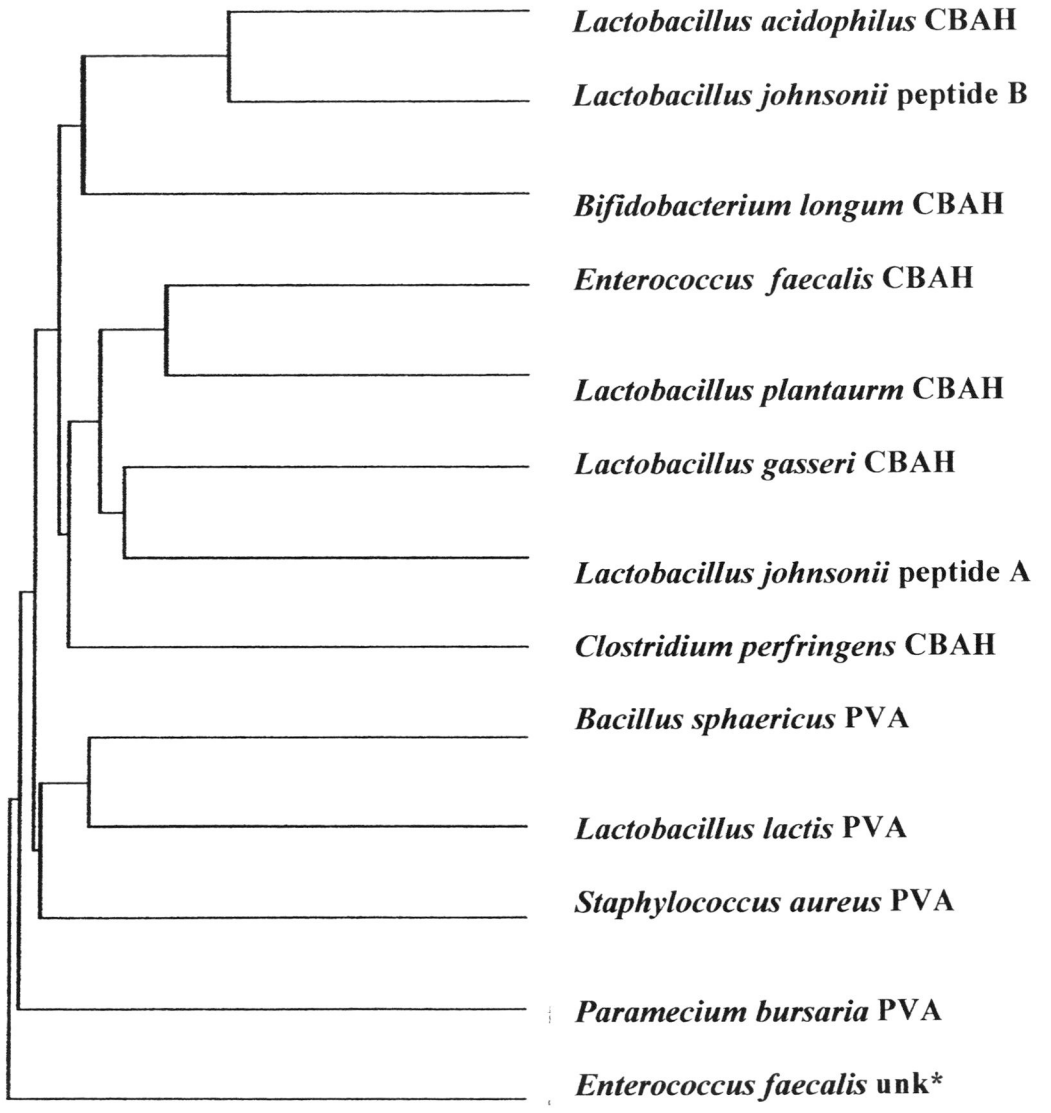


Figure 25. Dendrogram. The *E. faecalis* CBAH peptide sequence exhibits the greatest similarity to *L. plantarum, L. gasseri, L. johnsoniiK,* and *C. perfringens* CBAH sequences. unk*=unknown function.

DISCUSSION

Since the first conjugated bile acid hydrolase (from *Clostridium perfringens*) was characterized by Nair et al. (1967), numerous enzymes from different species of bacteria have been identified, and, as can be seen from Table 2, many of the characteristics of conjugated bile acid hydrolases are quite similar. Previously characterized penicillin V amidases also exhibit the same similarities, not only to themselves, but also, to conjugated bile acid hydrolases.

Both Aries and Hill (1969) and Kobashi et al. (1978) reported finding CBAH activity in strains of *E. faecalis*, but this activity has yet to be isolated. This work describes the successful cloning and characterization of the CBAH gene from *E. faecalis* strain DK. Numerous assays, as well as sequence analysis, have provided evidence to support the hypothesis that the cloned gene is that of a CBAH and not a penicillin V amidase.

Sequence analysis of the TIGR database using the previously cloned *C. perfringens* CBAH gene, provided evidence for the presence of the gene in *E. faecalis*. An open reading frame of approximately 975 base pairs was discovered, predicting a peptide of approximately 325 amino acids. The calculated protein size of the peptide, achieved by purification and SDS-PAGE, is near 37 kDa. This is similar to findings for *B. vulgatus* (Kawamoto et al., 1989), *L. plantarum* (Christiaens et al., 1992), *B. longum* (Tanaka et al., 2000), the B peptide of *L. johnsonii* (Lundeen and Savage, 1992), *C. perfringens* (Coleman and Hudson, 1995), and the *B. sphaericus* PVA (Pundle et al.,

1997). The *E. faecalis* sequence obtained from TIGR (strain V583) differs from the sequence obtained by our own automated sequencing (DK) by 3 nucleotides, a C to a T at position 83, a G to an A at position 725, and a C to a T at position 859 (Fig. 31). These single base-pair substitutions result in only 2 amino acid substitutions, a Val to an Ala at position 28, and a Glu to a Gly at position 242. The third nucleotide substitution is a conserved one where the Tyr is not altered at position 286.

The greatest activity was observed in clones of *E. coli* strain NM522, and extract produced from this organism was used for enzyme purification. Purification of the CBAH was achieved by first subjecting the crude extract to ammonium sulfate precipitation. Highest activity was observed in the 40-70% pellet. When applied to the hydrophobic interaction column, the enzyme eluted in the later fractions. Since proteins elute from the column in order of increasing hydrophobicity, it appears that the enzyme is somewhat hydrophobic. The quaternary structure of our enzyme have not been determined, whatever the organization may be, it should consist of subunits approximately 37 to 40 KDa in size, since this was the only band obtained from the SDS-PAGE. Calculations based on comparisons of activity between CBAH-containing NM522 crude extract and the purified enzyme give evidence of a 15-fold purification of the enzyme activity. Calculations based on activity differences between *E. faecalis* crude extract and the purified enzyme produce a specific activity of 0.01 U/mg for the crude, and 32.8 U/mg for the purified CBAH. A fold-purification estimation of 3280X is achieved, and an estimation of abundance of the CBAH is around 0.03% of the total *E. faecalis* protein.

Table 2. Characteristics of known CBAH and penicillin V amidases

Organism	Optimum pH	Molecular weight (Da)	Subunit molecular weight (Da)	Native state	Substrate specificity
Bacteroides vulgatus	5.6-6.4	140,000	36,000	Tetramer	Taurine conjugates only
Lactobacillus plantarum	4.7-5.5	NA	37,078	Tetramer	Glycine > Taurine
Bifidobacterium longum BB536	5.5-6.5	250,000	40,000	Hexamer	Glycine/Taurine
Bifidobacterium longum SBT2928	5.0-7.0	130,000	35,024	Tetramer	Glycine/Taurine
Bacteroides fragilis	4.5	250,000	32,500	Octamer	Glycine/Taurine
Lactobacillus johnsonii peptide A	4.2-4.5	115,000	42,000	Trimer	Glycine/Taurine
Lactobacillus johnsonii peptide B	4.2-4.5	105,000	38,000	Trimer	Glycine/Taurine
Clostridium perfringens MCV815	5.8-6.4	250,000	56,000	Tetramer	Glycine > Taurine
Clostridium perfringens 13	NA	147,000	36,100	Tetramer	Glycine/Taurine
Lactobacillus acidophilus	3.5-5.5	126,000	NA	NA	Glycine > Taurine
Enterococcus faecalis	**5.0**	**NA**	**36,932**	**NA**	**Glycine > Taurine**
Fusarium SKF PVA	6.5	83,200	83,200	Monomer	Penicillin V
Bacillus sphaericus PVA	NA	138,000	35,000	Tetramer	Penicillin V

The optimum pH for bile acid deconjugation was 4.5-5.5 for the crude extract, and 5.0 for the purified enzyme. The range of pH is similar to that previously reported for enzymes from other sources (Table 1). The broad range of pH seen in the crude extract may be due to any of several factors, one being that a second gene could be another CBAH enzyme acting optimally at a different pH than the first. It is possible that other molecules contained in the *E. faecalis* crude extract could aid in hydrolysis and thus enable the enzyme to exhibit greater activity under a wider range of pH values.

Substrate specificicty assays show that the *E. faecalis* CBAH has the greatest affinity for glycine conjugates, and can cleave taurine conjugates to a lesser extent. Minimal activity against penicillin V was observed in the crude extract, leading to the possibility that penicillin V amidase activity exists in *E. faecalis*. Sequence analysis data points to this possibility since the second nucleotide sequence and its translated peptide sequence does not exhibit any significant similarity to any known CBAH sequences. The second enzyme shows greater similarity to known penicillin V amidase peptide sequences.

Kinetic data have been obtained for several previously isolated CBAH and penicillin V amidases. Table 3 lists known Km values. Table 1 lists the K_m and V_{max} values calculated for the purified *E. faecalis* CBAH. The K_m's obtained are the highest reported to-date for GCA and GDCA. The taurine conjugates could only be estimated, but they fall within other known values (Stellwag and Hylemon, 1976; Nair et al., 1967). The *E. faecalis* enzyme has the highest binding affinity for the conjugate of deoxycholic acid, and lowest binding affinity for that of cholic acid, among the glycine conjugates.

Table 3. Known Km values (mM)

Organism	GCA	GDCA	GCDCA	TCA	TDCA	TCDCA	Penicillin V
B. fragilis	0.35	0.20	0.26	0.45	0.17	0.29	
L. plantarum		0.22					
L. johnsonii peptide A				0.76			
L. johnsonii peptide B				0.95			
E. faecalis	**11.4**	**4.0**	**11.0**	**1.1**	**0.7**	**1.8**	
C. perfringens MCV 815	0.50						
C. perfringens 19574	3.6	1.2	14.0	37.0	3.5	3.0	
Chiania PVA							17.1
Fusarium SKF PVA							10.0

These findings are similar to those for the *Bacteroides fragilis* CBAH by Stellwag and Hylemon (1976) and Nair et al. (1967). It is also interesting to note the relatively high K_m values of the penicillin V amidases.

The conjugated bile acid hydrolase gene appears to be present in two other *Enterococcus* species, *E. hirae* and *E. faecium*. At first glance, the Southern analysis and PCR assay may seem to provide conflicting results concerning *E. hirae*. The PCR assay clearly shows a band around 1 Kb identical those found in *E. faecalis* DK and *E. faecium* ATCC 6057. In the Southern blot a very faint band is present in the *E. hirae* lane, but not nearly as pronounced as for the positive *Enterococcus* strains. This may be due to sequence differences in the gene itself which could ultimately lead to hindered annealing by the probe, and a diminished signal in the Southern blot. The PCR assay and ninhydrin assays also confirm the CBAH presence in these two *Enterococcus* strains

Sequence analysis shows that the peptide sequence of the *E. faecalis* CBAH shows most similarity to the *Lactobacillus plantarum* CBAH isolated by Christaens et al. (1992) exhibiting 70% identity and 82% similarity. The nucleotide sequence of the *E. faecalis* strain DK gene obtained from automated sequencing matched all but 3 residues of the *E. faecalis* strain V583 sequence from TIGR. A possible Shine-Dalgarno sequence is found nine base-pairs upstream of the initiator codon, as expected, and the nucleotide sequence consists of 975 nucleotides.

In summary, a PVA/CBAH family gene from *Enterococcus faecalis* has been cloned, the nucleotide and peptide sequence has been determined, and the enzyme has

been purified and characterized. Substrate specificity and sequence comparisons demonstrate it to be a CBAH rather than a PVA.

REFERENCES

Aries, V. and Hill, J. 1969. Degradation of steroids by intestinal bacteria. I. Deconjugation of Bile Salts. Biochim. Biophys. Acta. 202:526-534.

Aries, V. and Hill, J. 1970. Degradation of steroids by intestinal bacteria. II. Enzymes catalyzing the oxidoreduction of the 3α, 7α, 12α-hydroxyl groups in cholic acid, and the dehydroxylation of the 7-hydroxyl group. Biochim. Biophys. Acta. 202:535-543.

Arroyo, M., Torres-Guzman, R., de la Mata, I., Castillon, M., and Acebal, C. 2000. Activation and stabilization of penicillin V acylase from *Streptomyces lavendulae* in the presence of glycerol and glycols. Biotech. Progress. 16:368-371.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (ed.). 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.

Chauhan, S., Nichkawade, A., Iyengar, M., and Chattoo, B. 1998. *Chainia* penicillin V acylase: strain characteristics, enzyme immobilization, and kinetic studies. Current Microbiol. 37:186-190.

Christiaens, H., Leer, R., Pouwels, P., and Verstraete, W. 1992. Cloning and Expression of a Conjugated Bile Acid Hydrolase Gene from *Lactobacillus plantarum* by Using a Direct Plate Assay. Appl. Environ. Microbiol. 58:3792-3798.

Coleman, J. and Hudson, L. 1995. Cloning and Characterization of a Conjugated Bile Acid Hydrolase Gene from *Clostridium perfringens*. Appl. Environ. Microbiol. 61:2514-2520.

Corzo, G. and Gilliland, SE. 1999. Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus*. J. Dairy Sci. 82:472-480.

Eneroth, P. 1963. Thin-layer chromatography of bile acids. J. Lipid Research. 1:11-16.

Gopal-Srivastava, R. and Hylemon, P. 1988. Purification and characterization of bile salt hydrolase from *Clostridium perfringens*. J. Lipid Res. 29:1079-1085.

Grill, JP., Schneider, F., Crociani, J., and Ballongue, J. 1995. Purification and Characterization of Conjugated Bile Salt Hydrolase from *Bifidobacterium longum* BB536. Appl. Environ. Microbiol. 61:2577-2582.

Hofmann, A. and Roda, A. 1984. Physiochemical properties of bile acids and their relationship to biological properties: an overview of the problem. J. Lipid Res. 25:1477-1489.

Hylemon, P. 1985. Metabolism of bile acids in intestinal microflora. In Danielsson and Sjovall (Eds.) Sterols and Bile Acids. Elsevier Science Publishers B.V., New York.

Javitt, N. 1997. Bile Acids. In Encyclopedia of Human Biology 2nd Ed. Vol. 1. Academic Press, New York.

Kawamoto, K., Horibe, I., and Uchida, K. 1989. Purification and Characterization of a New Hydrolase for Conjugated Bile Acids, Chenodeoxycholyltaurine Hydrolase, from *Bacteroides vulgatus*. J. Biochem. 106:1049-1053.

Kerr, D. 1993. A colorimetric Assay for Penicillin-V Amidase. Anal. Biochem. 209:332-334.

Kobashi, K., Nishizawa, I., Yamada, T., and Hase, J. 1978. A New Hydrolase Specific for Taurine-conjugates of Bile Acids. J. Biochem. 84:495-497.

Kolb, V., Bernlohr, R. 1977. A new spectrophotometric assay for protein in cell extracts. Anal. Biochem. 82:362-371.

Lee, Y. and Takahashi, T. 1966. An improved colorimetric determination of amino acids with the use of ninhydrin. Anal. Biochem. 14:71-77.

Lowe, D., Romancik, G., Elander, R. 1986. Enzymatic hydrolysis of penicillin-V to 6-aminopenicillanic acid by *Fusarium oxysporum*. Biotechnol. Lett. 8:151-156.

Lundeen, S. and Savage, D. 1990. Characterization and Purification of Bile Salt Hydrolase from *Lactobacillus* sp. Strain 100-100. J. Bacteriol. 172:4171-4177.

Lundeen, S. and Savage, D. 1992. Multiple Forms of Bile Saly Hydrolase from *Lactobacillus* sp. Strain 100-100. J. Bacteriol. 174:7217-7220.

Maniatis, T., Fritsch, E., and Sambrook, J. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Midtvedt, T. 1974. Microbial bile acid transformation. Am. J. Clin. Nutr. 27:1341-1347.

Nair, P., Gordon, M., and Reback, J. 1967. The Enzymatic Cleavage of the Carbon-Nitrogen Bond in 3α, 7α, 12α-Trihydroxy-5β-cholan-24-oylglycine. J. Biol. Chem. 242:7-11.

Olsson, A., Hagstrom, T., Nilsson, B., Uhlen, M., and Gatenbeck, S. 1985. Molecular Cloning of *Bacillus sphaericus* Penicillin V Amidase Gene and Its Expression in *Escherichia coli* and *Bacillus subtilis*. Appl. Environ. Microbiol. 49:1084-1089.

Olsson, A. and Uhlen, M. 1986. Sequencing and heterologous expression of the gene encoding penicillin V amidase from *Bacillus sphaericus*. Gene. 45:175-181.

Playoust, M. and Isselbacher, K. 1964. Studies on the Transport and Metabolism of Conjugated Bile Salts by Intestinal Mucosa. J. Clin. Invest. 43:467-476.

Pundle, A. and SivaRaman, H. 1997. *Bacillus sphaericus* Penicillin V Acylase: Purification, Substrate Specificity, and Active-Site Characterization. Current Microbiol. 34:144-148.

Russell, W. and Klaenhammer, T. 2001. Identification and Cloning of *gusA*, Encoding a New β-Glucuronidase from *Lactobacillus gasseri* ADH. Appl. Environ. Microbiol. 67:1253-1261.

Schiff, E., Small, N., and Dietschy, J. 1972. Characterization of the kinetics of the passive and active transport mechanisms for bile acid absorption in the small intestine and colon of the rat. J. Clin. Invest. 51:1351-1362.

Stellwag, E. and Hylemon, P. 1976. Purification and Characterization of Bile Salt Hydrolase from *Bacteroides fragilis* subsp. fragilis. Biochim. Biophys. Acta. 452:165-176.

Sudhakaran, V. and Shewale, J. 1995. Purification and characterization of extracellular penicillin V acylase from *Fusarium* sp. SKF. Hindustan Antibiotics Bulletin. 37:9-15.

Sung, J., Shaffer, E., and Costerton, J. 1993. Antibacterial Activity of Bile Salts Against Common Biliary Pathogens. Dig. Dis. Sci. 38:2104-2112.

Suresh, C., Pundle, A., SivaRaman, H., Rao, K., Brannigan, J., McVey, C., Verma, C., Dauter, Z., Dodson, E., and Dodson, G. 1999. Penicillin V acylase crystal structure reveals new Ntn-hydrolase family members. Nature. 6:414-416.

Tanaka, H., Hashiba, H., Kok, J., and Mierau, I. 2000. Bile salt hydrolase of *Bifidobacterium longum*-biochemical and genetic characterization. Appl. Environ. Microbiol. 66:2501-2512.

Torres, R., Ramon, F., de la Mata, I., Acebal, C., and Castillon, M. 1999. Enhanced production of penicillin V acylase from *Streptomyces lavendulae*. Appl. Microbiol. Biotech. 53:81-84.

Tung, W. and Chow, K. 1995. A modified medium for efficient electrotransformation of *E. coli.* Trends in Genetics. 11:128-129.

Valle, F., Balbas, P., Merino, E., and Bolivar, F. 1991. The role of penicillin amidases in nature and in industry. TIBS. 16:36-40.

Vandamme E. and Voets, J. 1975. Properties of the purified penicillin V-acylase of *Erwinia aroideae*. Experientia. 31:140-143.

Whiteman, P. and Abraham, E. 1996. Phenoxymethylpenicillin amidohydrolases from *Penicillium chrysogenum*. FEBS Lett. 394:31-33.

Xu, Y., Jiang, L., Murray, B., and Weinstock, G. 1997. *Enterococcus faecalis* Antigens in Human Infections. Infect. Immun. 65:4207-4214.

Xu, Y., Murray, B., and Weinstock, G. 1998. A Cluster of Genes Involved in Polysaccharide Biosynthesis from *Enterococcus faecalis* OG1RF. Infect. Immun. 66:4313-4322.