8-1-1998

Study of the baiE Gene in Bile Acid 7α-Dehydroxylating Bacteria

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STUDY OF THE *baiE* GENE IN BILE ACID 7α-DEHYDROXYLATING BACTERIA

A Thesis

Presented to

the Faculty of the Department of Biology

Western Kentucky University

Bowling Green, Kentucky

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

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August 1998

STUDY OF THE *baiE* GENE IN BILE ACID 7α-DEHYDROXYLATING BACTERIA

Date Recommended June 23, 1998

Director of Thesis

Elmer Gray 8/17/98

Dean, Graduate Studies and Research Date

ACKNOWLEDGMENTS

To the following persons in the Department of Biology at Western Kentucky University, I would like to express my extreme gratitude:

Dr. Kinchel C. Doerner, my major advisor for answering all my questions, his guidance, and patient assistance during my research and thesis writing. I would like to thank Dr. Larry P. Elliott for his support and encouragement in many endeavors in microbiology. I would like to thank Dr. Sigrid Jacobshagen for serving on my committee and for her help and support in my research and thesis writing. To Dr. Claire Reinhart and John Andersland, thank you for your help and assistance in my thesis writing.

I wish to thank other professors in the Department of Biology for all the extra time they invested in my progress through the program.

I would also like to thank my family for all their help and support to get me through graduate school. To my boyfriend, Robert G. Green, thank you for your support, help and patience. To Michael David, thank you for all your help, and your wonderful friendship.

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STUDY OF THE *baiE* GENE IN BILE ACID 7α-DEHYDROXYLATING BACTERIA

YASEMIN H. KAYA August 1998 39 Pages

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Intestinal bile acid 7α-dehydroxylating bacteria have recently been implicated in cholesterol gallstone disease. *Eubacterium* sp. V.P.I. 12708, a bile acid 7α-dehydroxylating bacterium, contains multiple bile acid inducible (*bai*) genes which encode the enzymes responsible for bile acid 7α-dehydroxylation. The *baiE* gene encodes a bile acid dehydratase activity in *Eubacterium* sp.V.P.I. 12708. Using the polymerase chain reaction assay we determined the presence or absence of *baiE*-like genes in five clostridial bile-acid 7α-dehydroxylating strains: *Clostridium* sp. TO-931, *Clostridium* sp. HD-17, *Clostridium* sp. TN-271, *Clostridium* bifermentans I-55, and *Clostridium sordellii* ATCC 9714. Results from all the strains tested showed amplification at the predicted DNA fragment size. Partial DNA sequence analysis of the amplified *baiE*-like genes revealed 88-95% homology with the *baiE* gene of *Eubacterium* sp.V.P.I. 12708. These data suggest that *baiE*-like genes are present in the five bile acid 7α-dehydroxylating strains studied.

CHAPTER I

INTRODUCTION

The primary bile acids, cholic acid and chenodeoxycholic acid, are steroids synthesized from cholesterol in the liver and conjugated to either taurine or glycine. The conjugated bile acids are secreted into the bile, stored in the gallbladder, and secreted into small intestine after ingestion of a meal (25). In the small intestine bile acids aid in the absorption of dietary lipids and lipid-soluble vitamins (25). Bile acids are absorbed by the ileum and returned to the liver by portal blood. The circulation between the liver and small intestine is referred to as the enterohepatic circulation. During this process approximately 5% of bile acids escape ileal absorption and enter the large intestine (25), where bile acids are exposed to no less than 400 species of the resident colonic microorganisms (8).

The intestinal microflora produces at least 15-20 different bile acid metabolites from the primary bile acids (17,18,25). In humans, the quantitatively most significant bacterial bile acid biotransformation is the rapid 7α-dehydroxylation of primary bile acids to yield secondary bile acids. Humans synthesize the primary bile acids cholic acid and chenodeoxycholic acid which are 7α-dehydroxylated by some species of colonic bacteria to form the secondary bile acids deoxycholic acid and lithocholic acid, respectively (17,18,25). Approximately 30% of the secondary bile acids are passively absorbed through the colon wall, return to the liver by portal blood, and accumulate in the circulating bile acid pool (25).

However, under steady-state conditions, humans excrete 400-600 mg of bile acids in the feces per day. Deoxycholic acid normally comprises approximately 20-25% of the biliary acid pool of man; however this varies from 10-60% (25).

Compared to primary bile acids, secondary bile acids are more hydrophobic (25) and potent suppressors of hepatic cholesterol 7α-hydroxylase and HMG-CoA reductase which are the rate limiting enzymes in bile acid and cholesterol biosynthesis, respectively (9,14,22). Thus, in a normal healthy state, intestinal 7α-dehydroxylating bacteria may indirectly regulate host physiology.

Studies show that high levels of deoxycholic acid in bile are correlated with an increased risk of gallstone disease (21,23), but until recently studies implicating bile acid 7α-dehydroxylating bacteria were lacking. Berr et al. (2) report that fecal levels of bile acid 7α-dehydroxylating bacteria are approximately 1000-fold higher in recurrent cholesterol gallstone patients than control patients. The recurrent cholesterol gallstone patients also exhibit increased levels of serum deoxycholic acid compared to patients without recurrent gallstone disease. Feces from recurrent cholesterol gallstone patients exhibit increased bile acid 7α-dehydroxylation activity. When patients with recurrent cholesterol gallstone disease were treated with ampicillin, levels of 7α-dehydroxylating bacteria, bile acid 7α-dehydroxylation activity, and the biliary cholesterol saturation index all decreased to normal levels. The cholesterol saturation index is a ratio of cholesterol to the maximal cholesterol micellar-holding capacity of the bile. It has been reported that the biliary cholesterol saturation index is positively correlated with the amount of deoxycholate in the bile (23). These data suggest that increased levels of colonic 7α-dehydroxylating bacteria increase

levels of biliary deoxycholic acid and possibly increase the risk of cholesterol gallstone disease.

Intestinal bacterial species that catalyze bile acid 7α-dehydroxylation are some members of the genera *Clostridium* and *Eubacterium* (4,10,12,13,15,24). These genera are anaerobic, Gram-positive rod shaped *Bacteria*. The mechanism of bile acid 7α-dehydroxylation has been rigorously studied in *Eubacterium* sp. V.P.I. 12708 (3,5,19,20). In this strain 7α-dehydroxylation activity is induced by cholic acid and active towards chenodeoxycholic as well as cholic acid.

Eubacterium sp. V.P.I. 12708 exhibits a multi-step bile acid 7α-dehydroxylation pathway in which bile acids are first linked to CoenzymeA upon entering the cell (Fig. 1) (11,27) . The bile acid undergoes two oxidation reactions yielding 7α,12α-dihydroxy-3-oxo-4-cholenoic acid. This intermediate is then dehydrated by removal of the 7α-hydroxyl group forming 12α-hydroxy-3-oxo-4,6-choldienoic acid followed by three reduction steps. The final step is the separation of CoenzymeA and formation of deoxycholic acid. Most of the required enzymes for this pathway are encoded in a 10kb poly-cisteronic operon called the *bai* (bile acid inducible) operon (Fig. 2) (3,19,20). Detailed studies concerning the physiology and genetics of other bile acid 7α-dehydroxylating intestinal bacteria have not been reported. The development of DNA probes for detection and quantification of fecal 7α-dehydroxylating bacteria would be useful in studying the role of these bacteria in cholesterol gallstone disease. The purpose of the current study was to determine the presence of *baiE*-like genes in selected bile acid 7α-dehydroxylating bacterial strains.

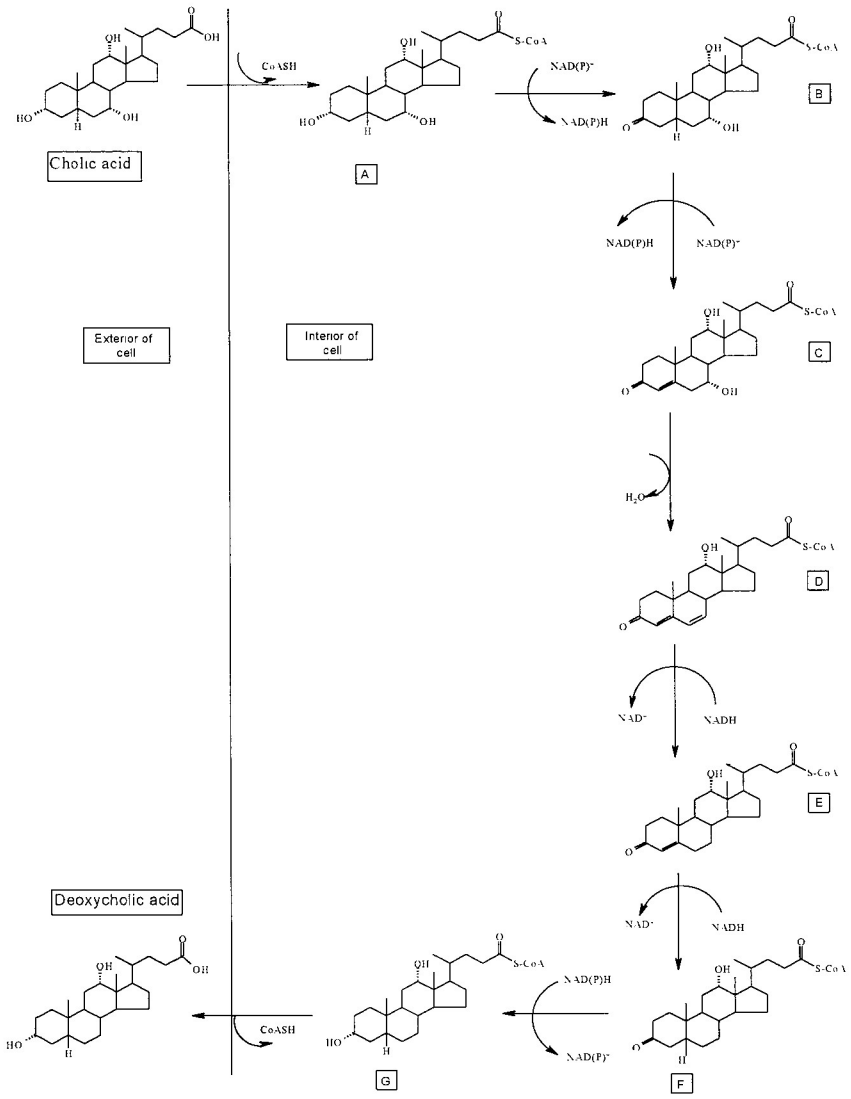

Figure 1. Pathway of 7-dehydroxylation of cholic acid to deoxycholic acid in *Eubacterium* sp.V.P.I. 12708. CoA: CoenzymeA; A: Cholic acid; B: 7α,12α-dihydroxy-3-oxo-5β-cholanoic acid; C: 7α,12α-dihydroxy-3-oxo-4-cholenoic acid; D: 12α-hydroxy-3-oxo-4,6-choldienoic acid; E:12α-hydroxy-3-oxo-4-cholenoic acid; F: 12α-hydroxy-3-oxo-5β-cholanoic acid; G: 3α12α-dihydroxy-5β-cholanoic acid (Redrawn from reference 6)

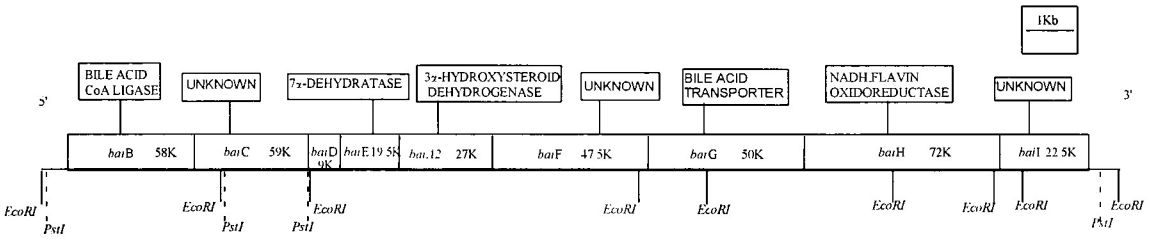

Figure 2. Partial restriction map and locations of open reading frames in the *bai* operon from *Eubacterium* sp.V.P.I. 12708 (Redrawn from reference 7). Enzymatic functions of the gene products are indicated in the boxes.

CHAPTER II

MATERIALS AND METHODS

Bacterial strains and culture conditions:

Eubacterium sp. V.P.I. 12708 was originally isolated from feces of a colon cancer patient by R.Hammann (Institute fur Medizinische Microbiologie und Immunologie der Universitat, Bonn, Germany). *Clostridium sordellii* ATCC 9714 was obtained from the American Type Culture Collection (Rockville, Md.). *Clostridium* sp. TO-931, *Clostridium* sp. HD-17, *Clostridium* sp. TN-271, and *Clostridium bifermentans* I-55 were gifted to our laboratory by F. Takamine (Laboratory of Microbiology, School of Health Sciences, Faculty of Medicine, University of Ryukyus, Okinawa, Japan).

Bile acid 7α-dehydroxylating bacterial strains were grown anaerobically at 37^0C in tryptic soy broth (Difco Laboratories, Detroit, MI) or brain heart infusion (Difco Laboratories, Detroit, MI) supplemented with 2 g fructose per liter. Anaerobic medium was prepared as previously described (16). In order to maintain anaerobic conditions, medium was boiled, flushed with nitrogen gas and reducing agent cysteine-HCl (1 gram per liter) was added. The pH of the medium was adjusted to 7.1, tubed anaerobically, and autoclaved at 121^0C, 15 psi (pounds per square inch) for no less than 20 minutes.

E.coli INVαF′ cells were grown in Luria-Bertaini (LB) medium, pH 7.5 (10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 5 g NaCl per liter) at 37^0C with moderate shaking. LB

medium was autoclaved as described above. When required ampicillin was added to a final concentration of 50 μg/ml to the sterilized medium. LB plates were prepared by adding 15 g of agar per liter to LB medium. Prior to inoculation, 40 μl of 40 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-gal) was added on to each plate.

Polymerase Chain Reaction (PCR) amplification of *baiE*-like gene:

Samples to be amplified were prepared as previously described (26). Cultures were centrifuged at 9000 x g, washed twice with phosphate-buffered saline (PBS), and once with water by centrifugation at room temperature at 9000 x g for 3 minutes (min). Pellets were resuspended in distilled water and diluted 1/100 (vol/vol) in 1% Triton X-100, boiled for 5 min, and immediately cooled in ice water. Primers targeting *Eubacterium* sp.V.P.I. 12708 *baiE* gene were designed by using Mac Vector software package (Oxford Molecular Group PLC). These primers allow amplification of a 483 base pair region of the *baiE* gene. The primers were 3599F (5'-GACATTAGAAGAGAGAGT TG-3') and 4081R (5-ATCGTGA TATGGATCTTTGG-3'). To ensure that the primers were complementary with the target gene but not with other known DNA sequences the GeneBank program BLAST was used. Bacterial cells from each strain (10μl) were directly added to 90 μl of PCR mixture containing at a final concentration of 20 μM each primer, 1.25 mM each deoxynucleoside triphosphate (dATP, dTTP, dCTP, and dGTP), 1x PCR buffer (Perkin Elmer, Branchburg, New Jersey), and 0.5 U of *Taq* polymerase. The PCR was conducted in Perkin Elmer GeneAmp PCR System 2400 (Foster City, CA.). The amplification conditions were one cycle of 94^{0}C for 2 min, 35 cycles of 94^{0}C for 30 seconds (s), 50^{0}C for 30 s and 74^{0}C for 35 s, and finally one cycle of 74^{0}C for 2 min and 45^{0}C for 2 s. Samples were then maintained

to 4^0C. PCR products (25 μl) were separated by 2% agarose gel electrophoresis at 100 V, and visualized by ethidium bromide staining (1).

Cloning *baiE*-like genes:

Following amplification and agarose gel electrophoresis analysis DNA fragments of the predicted size were excised from the gel and extracted with the Gene Clean reagents (Bio 101 Inc., Vista, CA.) as described by the manufacturer. The purified DNA fragment was ligated into the 3.9 kb pCR 2.1 vector (Invitrogen Corp., Carlsbad, CA). The ligation reaction was performed by adding 1 μl PCR (25 ng) product to 9 μl ligation reaction mixture that contained 1 x ligation buffer (6 mM Tris-HCl, 6 mM $MgCl_2$, 5 mM NaCl, 0.1 mg/ml bovine serum albumin, 7 mM β-mercaptoethanol, 0.1 mM ATP, 2 mM dithiothreitol, and 1 mM spermidine) supplied by the manufacturer, T4 DNA ligase (4.0 Weiss units/μl), and pCR 2.1 vector and incubated overnight at 14^0C. The inserted DNA fragment is flanked on each side by *EcoRI* sites. The ligation mixture was used to transform competent *E. coli* INVαF′ cells as described by the manufacturer. To select clones for further screening transformed *E. coli* INVαF′ cells were plated on LB plates containing ampicillin (50 μg/ml) and X-Gal (40 μl of 40 mg/ml) and incubated at 37^0C overnight. White colonies were picked for re-streaking for isolation. White colonies were selected because any DNA insert will distrupt *lacZ* gene present in the vector, which will prevent ß-galactosidase expression, and the cleavage of X-Gal. If the DNA insert is not present, ß-galactosidase will be expressed by *lacZ* gene, and cleave X-Gal and colonies will appear blue.

Purification of the plasmid containing the *baiE*-like gene fragment:

For purification of plasmid, *E.coli* INVαF' cells containing the pCR 2.1 vector were used to inoculate 5 ml of LB broth containing ampicillin (50 μg/ml). Cells were grown with shaking at 37^{0}C overnight. Cultures were harvested by centrifugation for two min at 12,000 x g and plasmid DNA was extracted and purified according to the instructions of the supplier (Qiagen Inc., Valencia, CA). After harvesting and resuspension, bacterial cells were lysed in NaOH/SDS (sodium dodecyl sulfate) in the presence of RNase A. The lysate was neutralized, adjusted to high-salt binding conditions by addition of the buffer supplied by the manufacturer and centrifuged at 12000 x g. Supernatants were applied to the QIAprep column and centrifuged for 30 seconds. Columns were washed twice with the appropriate buffers supplied by the manufacturer, and DNA was eluted by adding 50 µl of double distilled H_2O, and centrifuged for one minute. Purified plasmids were digested with *Eco*RI, analyzed by gel electrophoresis using 2% agarose in 1x TAE buffer (Tris-acetate-ethylenediamine tetraacetic acid [EDTA], 40 mM Tris acetate, 2 mM EDTA), and visualized by ethidium bromide staining to determine the presence of the cloned inserts.

DNA sequencing and sequence analysis:

DNA sequence determination was performed at the University of Kentucky, Macromolecular Structure Analysis Facility (Lexington, KY), using a Perkin-Elmer, Applied Biosystems 377 DNA Sequencer (Foster City, CA) utilizing dye-labeled ddNTP's from the dRhodamine Terminator Cycle Sequencing Ready Reaction with Amplitaq DNA polymerase, FS kit (Perkin-Elmer Corp., Foster City, CA). M13 reverse and T7 promoters were used as priming sites for sequencing both strands for *Clostridium bifermentans* I-55 and

Clostridium sordellii ATCC 9714. For sequencing one strand of DNA from *Clostridium* sp. TO-931, *Clostridium* sp. TN-271, and *Clostridium* sp. HD-17 only T7 promoter primer was used. The nucleotide sequences were analyzed by computer by comparing to the *baiE* gene from *Eubacterium* sp. V.P.I. 12708 as well as all other DNA sequences in the public domain DNA sequence databases using the GeneBank program BLAST.

Southern blot analysis of *baiE*-like gene:

Chromosomal DNA was extracted from overnight cultures of each bile acid 7α-dehydroxylating bacterial culture using the Qiagen genomic-tip columns (Qiagen, Chatsworth, CA.), and 2 μg of DNA was digested with *EcoRI* and *Pst*I at 37^{0}C overnight in the appropriate buffer. Penicillin-G (0.1 mg/ml) was added to cultures 4 hours prior to extraction of DNA. Lysozyme was used to hydrolyze the cell wall at a final concentration of 100 mg/ml during the extraction of genomic DNA. DNA fragments were separated electrophoretically using 1% agarose in 1 X TAE buffer. Gels were treated with 0.25N HCl for 10 minutes to fragment the DNA. To denature the DNA, gels were equilibriated in 1.0 M NaCl/0.5 NaOH twice for 20 minutes, then neutralized in 0.5 M Tris-HCl pH 7.5/1.5M NaCl twice for 20 minutes. Gels were equilibriated in 10 X SSC (1.5 M NaCl, 0.15 M Na_3citrate.$2H_2O$, pH 7.0) and blotted overnight as previously described except 10 X SSC was used for the transfer solution (1). DNA fragments were transferred onto MagnaNT nylon membranes (Micron Separations, Inc., Westborough, MA) via capillary action and baked for 1 h at 80^{0}C (1). Cloned *baiE* genes from the bacterial strains encoding dehydratase activity required for the dehydratation step in the bile acid 7α-dehydroxylation were used as molecular probes. Hybridization using nick-translated probes were performed in

hybridization solution consisting of 4 X SSC, 50% formamide, 5 X Denhardt's solution (1% ficoll, 1% polyvinyl pyrrolidone, and 1% bovine serum albumin), 1% SDS, and 0.1mg/ml denatured salmon sperm DNA (Sigma Chem. Co, St. Louis, MO). Membranes were incubated for at least 2 hours in a heat sealable bag at 42^0C, prior to addition of the probe. DNA probes were radio-labeled with ^{32}P-dCTP using a commercially available nick-translation kit (Gibco BRL, Gaithersburg, MD). After labeling, unincorporated nucleotides were removed using gel filtration. Briefly, the bottom of a 1 ml syringe was closed with glass wool and placed into 15 ml disposable glass test tube. The syringe was filled with swollen Sephadex G-50 which was equilibrated in STE (10 mM Tris.Cl, pH 7.5, 10 mM NaCl, and 1 mM EDTA) buffer and centrifuged in clinical centrifuge at high speed. Probe labeling mixture was loaded on top of the column and centrifuged for 4 minutes, and the probe was collected in a microfuge tube. Nick-translated probe was boiled for 5 minutes and rapidly cooled on ice prior to addition to the hybridization solution. Hybridization was allowed to take place overnight with shaking at 42^0C. Membranes were washed at room temperature with 2 X SSC (5 min), 2 X SSC with 0.1% SDS (30 min), 0.1 X SSC with 0.1% SDS (30 min), then 0.1 X SSC (30 min). Membranes were air dried and exposed to x-ray film at room temperature for seven days. Films were developed in 1 X Kodak GBX developer for 5 minutes, washed in water for 30 seconds and fixed in 1 X Kodak GBX fixer for 4 minutes (Eastman Kodak Company, Rochester, New York).

CHAPTER III

RESULTS

PCR amplification of *baiE*-like genes:

The amplified *baiE*-like genes were analyzed in 2% agarose gel electrophoresis following PCR for the six bacterial strains tested (Fig. 3). All the strains tested yielded a DNA product at the predicted size of 483 bp including the positive control, *Eubacterium* sp. V.P.I. 12708. *Clostridium* sp. TN-271 exhibited two amplification products, 483 bp and 450 bp. The negative control which was *Eubacterium* sp. V.P.I. 12708 cells with only the 3599F primer did not show amplification.

Cloning and screening of the *baiE*-like gene fragment:

The results of the transformations when 50 µl and 200 µl aliquots of transformation mixtures (*E.coli* INVαF′ cells containing the insert) were inoculated to LB plates in the presence of ampicillin (50 µg/ml) are shown in Table 1. LB plates that had the 50 µl of transformation mixture had 3 blue and 2 white colonies for *Clostridium* sp. HD-17 clone, 1 blue and 1 white colony for *Clostridium* sp. TO-931 clone, 4 blue and 2 white colonies for *Clostridium bifermentans* I-55 clone, 1 blue and 4 white colonies for *Clostridium sordellii* ATCC 9714 clone, and 2 blue and 3 white colonies for *Clostridium* sp. TN-271 clone. LB plates that had the 200 µl of transformation mixture had 8 blue and 3 white colonies for *Clostridium* sp. HD-17 clone, 6 blue and 6 white colonies for *Clostridium* sp. TO-931 clone,

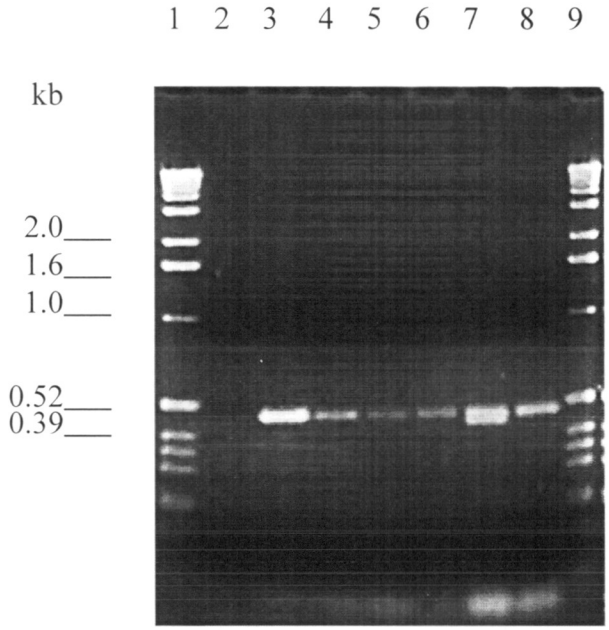

Figure 3. PCR amplification of *baiE*-like genes from various bile acid 7-dehydroxylating bacteria. Lane 1 and 9, molecular weight standards; lane 2 PCR reaction *Eubacterium* sp. V.P.I. 12708 with one primer (negative control); lane 3, *Eubacterium* sp. V.P.I. 12708; lane 4, *Clostridium* sp. HD-17; lane 5, *Clostridium* sp. TO-931; lane 6, *Clostridium bifermentans* I-55; lane 7, *Clostridium* sp. TN-271; lane 8, *Clostridium sordellii* ATCC 9714. This primer pair is predicted to amplify a 483 bp fragment from *Eubacterium* sp. V.P.I. 12708.

Table 1. Number of transformants when transformation mixtures were plated onto LB medium containing ampicillin (50 μg/ml).

Transformation mixtures *	Plate 1 (50 μl inoculum)		Plate 2 (200 μl inoculum)	
	Blue colonies	White colonies	Blue colonies	White colonies
Clostridium sp. HD-17	3	2	8	3
Clostridium sp. TO-931	1	1	6	6
Clostridium bifermentans I-55	4	2	4	1
Clostridium sordellii ATCC 9714	1	4	3	5
Clostridium sp. TN-271	2	3	3	3

* Transformation mixture contains *E.coli* INVαF′ transformed with the ligation mixtures of the *baiE* gene fragments and pCR 2.1 vector.

4 blue and 1 white colonies for *Clostridium bifermentans* I-55 clone, 3 blue and 5 white colonies for *Clostridium sordellii* ATCC 9714 clone, and 3 blue and 3 white colonies for *Clostridium* sp. TN-271 clone. From each transformation plate a single colony was picked for plasmid preparation. Plasmids were digested with *EcoRI* and the products were visualized using agarose gel electrophoresis and ethidium bromide staining. Transformants exhibited a DNA fragment at 3.9 kb that indicates the plasmid. Also for all the clones studied a DNA fragment at the predicted size was observed, except for one clone of *Clostridium bifermentans* I-55. For this *Clostridium bifermentans* I-55 transformant a DNA fragment at 3.0 kb was observed and the insert was not determined. Results for the restriction digest reactions are shown in Figure 4.

Southern blot analysis of bile acid 7-dehydroxylating bacterial chromosomes:

For control purposes it was determined whether the cloned *baiE* gene fragments are present in the chromosome of the strain from which the gene fragment was cloned. For this experiment, we employed the Southern blot technique.

Figure 5 shows the Southern blotting results from *Clostridium sordellii* ATCC 9714, *Clostridium* sp. TO-931, and *Eubacterium* sp. V.P.I. 12708 probed with the *baiE* gene fragment of *Clostridium sordellii* ATCC 9714. *Eubacterium* sp. V.P.I. 12708 chromosome, that was digested with *EcoRI* exhibited hybridization to the probe; however, the probe did not hybridize to any *Clostridium sordellii* ATCC 9714 chromosomal fragment. No cross hybridization has been determined between the probe and *Clostridium* sp. TO-931 chromosomal fragments.

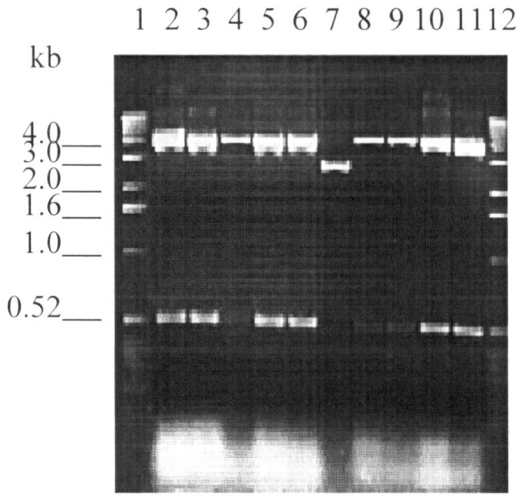

Figure 4. Restriction digest of cloned *baiE*-like genes with *EcoRI*. Lane 1 and 12, molecular weight standards; lane 2 and 3, *Clostridium* sp. HD-17; lane 4 and 5, *Clostridium* sp. TO-931; lane 6 and 7, *Clostridium bifermentans* I-55; lane 8 and 9, *Clostridium* sp. TN-271; lane 10 and 11 *Clostridium sordellii* ATCC 9714.

Figure 5. Autoradiogram of selected bacterial chromosomes probed with *baiE* gene fragment cloned from *Clostridium sordellii* ATCC 9714. Equivalent quantities (2 μg) of chromosomal DNA from each strain were digested with *EcoRI* or *Pst*I. Lane 1, molecular weight standard, lane 2, *Eubacterium* sp. V.P.I. 12708 digested with *EcoRI*; lane 3, *Eubacterium* sp. V.P.I. 12708 digested with *Pst*I; lane 4, *C. sordellii* ATCC 9714 digested with *EcoRI*; lane 5, *C. sordellii* ATCC 9714 digested with *Pst*I; lane 6, *C.* sp. TO-931 digested with *EcoRI*; lane 7, *C.*sp. TO-931 digested with *Pst*I.

Figure 6 shows the results when chromosomal DNA of the *Eubacterium* sp. V.P.I. 12708, *Clostridium* sp. HD-17, *Clostridium* sp. TN-271, *Clostridium* sp. TO-931, *Clostridium bifermentans* I-55, and *Clostridium sordellii* ATCC 9714 were probed with the *baiE* gene fragment of *Clostridium* sp. HD-17. The *baiE*-like gene hybridized to *Eubacterium* sp. V.P.I. 12708 and *Clostridium* sp. TN-271 chromosomal fragments, but did not hybridize to *Clostridium* sp. HD-17, *Clostridium bifermentans* I-55, *Clostridium* sp. TO-931, or to *Clostridium sordellii* ATCC 9714 chromosomal fragments.

Figure 7 shows the data from chromosomal DNA of *Eubacterium* sp. V.P.I. 12708, *Clostridium bifermentans* I-55, *Clostridium* sp. TO-931, *Clostridium* sp. HD-17, *Clostridium* sp. TN-271, and *Clostridium sordellii* ATCC 9714 probed with the *baiE* gene fragment from *Clostridium bifermentans* I-55. The probe hybridized to *Eubacterium* sp. V.P.I. 12708 and *Clostridium* sp. TN-271 chromosome fragments. The *baiE* gene fragment did not hybridize to *Clostridium bifermentans* I-55, *Clostridium sordellii* ATCC 9714, *Clostridium* sp. HD-17, or to *Clostridium* sp. TO-931 chromosomal fragments.

Figure 8 shows the results from chromosomal DNA of *Eubacterium* sp. V.P.I. 12708, *Clostridium* sp. TN-271, *Clostridium bifermentans* I-55, *Clostridium* sp. TO-931, *Clostridium sordellii* ATCC 9714, and *Clostridium* sp. HD-17 probed with the *baiE* gene fragment of *Clostridium* sp. TN-271. Both *Eubacterium* sp. V.P.I. 12708 and *Clostridium* sp. TN-271 chromosomal fragments hybridized to the probe. The *baiE* gene fragment did not hybridize to chromosomal DNA fragments from *Clostridium bifermentans* I-55, *Clostridium* sp. TO-931, *Clostridium* sp. HD-17, and *Clostridium sordellii* ATCC 9714.

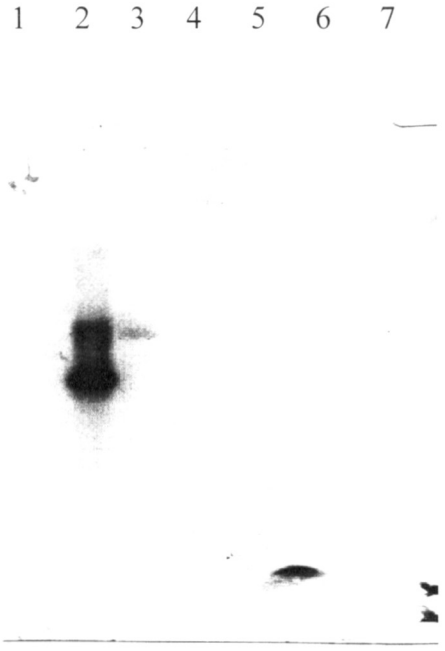

Figure 6. Autoradiogram of selected bacterial chromosomes probed with the *baiE* gene fragment cloned from *Clostridium* sp. HD-17. Equivalent quantities (2 μg) of chromosomal DNA from each strain were double digested with *EcoRI* and *Pst*I. Lane 1, molecular weight standard; lane 2, *Eubacterium* sp. V.P.I. 12708; lane 3, *C.* sp. TN-271; lane 4, *C. bifermentans* I-55; lane 5, *C.* sp. HD-17; lane 6, *C.* sp. TO-931; lane 7, *C. sordellii* ATCC 9714.

Figure 7. Autoradiogram of selected bacterial chromosomes probed with the *baiE* gene fragment cloned from *Clostridium bifermentans* I-55. Equivalent quantities (2 μg) of chromosomal DNA from each strain were double digested with *EcoRI* and *Pst*I. Lane 1, molecular weight standard; lane 2, *Eubacterium* sp. V.P.I. 12708; lane 3, *C.* sp. TN-271; lane 4, *C. bifermentans* I-55; lane 5, *C.* sp. HD-17; lane 6, *C.* sp. TO-931; lane 7, *C. sordellii* ATCC 9714.

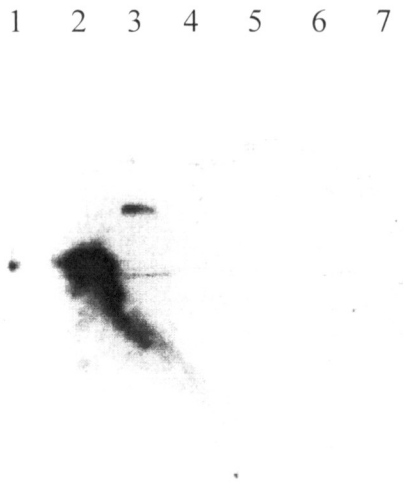

Figure 8. Autoradiogram of selected bacterial strains studied probed with the *baiE* gene fragment cloned from *Clostridium* sp. TN-271. Equivalent quantities (2 μg) of chromosomal DNA from each strain were double digested with *EcoRI* and *Pst*I. Lane 1, molecular weight standard; lane 2, *Eubacterium* sp. V.P.I. 12708; lane 3, *C.* sp. TN-271; lane 4, *C. bifermentans* I-55; lane 5, *C.* sp. HD-17; lane 6, *C.* sp. TO-931, lane 7, *C. sordellii* ATCC 9714.

Figure 9 shows the results when chromosomal DNA from *Clostridium sordellii* ATCC 9714 and *Clostridium* sp. TO-931 were probed with *baiE* gene fragment of *Clostridium* sp. TO-931. Only one *Eubacterium* sp. V.P.I. 12708 chromosomal fragment exhibited hybridization with the probe. No cross-hybridization was detected between the probe and the chromosomal DNA from *Clostridium sordellii* ATCC 9714 or *Clostridium* sp. TO-931.

DNA sequence analysis:

The sequence analysis of one strand of *Clostridium sordellii* ATCC 9714 showed 90% identity to *Eubacterium* sp. V.P.I. 12708 sequence when the T7 promoter was used as a primer. The sequencing result of the complementary strand of *Clostridium sordellii* ATCC 9714 when the M13 Reverse was used as a primer exhibited the same sequence that was derived from T7 promoter. Both 3599F and 4081R primers were identified in the DNA sequence obtained from both M13 Reverse and T7 promoter sequence reactions.

The results of sequencing reaction for *Clostridium bifermentans* I-55 from one strand of DNA derived by T7 promoter showed 91% identity to *Eubacterium* sp. V.P.I. 12708 sequence. The complementary strand sequence result derived by M13 Reverse primer exhibited the same sequence which was derived by T7 promoter, and exhibited 95% identity to *Eubacterium* sp. V.P.I. 12708. Both 3599F and 4081R primers were identified in the DNA sequence obtained from both M13 Reverse and T7 promoter sequence reactions.

Clostridium sp. TN-271 sequencing results from one strand of DNA derived by T7 promoter showed 90% identity to *Eubacterium* sp. V.P.I. 12708 sequence.

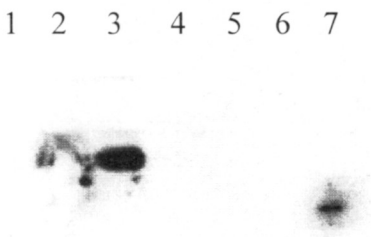

Figure 9 Autoradiogram of selected bacterial chromosomes probed with *baiE* gene fragment cloned from *Clostridium* sp. TO-931. Equivalent quantities (2 µg) of chromosomal DNA from each strain were digested with *EcoRI* or *Pst*I. Lane 1, molecular weight standard, lane 2, *Eubacterium* sp. V.P.I. 12708 digested with *EcoRI*; lane 3, *Eubacterium* sp. V.P.I. 12708 digested with *Pst*I; lane 4, *C. sordellii* ATCC 9714 digested with *EcoRI*; lane 5, *C. sordellii* ATCC 9714 digested with *Pst*I; lane 6, *C*. sp. TO-931 digested with *EcoRI*; lane 7, *C*. sp. TOdigested with *Pst*I.

Clostridium sp. HD-17 sequence results from one strand of DNA derived by T7 promoter showed 88% identity to *Eubacterium* sp. V.P.I. 12708 sequence.

The data from sequencing reaction of *Clostridium* sp. TO-931 from one strand of DNA derived by T7 promoter exhibited 92% identity to *Eubacterium* sp. V.P.I. 12708 sequence.

Table 2 shows the summary of the results of the number of bases compared to *Eubacterium* sp. V.P.I. 12708 sequence and the % identities.

Table 2. Results of the sequence comparisons of the cloned inserts from the stated bacterial strains to *Eubacterium* sp. V.P.I. 12708 *baiE* sequence.

Clone	T7 Promoter		M13 Reverse	
	# bases	% identity with 5′ end of *baiE**	# bases	% identity with 5′ end of *baiE*
Clostridium sp. HD-17	204	88	_♀	_
Clostridium sp. TO-931	269	92	_	_
Clostridium sp. TN-271	265	90	_	_
Clostridium bifermentans I-55	472	91	469	95
Clostridium sordellii ATCC 9714	444	90	550	95

* Percent identity of 5′ end of the *baiE* gene from *Eubacterium* sp. V.P.I. 12708

♀ Indicates the sequence data was not obtained.

CHAPTER IV

DISCUSSION

Bile acid 7α-dehydroxylation has been studied for many years. Bacterial strains that exhibit bile acid 7α-dehydroxylation activity are the members of the genera *Clostridium* and *Eubacterium* (4,10,12,13,15,24). Most studies have focused on *Eubacterium* sp. V.P.I. 12708 but not with the other intestinal bile acid 7α-dehydroxylating bacteria. The purpose of this study was to determine the presence or absence of *baiE*-like gene that encodes the bile acid 7α-dehydratase enzyme in selected bile acid 7α-dehydroxylating bacterial strains.

PCR primers were designed targeting *Eubacterium* sp. V.P.I. 12708 *baiE* gene. For these experiments to be successful, the nucleotide sequence of the *baiE*-like genes in the five bacterial strains tested must be similar enough to *Eubacterium* sp. V.P.I. 12708 *baiE* gene sequence to allow amplification. The PCR results for all the strains tested, including *Eubacterium* sp. V.P.I. 12708, showed an amplification at the predicted size. This data suggested that the *baiE*-like gene is present in all the bacterial strains tested.

Following PCR amplification, *Clostridium* sp. TN-271 exhibited two DNA fragments, 483 bp and 450 bp. This result could be because the primer pair is amplifying at different locations on the chromosome. This other amplification site might be further down the *baiE* gene. In addition, other genes in the *bai* operon are known to be homologous to the *baiE* gene, thus sequences flanking the target gene could also contain priming sites.

The transformation assay yielded few white colonies; however only one colony was necessary to prepare plasmids. Thus, in our tests we picked one colony from each transformation plate.

To date one sequencing reaction per clone of *Clostridium* sp. TO-931, *Clostridium* sp. HD-17, and *Clostridium* sp. TN-271 revealed 88-94% homology with the *baiE* gene of *Eubacterium* sp. V.P.I. 12708, suggesting that the *baiE*-like genes exist in the these bacterial strains. The sequencing reaction from the other direction for these bacterial strains did not work. There might be several reasons for this problem. First, the plasmid yield may have been too low to provide sufficient DNA for the sequencing reaction. Second, water used in the plasmid isolation was not double distilled; contaminants in the water may have affected the reaction. Third, the DNA may have degraded or become contaminated during shipment to the University of Kentucky sequencing laboratory.

The sequencing results from *Clostridium bifermentans* I-55 and *Clostridium sordellii* ATCC 9714 exhibited the same DNA sequence on both the 5′ and 3′ ends of the clones which is troubling. These confusing data might be due to having a mixed culture while preparing the plasmid preparations from isolated colonies of the transformants. Two different colonies of the same transformant might have appeared as one colony, and this colony might have been picked for plasmid preparations. Since the PCR product can ligate into the vector in either direction, the two colonies may have had sequences ligated in opposite directions. In preparing plasmids for the forward sequencing reaction, one colony may have predominated, while in the other preparation for the reverse reaction, the other colony may have predominated. These problems could be prevented by re-streaking the colonies from the transformation reactions to ensure the colony was a single isolate.

In the Southern blot reactions *Clostridium* sp. TN-271 hybridized to *Clostridium* sp. TN-271 chromosome and *Eubacterium* sp. V.P.I. 12708 chromosome suggesting the presence of the gene. The hybridization of *Clostridium* sp. TN-271 to its own chromosome weakly, but to *Eubacterium* sp. V.P.I. 12708, strongly could be because there might be more

DNA for *Eubacterium* sp. V.P.I. 12708 than *Clostridium* sp. TN-271. In addition, in *Eubacterium* sp. V.P.I. 12708 there might be more than one *baiE* gene that is located at another place than the *bai* operon. Therefore, in this case the hybridization to *Eubacterium* sp. V.P.I. 12708 will be stronger.

*Clostridium*sp. TO-931, *Clostridium bifermentans* I-55, *Clostridium* sp. HD-17, and *Clostridium sordellii* ATCC 9714 *baiE* gene fragments not hybridizing to their own chromosomes but to *Eubacterium* sp. V.P.I. 12708 could be because these strains might not have enough DNA loaded on the gel, or there could be sub-optimal hybridization conditions.

Taken together, sequencing, and Southern blotting results indicate the presence of *baiE*-like gene in *Clostridium* sp. TN-271 although in a previous study it has been shown that this strain does not have the *baiE* gene (7). In the previous study the entire *Eubacterium* sp. V.P.I. 12708 *baiE* gene fragment was used as a probe. Although *Clostridium* sp. TN-271 shows high sequence identity to *Eubacterium* sp. V.P.I. 12708, few mismatches could prevent hybridization of full length *baiE* gene probe to the bacterial chromosome tested. In this study I did not use the entire gene as a probe, but rather amplified the gene using two, 20 nucleotides long PCR primers. This approach was successful. This result suggests the *baiE* gene is truely present in *Clostridium* sp. TN-271.

Although *Clostridium bifermentans* I-55. *Clostridium sordellii* ATCC 9714, *Clostridium* sp. HD-17, and *Clostridium* TO-931 all showed a sequence similarity to *Eubacterium* sp. V.P.I. 12708 in southern blot reactions the respective probes did not show hybridizations to their own chromosomes possibly because of sub-optimal hybridization conditions or the *baiE* gene does not exist in these bacterial strains.

For future studies, having *Clostridium* sp. TN-271 *baiE*-like gene sequenced from both directions may be helpful in showing the gene does exist in that strain.

APPENDIX

GACATTAGAAGAGAGAGTTGAAGCATTAGAAAAAAGAATTGCAGGA 46

GATGAAGGATATTGAGGCAATCAAGGAAACTGAAAGGAAAGTATTT 92

CCGCTGCCTGGACGGAAANATGTGGGATGAGCTGGAGACCACCCTG 138

TCACCAAATATCGTAACCTCTTATTCCAACGGGAAACTGGTATTCC 184

NTAGCCCGAAGGAATTACCGATTACTTAAAGAGCTCGATGCCAAAA 230

NAATAAATCNGCATGCATATGGGCCCCCCGCCGGANATC

Figure 1A. Nucleic acid sequence of *Clostridium* sp. TO-931 when T7 promoter was used. The sequence in bold is the 3599F PCR primer.

ATCGTGATATGGATCTTTGGGATCACGCATGAAAATGTTCTTCATA 46

GAATTCGTACATAGCCTGTTTNCAANGGAATGTTACCACTGGGCCT 92

TCTAATTTTCCTCGTAATTGGTCTGTATAAGAAACGCCGCCCGCCG 138

GTTAAATCCCCACATCTTTGTTACTTGCCCGTCCGTAAAAGATCAG 184

TTTTATCTTCCAGAATACCATCTGCCCGTAGCCGTAGTCTCGCTGT 230

CAATGGTGATCTCCGGCGTGTGGCCCATATGCATGCTGATCTCTTC 276

TTTTGGCATTGTGCTCTTTAAAGTAATCGGTAACTTCCTTCGGGGCT 322

ATGGAAATACCAGTTTACCGTTGGGAATAAGAAGTTACGAATATTT 368

GGTGACAGAATGGTCTCCAGTTCATCCCACATCTTTCCGTCCAAGC 414

AGCGGGAAATACTTCCCTTTCAGTTCTTTGATCGCCTCGANATCCT 460

TCATCTTCTGCAGTTCTTTTNCNAAATGCTCCACTCTCACTTCTAA 506

TGTN

Figure 1B. Nucleic acid sequence of *Clostridium bifermentans* I-55 when T7 promoter was used. The sequence in bold is the 4081R PCR primer. The underlined sequence is the 3599F PCR primer.

GACATTAGAAAGAGAGAGTTGAAGCATTAGAAAAAGAACTGCAGAA 46

AGATGAAGGATATCGAGGCGATCAAAGAAACTGAAAGGAAAGTATT 92

TCCGCTGCCTGGACGGAAAAGATGTGGGATGAACTGGAGACCACTC 138

TGTCACCAAATATCGTAACCTCTTATTCCAACGGTAAACTGGTATT 184

CCATAGCCCGAAGGAAGTTACCGATTACTTAAAGAGCACAATGCCA 230

AAAGAANAGATCAGCATGCATATGGGCCACACGCC

Figure 1C. Nucleic acid sequence of *Clostridium* sp. TN-271 when T7 promoter was used. The sequence in bold is the 3599F PCR primer.

ATCGTGATATGGANGGGTGGATCACGCATGANAATGTNCTTCATAG 46

AATTCGTACATAGCCNGTNTCANGGATGTACCACTGGCCTTCTATT 92

TTCTCGTATTTGTCTGTATAGAAACGCGCCGCCGTTAATCCCCACA 138

TCTTTGTACTTGCCGTCCGTAAAGATCAGTTTATCTTCCAGATACC 184

ATCTGCCCGTAGCCGTAGTCTCGCTGTCAATGGTGATCTCCGGCGT 230

GTGGCCCATATGCATGCTGATCTCCGGCGTGTGGCCCATATGCATG 276

CTGATCTCTTCTTTNGGCATGGTGCTCTTTAAGTAATCGGTAACTT 322

CCTTCGGGCTATGGAATACCAGTTTACCGTNGGAATANGAGGTACN 368

ATATTGGGTGACAGANTGGTCTCCAGTTCATCCCACATCTTCCGTC 414

CAGCACGGAAATACTTCCTTCCAGTTCTTTGATCGCCTCGAATATC 460

CTTCCATCTTCTGCAGTTCCTTTNCCTAATGCCTCAACTCTCTCCTT 506

CTAATGTC

Figure 1D. Nucleic acid sequence of *Clostridium sordellii* ATCC 9714 when T7 promoter was used. The sequence in bold is the 4081R PCR primer. The underlined sequence is the 3599F PCR primer.

GACATTAGAAGAGAGAGTTCAAGCATTAGAAAANGANTTGCAGGAT 46

ATGANTGATNTTGAGGCCNTCNNGGAACTGAAAGGANNGTATTTCC 92

NCTGCCTGGACAGATANATGTGGGATGAGCTGGAGACCTCCCTGTC 138

ACCAAATANCNTANCCTCTTATTCCANCTGGGAAACTGGTATTCCN 184

TACCCCGAAGGAATTTACCG

Figure 1E. Nucleic acid sequence of *Clostridium* sp. HD-17 when T7 promoter was used. The sequence in bold is the 3599F PCR primer.

ATCGTGATATGGATCTTTGGATCACGCATGAAATGTTCTTCATAGA 46

TTCGTACATAGCCTGTTTCAAGGATGTACCACTGGCCTTCTATTTT 92

CTCGTATTTGTCTGTATAGAACGCGCCGCCGTTAATCCCCACATCT 138

TTGTACTTGCCGTCCGTAAAGATCAGTTTATCTTCCAGATACCATC 184

TGCCCGTAGCCGTAGTCTCGCTGTCAATGGTGATCTCCGGCGTGTG 230

GCCCATATGCATGCTGATCTCTTCTTTTGGCATTGTGCTCTTTAAG 276

TAATCGGTAACTTCCTTCGGGCTATGGAATACCAGTTTACCGTTGG 322

AATAAGAGGTTACGATATTTGGTGACAGAGTGGTCTCCAGTTCATC 368

CCACATCTTTCCGTCCAGGCAGCGGAAATACTTTCCTTTCAGTTCT 414

TTGATCGCCTCGATATCCTTCATCTTCTGCAGTTCTTTTTCTAATG 460

CTTCAACTCTCTCTTCTAATGT

Figure 1F. Nucleic acid sequence of *Clostridium bifermentans* I-55 when M13 Reverse primer was used. The sequence in bold is the 4081R PCR primer. The underlined sequence is the 3599F PCR primer.

ATCGTGATATGGATCTTTGGATCACGCATGAAATGTTCTTCATAGA 46

TTCGTACATAGCCTGTTTCAAGGATGTACCACTGGCCTTCTATTTT 92

CTCG TATTTGTCTGTATAGAACGCGCCGCCGTTAATCCCCACATCT 138

TTGTACTTGCCGTCCGTAAAGATCAGTTTATCTTCCAGATACCATC 184

TGCCCGTAGCCGTAGTCTCGCTGTCAATGGTGATCTCCGGCGTGTG 230

GCCCATATGCATGCTGATCTCTTCTTTTGGCATTGTGCTCTTTAAG 276

TAATCGGTAACTTCCTTCGGGCTATGGAATACCAGTTTACCGTTGG 322

AATAAGAGGTTACGATATTTGGTGACAGAGTGGTCTCCAGTTCATC 368

CCACATCTTTCCGACCAGGCAGCGGAAATACTTTCCTTTCAAGTTC 414

TTTGATCGCCTCGATATCCTTCATCTTCTGCAGTTCTTTTTCTAAT 460

GCTTCAACTCTCTTCTAATGTCAAGCCGAATTCTGCANATATCCAT 506

CACACTGGCGGNCGCTCGAACATGCATCTAGAGGGCCCAATTCG

Figure 1G. Nucleic acid sequence of *Clostridium sordellii* ATCC 9714 when M13 Reverse primer was used. The sequence in bold is the 4081R PCR primer. The underlined sequence is the 3599F PCR primer.

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