ENZYMEOLOGY AND MOLECULAR BIOLOGY OF BILE ACID 7alpha- AND 7beta- DEHYDROXYLATION BY THE INTESTINAL BACTERIA CLOSTRIDIUM SCINDENS AND CLOSTRIDIUM HYLEMONAE

Jason Michael Ridlon
Virginia Commonwealth University

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ENZYMEOLOGY AND MOLECULAR BIOLOGY OF BILE ACID 7α- AND 7β-
DEHYDROXYLATION BY THE INTESTINAL BACTERIA CLOSTRIDIUM
SCINDENS AND CLOSTRIDIUM HYLEMONAE

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy at the
Virginia Commonwealth University

By

Jason Michael Ridlon
B.S., Bridgewater College, 2002

Major Director: Dr. Phillip B. Hylemon
Professor of Microbiology and Immunology and Medicine

Virginia Commonwealth University
Richmond, Virginia
May, 2008
DEDICATION

To my loving wife Liz for her constant support and encouragement to succeed at this tremendous undertaking. To my mother and grandmother for all their love and support.
ACKNOWLEDGEMENTS

I would like to express my utmost and sincere gratitude to Dr. Phillip B. Hylemon for his guidance, patience and support before and during this work. I cherish our many wonderful conversations on science, life and current events. I would also like to extend many thanks to the members of my graduate committee, Drs. D. Peterson, C. Cornelissen, R. Marconi and W.M. Pandak for all of their encouragement and valuable suggestions.

I would also like to thank the Hylemon lab, particularly Pat Bohden, Emily Gurley and Elaine Studer for keeping the lab running and for their friendship and support.

I must give special thanks and appreciation to DJ Kang for his friendship, constant help and support and his expertise on protein expression and purification.

I would also like to thank Dr. Hylemon and the Department of Microbiology and Immunology whose financial support allowed completion of this project.
Man's liver is a brownish blob
That does a most prodigious job.
  It manufactures gall, or bile
  And normally keeps some on file
Stored neatly in a pear-shaped sac.
From there the liver's yields attack
The food man eats, to change its state
  By methods man can't duplicate,
  Or even halfway understand.
He ought to treat this outsize gland,
  With due respect and loving care
To keep it in top-notch repair,
  Because to get along at all
Man needs an awful lot of gall.

Irene Warsaw, JAMA, 1975, v. 231, p. 1260
The known is finite, the unknown infinite; intellectually we stand on an islet in the midst of an illimitable ocean of inexplicability. Our business in every generation is to reclaim a little more land.

- T.H. Huxley, 1887
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The collective microbial genomes within our gut
(microbiome) represent a powerful metabolic force, leading
many authors to call our GI flora an “organ within an
organ”, and the metagenomic sequencing of our microbiome,
“the second human genome project”. Bile acids,
endogenously produced by the host liver, represent both a
strong selective pressure for potential colonizers, as
well as substrates for microbial metabolism. Indeed,
microbes have evolved enzymes to deconjugate bile salts,
epimerize bile acid hydroxyl groups, and 7α-dehydroxylate
primary bile acids. The products of microbial 7α-
dehydroxylation, secondary bile acids, are suggested by numerous lines of evidence to be involved in promoting colon carcinogenesis. 7α-dehydroxylating activity is a multi-step pathway, genes of which have only been identified in a small number of organisms within the genus Clostridium. The biochemistry of this pathway has been largely worked out. The third step in the pathway is introduction of a Δ4-double bond; however, the gene product(s) responsible have not been identified. The baiCD and baiH genes were cloned, expressed and shown to have NAD^+‐dependent 3‐oxo‐Δ4‐steroid oxidoreductase activity showing stereospecificity for 7α‐hydroxy and 7β‐hydroxy bile acid, respectively.

In addition, bai genes were isolated from C. hylemonae TN271 by bidirectional genome‐walking by PCR. This represents the first report of bai genes from a “low activity” 7α‐dehydroxylating bacterium. The gene organization and sequence of the baiBCDEFGHI operon was highly conserved between C. hylemonae TN271 and the “high activity” 7α‐dehydroxylating bacterium C. scindens VPI 12708. The baiA gene was located by PCR using degenerate oligonucleotides. Bi‐directional genome‐walking revealed what appears to be several novel genes involved in bile acid metabolism which were also located in C. scindens VPI 12708. Expression of a 62 kDa flavoprotein and reaction with [24-14C] 3‐oxo‐DCA and NADP resulted in a product of greater hydrophilicity than deoxycholic acid. The identity of this product was not determined. A second gene appears to share a common evolutionary origin with...
the baiF gene. A hypothesis is offered regarding the function of these homologues as Type III CoA transferases recognizing 5β-bile acids, or 5α-bile acids (allo-bile acids). A third gene encodes a putative short chain reductase, similar in size and predicted function to the baiA gene, which may be involved in the final reductive step in the pathway. These novel genes also contained a conserved upstream regulatory region with the bai oxidative genes. Finally, two genes were identified which may serve as potential drug targets to inhibit bile acid 7α-dehydroxylation. The first is an ABC transporter which may be co-transcribed with the other novel bile acid metabolizing genes, and what appears to be a bile acid sensor/regulator similar to the Tryptophan-rich sensory protein (TspO)/mitochondrial peripheral benzodiazepine receptor (MBR) family of proteins.
INTRODUCTION

Bile salt metabolism by intestinal bacteria

The human large intestine harbors a complex microbial flora (1). Bacterial density in the human colon is among the highest found in nature, approaching $10^{12}$ bacteria/gram wet weight feces (2, 3). In contrast, the host suppresses significant bacterial colonization of the small intestine by a variety of mechanisms including rapid transit times, antimicrobial peptides, proteolytic enzymes, and bile (4). Failure of these mechanisms leads to bacterial overgrowth of the small intestine, resulting in malabsorption as bacteria compete with the host for nutrients. Under normal conditions, bacterial fermentation in the colon represents an important salvage mechanism. Complex carbohydrates, which are intrinsically indigestible, or which escape digestion and absorption in the proximal gut, are fermented by colonic bacteria to yield short chain fatty acids. It has been estimated that these short-chain fatty acids constitute 3-9% of our daily caloric intake (4). Colonic bacteria also contribute to salvage of bile salts that escape
active transport in the distal ileum. The major bile salt modifications in the human large intestine include deconjugation, oxidation of hydroxy groups at C-3, C-7 and C-12, and \(7\alpha/\beta\)-dehydroxylation (Figure 1). Deconjugation and \(7\alpha/\beta\)-dehydroxylation of bile salts increases their hydrophobicity and raises their pKa, thereby permitting their recovery via passive absorption across the colonic epithelium. However, the increased hydrophobicity of the transformed bile salts also is associated with increased toxic and metabolic effects. Elevated concentrations of secondary bile acids in feces, blood and bile have been linked to the pathogenesis of cholesterol gallstone disease and colon cancer (5). The following is a current review of the microbiology of bile acid metabolism in the human gastrointestinal tract focusing on understanding the biochemical mechanisms and physiological consequences of such metabolism on both the bacterium and the human host.

**The enterohepatic circulation of bile acids**

Bile acids are saturated, hydroxylated C-24 cyclopentanephenanthrene sterols synthesized from cholesterol in hepatocytes. The two primary bile acids
Figure 1. Bacterial bile salt-biotransforming reactions in the human intestinal tract. Hydroxy group carbons of cholate are numbered and the AB rings are identified. The 3, 7, and 12 carbons of cholic acid (CA) are numbered. Nomenclature is that of Hofmann et al. (160). BSH, bile salt hydrolase; HSDH, hydroxysteroid dehydrogenase.
Multi-step $\alpha$-dehydroxylation pathway

\[ \text{cholylglycine} \xrightarrow{\text{BSH}} \text{allodeoxycholic acid} \]

\[ \text{3-dehydrocholic acid} \xleftrightarrow{3\alpha\text{-HSDH}} \text{cholic acid} \xleftrightarrow{7\alpha\text{-HSDH}} \text{7-oxodeoxycholic acid} \]

\[ \text{7-epicholic acid} \]

\[ \text{isocholic acid} \]

\[ \text{Deoxycholic acid} \]

\[ \text{12-oxochenodeoxycholic acid} \xleftrightarrow{\text{12-HSDH}} \text{12-epicholic acid} \]
synthesized in the human liver are cholic acid (CA) (3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic acid (CDCA) (3α, 7α-dihydroxy-5β-cholan-24-oic acid). Bile acids are further metabolized by the liver via conjugation (N-acyl amidation) to glycine or taurine, a modification that lowers the pKa to around 5. Thus, at physiological pH, conjugated bile acids are almost fully ionized, and may be termed bile salts (6). Bile salts are secreted actively across the canalicular membrane and are carried in bile to the gallbladder where they are concentrated during the inter-digestive period. Following a meal, release of cholecystokinin from the duodenum stimulates the gallbladder to contract, causing bile to flow into the duodenum (7). Bile salts are highly effective detergents that promote solubilization, digestion and absorption of dietary lipids, and lipid soluble vitamins throughout the small intestine. High concentrations of bile salts are maintained in the duodenum, jejunum and proximal ileum, where fat digestion and absorption take place. Bile salts are then absorbed through high-affinity active transport in the distal ileum (6). Upon entering the bloodstream, bile salts are
complexed to plasma proteins and returned to the liver. Upon reaching the liver they are cleared efficiently from the circulation by active transporters on the sinusoidal membrane of hepatocytes and rapidly secreted into bile. This process is known as the enterohepatic circulation. Figure 2 depicts the enterohepatic circulation in the context of the gastrointestinal anatomy and also indicates the relative numbers and genera of the predominant bacteria inhabiting each section of the GI tract.

During the enterohepatic circulation, bile salts encounter populations of facultative and anaerobic bacteria of relatively low numbers and diversity in the small bowel. Bile salt metabolism by small bowel microbes consists mainly of deconjugation and hydroxy group oxidation. Ileal bile salt transport is highly efficient (~95%), but approximately 400-800 mg of bile salts escape the enterohepatic circulation daily and become substrate for significant microbial biotransforming reactions in the large bowel (6). Comparison of bile acid composition in the gallbladder and feces illustrates the extent of microbial bile acid metabolism in the large intestine (Figure 3). Secondary bile acids deoxycholic acid (DCA)
Figure 2. Anatomy, physiology, and microbiology of the gastrointestinal tract. Large arrows denote the enterohepatic circulation of bile acids, which begins with contraction of the gallbladder, releasing bile into the duodenum. Small arrows denote the passive absorption of bile acids that escape active transport. * Secondary bile acids produced by 7α-dehydroxylation are passively absorbed in the large intestine and returned to the liver (see Fig 3). The genera of predominant bacteria isolated from each region of the lower gastrointestinal tract are listed.
A: Duodenum (25cm)
  pH 5.7~6.4
  \( \sim 10^3 \) bacteria/ml
  Lactobacillus
  Streptococcus

B: Jejunum (1.0m)
  pH 5.9~6.8
  \( \sim 10^4 \) bacteria/ml
  Lactobacillus
  Streptococcus
  Staphylococcus
  Veillonella

C: Ileum (2.0m)
  pH 7.3~7.7
  \( 10^6-10^8 \) bacteria/ml
  Enterobacteria
  Enterococcus
  Bacteroides
  Clostridium
  Lactobacillus
  Veillonella

D: Cecum/Colon (150cm)
  pH 5.7~6.8
  \( \sim 10^{11} \) bacteria/g
  Bacteroides, Eubacterium,
  Bifidobacterium,
  Ruminococcus,
  Peptostreptococcus,
  Propionibacterium,
  Clostridium, Lactobacillus,
  Escherichia,
  Streptococcus,
  Methanobrevibacter
Figure 3. Composition of bile acids in the gallbladder and feces of healthy individuals. "Other" bile acids refer to oxo- and 3β-hydroxy derivatives of secondary bile acids. Values were derived from published sources (6, 93, 161, 162). CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid.
Biliary Bile acid Composition

- CA: 35%
- CDCA: 35%
- DCA: 25%
- UDCA: 2%
- LCA: 1%
- Other: 2%

Bacterial enzymes

Fecal Bile acid composition

- CA: 34%
- CDCA: 2%
- DCA: 28%
- UDCA: 2%
- LCA: 29%
- 12-oxo-LCA: 3%
- Other: 2%
(3α, 12α-dihydroxy-5β-cholan-24-oic acid) and lithocholic acid (LCA) (3α-hydroxy-5β-cholan-24-oic acid) are produced solely by microbial biotransforming reactions in the human large intestine. DCA accumulates in the bile acid pool (LCA to a much lesser extent) due to passive absorption through the colonic mucosa and the inability of the human liver to 7α-hydroxylate DCA and LCA to their respective primary bile acids. LCA is sulfated in the human liver at the 3-hydroxy position, conjugated at C-24 and excreted back into bile (6). The resultant bile acid sulfate is poorly reabsorbed from the gut. Even though 3-sulfo-LCA glycine and taurine conjugates are deconjugated and to some extent desulfated by intestinal bacteria, 3-sulfo-LCA/LCA is lost in feces and does not normally accumulate in the enterohepatic circulation (8).

**Deconjugation of Bile Salts**

**Characteristics of bile salt hydrolase(s) (BSH)**

Deconjugation refers to the enzymatic hydrolysis of the C-24 N-acyl amide bond linking bile acids to their amino acid conjugates. This reaction is substrate limiting and goes to completion in the large bowel. BSH
are in the choloylglycine hydrolase family (EC 3.5.1.24) and have been isolated and/or characterized from several species of intestinal bacteria (Table 1). The importance of the position, charge, shape, and chirality of various analogues of taurine/glycine conjugates on the rate of hydrolysis by BSH has also been investigated (9). BSH differ in subunit size and composition, pH optimum, kinetic properties, substrate specificity, gene organization and regulation. BSH do, however, share in common several conserved active site amino acids (Cys2, Arg18, Asp21, Asn175, and Arg228) and share a high degree of amino acid sequence similarity with the penicillin V amidase (PVA) of Bacillus sphaericus (Figure 4). The conservation of Tyr82 in PVA and Asn82 in BSH are likely a result of differing steric requirements for their respective substrates (10). Recently, a bsh from Clostridium perfringens has been crystallized both in the apo-enzyme form and in complex with TDCA (hydrolyzed product) at resolutions of 2.1 and 1.7 Å, respectively (11). The structure revealed the Cys2 residue is in position for nucleophilic attack of the N-acyl amide bond. Site directed mutagenesis of the Cys2 residue from the BSH
### Table 1. Characteristics of bile salt hydrolases (BSH) from intestinal bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>MW (kDa)</th>
<th>MW (kDa) Subunits</th>
<th>Apparent K&lt;sub&gt;m&lt;/sub&gt; (mM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pH optimum</th>
<th>Reference</th>
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<td><strong>Bacteroides fragilis</strong></td>
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<td>35</td>
<td>+ + + - - -</td>
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<td>36.1</td>
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<td><strong>Lactobacillus johnsonii 100-100</strong></td>
<td>42&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+ + + + ND ND ND ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9-4.5</td>
<td>16,17, 23</td>
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<tr>
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<td>42</td>
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<tr>
<td>isoenzyme B</td>
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<td>0.95 + + + ND ND</td>
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<tr>
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<tr>
<td>isoenzyme D</td>
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<td><strong>Lactobacillus plantarum</strong></td>
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<td>4.7-5.6</td>
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<td><strong>Lactobacillus acidophilus</strong></td>
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<td>+ ND ND + ND ND</td>
<td>ND</td>
<td>17,27</td>
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<td>0.875 1.61 0.516 1.33 1.4 1.37</td>
<td>5.5-6.5</td>
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<tr>
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<td>1.2 0.33 0.79 0.16 0.13 0.28</td>
<td>5.0-7.0</td>
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<tr>
<td><strong>Bifidobacterium bifidum</strong></td>
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<td>35</td>
<td>+ + + ++ ++ ++</td>
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<td>18</td>
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<tr>
<td><strong>Bifidobacterium animalis</strong></td>
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<td>ND ND ND ND ND ND ND</td>
<td>ND</td>
<td>19</td>
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<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td>ND</td>
<td>38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND ND ND ND ND ND ND</td>
<td>ND</td>
<td>21,163</td>
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<sup>a</sup> TCA, taurocholate; GCA, glycocholate; TDCA, taurodeoxycholate; GDCA, glycodeoxycholate; TCDDA, taurochenodeoxycholate; GCDDA, glycochenodeoxycholate. + activity detected; - no activity detected; TR, trace of activity.
<sup>b</sup> ND, not determined.
<sup>c</sup> Value derived from Protparam program (http://www.expasy.org/tools/protparam.html) using deduced amino acid sequence.
Multiple sequence alignment of cholylglycine hydrolases. Protein sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Alignments were made with the ClustalW program (http://www.ebi.ac.uk/clustalw/) using the GONNET 250 matrix. Residues highlighted in yellow are predicted active site amino acids based on the crystal structure of the BSH from C. perfringens (11) as well as on site-directed mutagenesis and biochemical data (10, 12-14). Residues highlighted in gray correspond to residues involved in substrate binding in the BSH from C. perfringens (11). The secondary structural elements, which are based on the conjugated bile acid hydrolase from C. perfringens (CBAH-1) crystal structure, are shown above the alignment. The a and b designations of Lactobacillus johnsonii refer to the two isoforms of the genes found in this bacterium.
of *Bifidobacterium longum* and *Bi. bifidum* (9, 17) as well as sulfhydryl inhibition of several BSH have shown the importance of this residue in catalysis (10, 12, 13). Alignment of amino acid sequences from BSH shows the Cys2 residue is conserved in all BSH characterized to date (Figure 4). The broad substrate specificities reported (Table 1) are potentially a function of a lack of conservation observed in residues making up the substrate binding pocket of the conjugated bile acid hydrolase gene product (CBAH-1) of *C. perfringens* and the corresponding residues predicted in amino acid multiple sequence alignment with other BSH (Figure 4). The sterol moiety is bound primarily through hydrophobic interactions in the CBAH-1 of *C. perfringens* (residues highlighted in grey in Figure 4) as well as hydrogen bonds to the carboxylate group. While the crystal structure of CBAH-1 did not reveal specific recognition of the taurine/glycine moiety, kinetic data from several BSH suggest the conjugates are important in substrate specificity (Table 1). Therefore, additional crystallization and site-directed mutagenesis (preferably with mutagenesis of Cys2) of BSH from
different species will be helpful in explaining kinetic observations of substrate specificity.

**Distribution, genetic organization, and regulation of BSH**

Genes encoding BSH have been cloned from *C. perfringens* (14), *Lactobacillus plantarum* (15), *La. johnsonii* (16, 17), *Bi. longum* (10), *Bi. bifidum* (18), *Bi. adolescentis* (19), and *Listeria monocytogenes* (20, 21). Homologues and putative *bsh* genes have also been identified recently through microbial genome analysis. The organization and regulation of genes encoding BSH differs between species and genera. Monocistronic BSH genes have been reported in *La. plantarum* (15), *La. johnsonii* (17), *Li. monocytogenes* (21), and *Bi. bifidum* (18). A gene encoding BSH (CBAH-1) cloned from *C. perfringens* (14) differed significantly in size and amino acid sequence from a BSH purified from a different strain of *C. perfringens* (12). The inactivation of the gene encoding CBAH-1 resulted in only partial reduction in BSH activity (BSH activity 86% of WT) suggesting multiple BSH genes in *C. perfringens*. Furthermore, the crystal structure showed that the enzyme encoded by the CBAH-1 gene forms an active
homotetramer (11). These observations, coupled with detection of both intracellular and extracellular BSH provides further evidence for multiple isoforms though organization and regulation of the bsh gene(s) from C. perfringens are not presently known (22). Polycistronic operons encoding three genes involved in bile salt deconjugation (cbsT1, cbsT2, cbsHβ) have been characterized in La. johnsonii and La. acidophilus (17). Genes cbsT1 and cbsT2 appear to be gene duplications each of which encode TCA/CA antiport proteins of the major facilitator superfamily while cbsHβ encodes the BSH β-isoform (23). In addition, an uncharacterized extracellular factor has been detected in La. johnsonii 100-100 which stimulates BSH activity and uptake of conjugated bile salts during stationary growth phase (17, 24). BSH expression is also growth phase dependent. Stationary phase expression has been reported in Bacteroides fragilis (25) while exponential phase expression was reported for Bi. longum (10).

Benefits of BSH to the bacterium
BSH appear to enhance the bacterial colonization of the lower GI tract of higher mammals. The physiological advantages of BSH are not fully understood and may vary between bacterial species and genera. It has been hypothesized that deconjugation may be a mechanism of detoxification of bile salts. De Smet et al. (1995) observed significantly higher rates of deconjugation of GDCA over TDCA in La. plantarum (26). Mutants lacking functional BSH (bsh⁻) exhibited pH and concentration dependent toxicity of GDCA as compared to wild type cells; whereas, this effect was not demonstrated with TDCA. De Smet et al. (1995) hypothesized that the difference in dissociation constants between GDCA and TDCA resulted in the collapse of cellular proton motive force by intracellular deprotonation with the glycine-conjugate (26). The presence of a functional BSH results in the intracellular accumulation of free bile acids which become protonated in a stoichiometric fashion decreasing energy dependent H⁺ ATPase driven proton efflux. BSH from human intestinal lactobacilli generally have higher affinity for glycine conjugates (26-29). This observation may lend weight to the hypothesis of De Smet et al. (1995) or the
higher affinity of BSH for glycine conjugates may have evolved because glycine conjugates are generally higher in proportion (3:1) than taurine conjugates in human bile (6). Tannock et al. (1989) argued against the hypothesis of deconjugation as a means of detoxification in lactobacilli, pointing to the fact that free bile acids are more cytotoxic than their conjugates (30). However, when the free bile acids become 7-dehydroxylated by other intestinal bacteria in vivo, the resultant secondary bile acids tend to precipitate (extent depending on luminal pH) and bind to insoluble fiber or may be absorbed through the colonic membrane, and may exist in low concentrations in the bacterium’s microenvironment. Therefore, additional studies comparing various characteristics of bsh knockouts with their isogenic parent strain will be needed to determine the function of deconjugation in Lactobacillus spp.

Strategies to resist bile salt toxicity have been observed in pathogens that colonize the intestinal tract (31-33). Recently, a BSH from L. monocytogenes was shown to be a novel virulence factor (21). Comparative genome analysis revealed the absence of a bsh gene in the closely
related non-virulent *Li. innocua* (20). The *bsh* gene is positively regulated by PrfA, which is a transcriptional activator of numerous virulence genes in *Li. monocytogenes*. Deletion of the *bsh* gene results in decreased resistance to bile salts, and significantly reduced infectivity *in vivo*. These results demonstrate the importance of BSH activity for survival *in vivo* and infection in the intestinal and hepatic phases of listeriosis. The mechanism by which BSH activity in *L. monocytogenes* enhances survival and virulence is currently unknown.

Deconjugation may provide a means of obtaining cellular carbon, nitrogen, and sulfur for some bacterial species. This has been demonstrated in bacteroides (34) and is suggested in *Bi. longum* (10). In fact, the *bsh* gene from *Bi. longum* is co-transcribed with the gene encoding glutamine synthetase adenylyltransferase (*glnE*); a component of the nitrogen regulation cascade (10). In this regard, hydrolysis of the conjugated bile acid may provide amino nitrogen; providing a possible explanation for coordinate regulation of these seemingly physiologically unrelated genes (10). Taurine utilization
is also widespread and can serve as an energy source under both aerobic and anaerobic conditions (35). Glycine can be utilized as an energy source by certain clostridia by the Stickland reaction (36). The Stickland reaction is a form of amino acid fermentation where one amino acid donates electrons which are accepted by another amino acid distinct from the electron donor. Another hypothesis suggests BSH are detergent shock proteins enabling survival during stress (37). De Smet et al. (1995) found no evidence for this in lactobacilli after growth with various detergents (26).

The widespread distribution of BSH across gram negative and gram positive intestinal bacteria coupled with a wide range of substrate specificities, genetic regulation and the occurrence of multiple isoforms in certain strains have created conflicting reports regarding the physiological benefit to the bacterium in hydrolyzing bile acid conjugates. Determining the mechanism(s) by which BSH aid bacteria in colonization of the mammalian intestine will be of great interest, especially in regards to bacterial pathogenesis.
Taurine, hydrogen sulfide production and colon cancer

The bile acid conjugates glycine and taurine serve as substrate in microbial metabolism. Unlike glycine, taurine contains a sulfonic acid moiety which is reduced and dissimilated to hydrogen sulfide following deconjugation (38, 39). Hydrogen sulfide is highly toxic, and has been shown to increase colonocyte turnover (40). Activation and upregulation of the ERK 1/2 signaling pathway has been suggested as a possible mechanism for sulfide induced colonocyte proliferation (41). Hydrogen sulfide also inhibits butyrate metabolism in colonocytes, a key nutrient and regulator of cell turnover in the gut (40). Levitt et al (1999) demonstrated that colonocytes have evolved a highly efficient mechanism to detoxify volatile reduced sulfides through oxidation to thiosulfate (42). Defects in this detoxification system are suggested to play a role in the etiology of ulcerative colitis, a known risk factor for colon cancer (42, 43). Recently, sulfide has been implicated in preventing apoptosis in the adenocarcinoma cell line HCT116 after exposure of cells to β-phenylethyl isothiocyanate (PEITC), a phytochemical
found in cruciferous vegetables, which has been shown to prevent colon carcinogenesis (44, 45).

A diet high in meat has been shown to significantly increase the levels of both taurine conjugation to bile acids (46, 47), and the production of hydrogen sulfide in the colon (48). A relationship exists between generation of hydrogen sulfide in the colon and chronic gastrointestinal illness such as inflammatory bowel disease and colon cancer (5, 49). Populations such as native black Africans with low incidence of colon cancer consume low meat diets (50). Native black Africans also have low ratios of taurine to glycine conjugation (1:9) and low hydrogen sulfide production when compared to populations consuming a "Western diet" (46, 47). In human fecal slurries obtained from individuals consuming a "Western diet", taurine addition generated some of the highest sulfide levels of any organic or inorganic sulfur source added (43). Taurine addition to a co-culture of a species of bacteroides and an unidentified 7α-dehydroxylating bacterium resulted in significant sulfide production, which stimulated increased rates of DCA production (34).
While the extent to which taurine metabolism contributes to total colonic sulfide production has yet to be established, several key points have been made: 1) the extent of taurine conjugation in the bile acid pool is largely affected by diet; 2) the same dietary factors which increase taurine conjugation are hypothesized to increase colon cancer risk; 3) taurine metabolism by intestinal bacteria results in hydrogen sulfide generation; 4) sulfide generation is linked to the carcinogenesis process through enhanced cell proliferation, inhibition of butyrate metabolism and activation of cell signaling pathways; 5) sulfide generation may enhance DCA formation in the gut through stimulation of the microbial bile acid 7α-dehydroxylation pathway.

**Microbial bile acid hydroxysteroid dehydrogenase(s) (HSDH)**

**Oxidation and epimerization**

Oxidation and epimerization of the 3-, 7-, and 12-hydroxy groups of bile acids in the gastrointestinal tract are carried out by HSDH expressed by intestinal bacteria (Figure 1). Epimerization of bile acid hydroxy groups is
the reversible change in stereochemistry from α to β configuration (or vice versa) with the generation of a stable oxo-bile acid intermediate. Epimerization requires the concerted effort of two position-specific, stereochemically distinct HSDH of intraspecies or interspecies origin. For example, the presence of both 7α- and 7β-HSDH in *C. absonum* allow epimerization by a single bacterium (51); whereas epimerization also can be achieved in co-cultures of intestinal bacteria, one possessing 7α-HSDH and the other 7β-HSDH (52, 53).

The extent of the reversible oxidation and reduction of bile acid hydroxy groups by HSDH depends in part on the redox potential of the environment. Addition of oxygen to the culture medium increases the accumulation of oxo-bile acids (51). Generation of oxo-bile acids may be more favorable under the higher redox potentials found on the mucosal surface (4); while reduction of oxo-bile acids may be more favorable under the low redox potential (-200 to -300mV) in the large intestinal lumen. Thus, while the redox potential of the colon is net reductive, microenvironments at the mucosa may provide oxidizing conditions favorable for certain microbial reactions.
HSDH differ in their reductive and oxidative pH optima, NAD(H) or NADP(H) requirements, M_r, and gene regulation (Table 2).

3α- and 3β-HSDH

3α/β-HSDH specifically catalyze the reversible, stereospecific oxidation/reduction between 3-oxo-bile acids and 3α- or 3β-hydroxy bile acids. 3α-HSDH have been detected in some of the most prevalent intestinal bacteria including C. perfringens (54), Peptostreptococcus productus (55) and Eggerthella lenta (formerly Eubacterium lentum) (56, 57) as well as intestinal bacteria present in lower numbers (≤ 10^5/g wet weight feces) including C. scindens (58), and C. hiranonis (59), and in non-intestinal bacteria including Pseudomonas testosteroni (60, 61). 3β-HSDH activity has been described in species of Clostridium and Rumminococcus (62-64). It appears that intraspecies 3-epimerization favors the 3α-position. In fact, growing cultures of C. perfringens in the presence of 3-oxo-CDCA formed CDCA (84%) preferentially over iso-CDCA (16%) under anaerobic conditions (65).
<table>
<thead>
<tr>
<th>Organism</th>
<th>Stereo Specificity</th>
<th>cofactor</th>
<th>pH optimum</th>
<th>Native MW (kDa)</th>
<th>Induction</th>
<th>Reference</th>
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<td>54, 66</td>
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<td>12α (CE)</td>
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<td>95</td>
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<td>53</td>
<td>Induced by 7-oxo bile acids</td>
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<td>NAD(H)</td>
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<td>110 (lactate)</td>
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<td>327</td>
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<td>NAD(H)</td>
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<td>9.6</td>
<td>120 (lactate)</td>
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<td>NAD(H)</td>
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<td>Induced by CDCA and CDCAexpressed by UDCA</td>
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<td>Clostridium scindens VPI 2708</td>
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<td>NAD(H)/NAD(P)H</td>
<td>5.0 - 9.0</td>
<td>27 (lactate)</td>
<td>Induced by CA, CDCA, CDCA, CDCA</td>
<td>58, 81</td>
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<td></td>
<td>7α (P)</td>
<td>NAD(H)</td>
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<td>124 (lactate)</td>
<td>Non-inducible</td>
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<td>Clostridium bifertorum</td>
<td>7β (PP)</td>
<td>NAD(P)H/NAD(H)</td>
<td>11</td>
<td>ND</td>
<td>Induced by 7-oxo-LCA, CDCA, DCA</td>
<td>84</td>
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<td>Clostridium leviense</td>
<td>7α (CE)</td>
<td>NAD(H)/NAD(P)H</td>
<td>10.5</td>
<td>ND</td>
<td>Induced by CA, CDCA, UDCA</td>
<td>76</td>
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<td>7β (CE)</td>
<td>NAD(P)H</td>
<td>10.5</td>
<td>ND</td>
<td>Induced by CA, CDCA, UDCA</td>
<td></td>
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<td>NAD(P)H</td>
<td>8.6 - 9.0</td>
<td>225</td>
<td>Non-inducible</td>
<td>90</td>
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<td>NAD(P)H</td>
<td>7.8 (oxid); 10 (nadh)</td>
<td>125</td>
<td>Non-inducible</td>
<td>92</td>
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</table>

* (CE) cell extract, (PP) partially purified, (P) purified, ND, not determined, BA, bile acids
Pyridine nucleotide cofactor requirements differ between 3α/β-HSDH. 3α-HSDH require NAD(H), with the exception of the enzyme purified from *C. perfringens*, which uses NADP(H) and that purified from *C. scindens* which can use either NAD(H) or NADP(H) (54, 56-58, 61). 3β-HSDH have been shown to preferentially require NADP(H), with the exception of *C. innocuum* which uses NAD(H) (62-64). Dihydroxy bile acids (DCA, CDCA and UDCA) are generally better substrates than trihydroxy bile acids (CA) (62, 65).

3α/β-HSDH characterized to date are constitutively expressed with the exception of *C. scindens* and *C. hiranonis* which are induced by primary bile acids CA and CDCA. In fact, three copies of 3α-HSDH genes (*baiA1*, *baiA2* and *baiA3*) have been identified from *C. scindens* (66-67) and the *baiA1* gene has been expressed in *E. coli* and characterized (58). The *baiA* gene products are unique among 3α/β-HSDH due to their high specificity toward CA-CoA and CDCA-CoA conjugates and relatively low activity toward free bile acids (58).

7α- and 7β-HSDH
7α/β-HSDH catalyze the reversible, stereospecific oxidation/reduction of the 7α- and 7β-hydroxyl groups of bile acids. While 7α/β-HSDH are common among intestinal bacteria, the extent of 7α/β-dehydrogenation in the intestine, or in mixed fecal suspensions is difficult to interpret due to the competing, and irreversible 7α/β-dehydroxylation of bile acids (see section 6) (68).

7α/β-HSDH are widespread among the bacteroides and clostridia as well as Escherichia coli, and Ruminococcus sp. (Table 2) (54, 64, 69-74). In addition, several intestinal clostridia express both 7α- and 7β-HSDH and have been shown to epimerize the 7α/β-hydroxy group (75, 76-78). 7α/β-HSDH have been partially purified from intestinal bacteria including Ba. fragilis (71, 79), Ba. thetaiotaomicron (74), C. scindens (80), C. sordellii (70), E. coli (73), as well as the soil isolates Xanthomonas maltophilia (81), C. absonum (82), and C. bifermentans (83). Interestingly, several intestinal bacteria with 7α/β-HSDH activity also possess BSH including: Ba. fragilis (25), C. sordellii (70), C. perfringens (84), C. innocuum (63), and the soil isolate C. bifermentans (83).
7α-HSDH generally utilize NADP(H) as a cofactor, with the exception of *E. coli* (85), and *Ba. thetaiotaomicron* (74). *C. bifermentans, C. absonum,* and *Ba. fragilis* 7α-HSDH can utilize either NAD(H) or NADP(H) as a cofactor (76, 79, 83). 7β-HSDH enzymes characterized to date utilize NADP(H) as a cofactor (53, 62, 75, 76). 7α/β-HSDH enzymes have higher affinity for dihydroxy bile acids (CDCA, 7-oxo-LCA) than trihydroxy (CA, 7-oxo-DCA). *C. limosum* cell extracts utilized both free and conjugated bile acids, while whole cells only oxidized free bile acids (75, 85). This is due to intracellular location of the 7α-HSDH, and the inability of the organism to take up a conjugated bile salt. The genes encoding 7α-HSDH have been cloned from *E. coli* (73), *C. scindens* (69), and *C. sordellii* (70). Sequence similarity suggests these enzymes belong to the short-chain polyol dehydrogenase family. Regulation of 7α/β-HSDH expression is generally growth phase dependent and inducible by bile acid substrates (Table 2). *C. scindens* and *E. coli* constitutively express 7α-HSDH and are non-inducible (69, 85). Unexpectedly, the non-substrate DCA can induce 7α-HSDH expression in *C. absonum* and *C. sordellii* though the
reason for this induction remains unclear (70, 76). Macdonald et al. (1983) observed the expression of five soluble and two membrane polypeptides upon exposure of C. absonum to DCA and CDCA in the culture medium although the functions of these additional polypeptides have not been determined (82).

Crystal structures of the *E. coli* 7α-HSDH binary and ternary complexes have been solved and a mechanism for 7α-dehydrogenation proposed (Figure 5) (87). Binding of 7α-hydroxy bile acids elicits major conformational changes at the substrate binding loop and C-terminal domain. A two step mechanism is proposed in which Tyr159 acts as a catalytic base removing the C-7 hydroxy hydrogen. Hydrogen bonding by Ser146 is hypothesized to stabilize the intermediate. Regeneration of the catalyst occurs through transfer of the acquired hydride from the phenolic group on Tyr159 to Lys163 to position 4 of NAD⁺. Lys163 serves two important roles: anchoring NAD⁺ through bifurcated hydrogen bonding, and indirect hydride transfer from Tyr159 to position 4 of NAD⁺ (87). Site directed mutagenesis confirmed the role of these amino acids in 7α-HSDH catalysis (88). Analysis of the 7-oxo-GLCA bile acid
Proposed catalytic mechanism of bile acid 7α-dehydrogenation based on the crystal structure and site-directed mutagenesis of active site amino acids of the 7α-dehydrogenation from *E. coli*. See text for a description of catalysis. Reprinted with permission from Tanaka et al. (87). Copyright © 1996 American Chemical Society.
substrate/enzyme complex revealed tight binding of the sterol with loose association for the glycine conjugate. Binding of glycine and taurine conjugates of CDCA were not significantly different from the free bile acid (87).

12α- and 12β-HSDH

12α/β-HSDH have been detected mainly among members of the genus *Clostridium*. NADP-dependent 12α-HSDH have been detected in *C. leptum* (89), *Clostridium* group P (89), while NAD-dependent 12α-HSDH activity was reported in *Eg. lenta* (56), and *C. perfringens* (54). 12β-HSDH have been detected in *C. tertium*, *C. difficile* and *C. paraputrificum* (91, 92). 12α/β-HSDH characterized to date are constitutively expressed, and non-inducible, with the exception of the 12β-HSDH from *C. paraputrificum* which is induced by 12-oxo-bile acid substrates (92). 12α/β-HSDH generally have higher affinity for dihydroxy (DCA) than trihydroxy bile acids (CA, iso-CA) and free vs. conjugated bile acids. The 12α-HSDH from *C. leptum* is an exception; demonstrating higher affinity for CA conjugates than free CA (89). 12α-HSDH appear to be repressed by the addition of bile acid substrates (DCA>CDCA>CA) to the growth medium
at 1 mM concentrations. It has been suggested that these enzyme activities should be repressed in bacteria colonizing the large intestine (56, 92); although 12-oxo-bile acids have been detected at low levels in the feces of healthy individuals (Figure 3) (93-94).

Benefits of bile acid hydroxysteroid oxidoreductases to the bacterium

The oxidation of bile acid hydroxyl groups generates reducing equivalents for cellular biosynthetic reactions and possibly electron transport phosphorylation. Bile acid dehydrogenation is hypothesized to generate energy in *Ba. thetaiotaomicron* (74). Sherod and Hylemon (1977) suggested that reduced pyridine nucleotides generated from 7α-hydroxy oxidation serve to generate ATP via a cytochrome-linked electron transport chain in the presence of electron acceptors (i.e., fumarate) (74).

Bile acids are potent antimicrobial agents provided the proper concentration and proportion of hydrophobic bile acids (CDCA, LCA, DCA) are present (95). Alteration of hydroxy group stereochemistry has marked influence on the physiochemical properties of bile acids (96, 97). The
epimerization of the 7α-hydroxy group of CDCA lowers the hydrophobicity and toxicity of the bile acid (97). Macdonald, White and Hylemon (1983) observed that C. absonum grew on plates containing 1 mM UDCA, though was unable to grow on plates containing 1 mM CDCA (82). Furthermore, when cultured in the presence of 7-oxo-bile acids only low concentrations of CA and CDCA were formed while the majority of 7-oxo-bile acids were reduced to 7-epi-CA and UDCA, respectively, by log phase C. absonum (51). UDCA has also been shown to act as a repressor of 7α/β-HSDH production in C. absonum suggesting UDCA is an end-product (76). The enzyme also displays markedly higher affinity for CDCA than CA, the former being more toxic. In summary, dehydrogenation may serve functions related to energy generation as well as attempts to maintain low concentrations of more hydrophobic bile acids in the bacterium’s microenvironment.

**Interplay between HSDH enzymes in human liver and intestinal bacteria**

The co-evolution between host and gut flora is evident when observing the interplay between liver and
bacterial biotransforming reactions. The liver synthesizes bile acids in which the hydroxy groups are in the \( \alpha \) orientation. In the \( \alpha \)-hydroxy orientation, one face of the molecule is hydrophobic while the other side is hydrophilic. This translates to efficient solubilization of lipid molecules through formation of mixed micelles capable of efficient emulsification while remaining soluble in aqueous environments. Generation of \( \beta \)-hydroxy-bile acids by microbial enzymes alters the efficiency of micelle formation due to hydrophilic groups on both faces of the sterol molecule. The differences observed between the composition of bile acids in serum and bile are a result of the continual interplay between liver and bacterial enzymes. Exposure of bile salts to intestinal bacteria results in roughly 50% of bile acids requiring reconjugation and low levels of bile acids returned to the liver in the \( 3\beta \)-hydroxy orientation (98). Without a means of epimerizing bile acid hydroxy groups in the human liver, \( \beta \)-hydroxy bile acids would accumulate in the bile acid pool. Interestingly, the liver seems to “allow” the accumulation of UDCA (7\( \beta \)-hydroxy) in the biliary pool as in the case of therapeutic administration of CDCA (99).
The protective effects of UDCA observed in clinical studies (100), as well as cell culture studies (97, 101) may provide an evolutionary explanation for this phenomenon.

The biochemistry and molecular biology of bile acid 7α/β-Dehydroxylation

Introduction

Secondary bile acids (DCA, LCA) predominate in human feces (Figure 3). Therefore, 7α-dehydroxylation is the most quantitatively important bacterial bile salt biotransformation in the human colon. The rapid rate of conversion of primary to secondary bile acids is surprising given that current estimates suggest this metabolic pathway is found in roughly 0.0001% of the total colonic flora (102-104). Human intestinal bacteria capable of bile acid 7α-dehydroxylation have been isolated (104, 105) and 16s rDNA phylogenetic analysis has led to their classification to the genus Clostridium (106-108).

Unlike bile acid oxidation and epimerization, 7α/β-dehydroxylation appears restricted to free bile acids. Removal of glycine/taurine bile acid conjugates via BSH enzymes is thus a prerequisite for 7α/β-dehydroxylation by
intestinal bacteria (109-112). Some intestinal bacteria are capable of both $7\alpha/\beta$-dehydroxylating activities (113), while $7\beta$-dehydroxylation activity is absent in other intestinal $7\alpha$-dehydroxylating bacteria (113). Epimerization of UDCA ($7\beta$-hydroxy) to CDCA ($7\alpha$-hydroxy) via $7\beta$-HSDH produced by members of the gut flora result in subsequent $7\alpha$-dehydroxylation. In this regard, it would appear the presence of $7\beta$-dehydroxylation activity is more of a luxury than a necessity.

**Elucidating the bile acid $7\alpha/\beta$-dehydroxylation pathway**

Samuelsson (1960) administered $[6\alpha^{-3}H, 6\beta^{-3}H, 8\beta^{-3}H]-[24^{14}C]$ CA to bile duct cannulated rabbits and rats (114). Analysis of the products recovered following exposure to intestinal bacteria revealed a differential loss of the $[6\beta^{-3}H]$ during $7\alpha$-dehydroxylation of CA. Previous work showed complete retention of the $7\beta^{-3}H$ in $[7\beta^{-3}H]-[24^{-14}C]$-CA during $7\alpha$-dehydroxylation in the rat intestine (115, 116). These data led Samuelsson (1960) to propose a mechanism for CA $7\alpha$-dehydroxylation involving two steps: diaxial trans-elimination of the $7\alpha$-hydroxy group and $6\beta$-hydrogen atom followed by reduction through trans-hydrogenation of the $6\beta$ and $7\alpha$ positions of the cholen-6-
oic acid intermediate forming DCA (114). Björkhem et al. (1989) showed the formation of a 3-dehydro-4-cholenoic acid intermediate following the differential loss of the 5βH in vitro and in vivo using a [3β-3H]-[24-14C] and [5β-3H]-[24-14C]-labeled CA (117). Hylemon et al. (1991) subsequently observed accumulation of multiple bile acid intermediates in cell extracts of Clostridium scindens induced by CA (Figure 6) (118). These radiolabeled CA intermediates were identified by mass spectrometry, then chemically synthesized and added to cell extracts of CA induced C. scindens. Each [24-14C] CA intermediate was converted into [24-14C] DCA in cell extracts prepared from CA induced cultures of C. scindens. These observations suggested that the 7α-dehydroxylation mechanism was more complex than the two-step mechanism proposed by Samuelsson et al (1960). Furthermore, these data demonstrated that bile acid 7α-dehydroxylation was a multi-step pathway in C. scindens and suggested the presence of multiple bile acid inducible genes (bai).

The induction of 7α-dehydroxylation activity in C. scindens by unconjugated C24 primary bile acids resulted in the appearance of several new polypeptides as observed on
Figure 6. Accumulation of [24- C]CA intermediates during 7α-dehydroxylation in cell extracts of C. scindens VPI 12708. Cell extracts were prepared from either CA-induced (I) or control (C) cells. This figure was modified from Hylemon et al. (118). Copyright© 1992 American Society for Biochemistry and Molecular Biology, Inc.
3-dehydroallo-DCA
3-dehydro-DCA
3-dehydro-4,6-DCA
3-dehydro-4-DCA
allo-DCA
DCA
3-dehydro-CA
7-oxo-DCA
3-dehydro-4-CA
CA
Origin
one and two-dimensional SDS-PAGE (119, 120). Purification and N-terminal sequencing of these bai polypeptides facilitated cloning of bai genes through design of degenerate probes (121-123). Northern blot analysis indicated the presence of a large CA inducible (≥ 10 kb) mRNA transcript and a smaller transcript (< 1.5 kb) in C. scindens (121, 124). These studies led to the discovery of a bai regulon encoding at least 10 open reading frames (Figure 7). Individual bai genes have been subcloned into E. coli and the functions of many determined (58, 67, 123, 125-130, Hylemon P.B., unpublished data). The proposed bile acid 7α/β-dehydroxylation pathway in C. scindens is shown in Figure 8. A bai operon has also been characterized from C. hiranonis (59), though the following discussion of the 7α/β-dehydroxylation pathway will center on C. scindens from which the functions of the gene products have been determined.

bai genes: a regulon for 7α/7β-dehydroxylation

The transport of unconjugated primary bile acids into C. scindens is facilitated by the baiG gene product which belongs to a major pump/facilitator superfamily of protein
Figure 7. Gene organization of the bile acid-inducible (bai) \(7\alpha/\beta\)-dehydroxylitation operons characterized in C. scindens VPI 12708. \(P\) indicates the promoter region.
Putative transcriptional regulators

barA 22 kDa
barB 46 kDa

bile acid CoA ligase 58 kDa

7α-dehydratase 19.5 kDa

H⁺ dependent bile acid transporter 50 kDa

unknown 72 kDa

Bile acid CoA transferase ? 47.5 kDa

7β-dehydratase ? 22 kDa

3α-HSDH 27 kDa

1 kb
transporters (126). The baiG gene has been cloned into E. coli and shown to encode a 50 kDa H+-dependent bile acid transporter (126). The BaiG facilitates transport of unconjugated CA and CDCA but not the secondary bile acids DCA or LCA (126). Computer-aided modeling suggests the baiG polypeptide contains 14 membrane spanning domains (126).

Following transport, ligation to coenzyme A (CoA) is the first step in activating CA and CDCA for 7α-dehydroxylation as several subsequent enzymatic steps are specific for CoA conjugates. The baiB gene was shown to encode a 58 kDa bile acid CoA ligase (125). CoA ligation is ATP, CoA, Mg2+ dependent and also requires a free carboxyl group on C24 bile acids (125). CoA ligation may function both to sterically hinder the constitutive 7α-HSDH committing the bile acid to 7α-dehydroxylation and trapping the bile acid inside the cell.

The 3α-hydroxy group is oxidized following CoA ligation. Oxidation of the 3-hydroxy group inhibits 7α-hydroxy group dehydrogenation; favoring 7-dehydroxylation over constitutively expressed 7α-HSDH in C. scindens (58, 80). The baiA gene products encode 27 kDa polypeptides
that have significant similarity with the short-chain alcohol/polyol dehydrogenase gene family (58, 124). Amino acid multiple sequence alignment and comparison between baiA gene products and other members of the short-chain alcohol dehydrogenase family revealed a possible NAD(P) binding site and catalytic active site (58). Three baiA genes have been cloned from C. scindens; the baiA1 and baiA3 genes are monocistronic while the baiA2 gene is part of the polycistronic bai operon (66, 67, 124). The baiA genes from C. scindens were cloned in E. coli and shown to encode 3α-HSDH (58). The enzymes only recognize bile acid CoA conjugates and can utilize either NAD⁺ or NADP⁺ as electron acceptors (58). Interestingly, the different 3α-HSDH of C. scindens share 92% amino acid sequence identity with one another suggesting gene duplication. The physiological importance of multiple baiA genes remains unclear.

The baiH gene encodes a 72 kDa polypeptide containing 661 amino acids (121). The BaiH protein exists as a homotrimer with NADH: flavin oxidoreductase activity (121). The baiH gene has been subcloned into E. coli, purified and shown to contain 1 mol FAD, 2 mol iron, and 1
mol copper per mol polypeptide subunit (129). The BaiCD polypeptide shows considerable amino acid sequence identity with the ‘Old Yellow Enzyme’ family, a putative NADH oxidase from *L. monocytogenes*, several 2,4-dienoyl CoA reductases, as well as the *baiH* from *C. scindens* (121, 129). The physiological function of the *baiCD* and *baiH* gene products is currently unknown.

The *baiF* gene encodes a 47.5 kDa polypeptide containing 426 amino acids which was shown to have bile acid CoA hydrolase activity (122, 130). However, the *baiF* gene is hypothesized to encode a CoA transferase due to reasons for energy conservation (Fig. 8) and homology to the type III family of CoA transferases (131). The first few cycles of 7α-dehydroxylation would require ATP hydrolysis (Figure 8); though ATP independent recycling of the thioesterase intermediates via transfer of CoA from an accumulated downstream intermediate, to incoming primary bile acids would significantly conserve energy. However, this hypothesis remains to be tested.

7α-Dehydration of 3-dehydro-4-chenoic acid and 3-dehydro-4-chenodeoxycholoenic acid results in the generation of a conjugated double bond in rings A and B
Figure 8. Proposed bile acid 7α-dehydroxylation pathway in \textit{C. scindens} VPI 12708 (see text).
forming a stable 3-dehydro-4,6-deoxycholdienoic acid and 3-dehydro-4,6-lithocholdienoic acid intermediates, respectively. The 7α-dehydration step results in the largest calculated energy change (-9.4 kcals/mol) of any reaction in this pathway, and the reverse reaction was not detected \textit{in vitro} (128). The 19.5 kDa bile acid 7α-dehydratase is encoded by the \textit{baiE} gene (128). This enzyme showed no activity with 3-dehydro-4-ursodeoxycholenoic acid (128). The \textit{baiE} gene product was modeled based on the crystal structure of protein homologues (secondary structure) ketosteroid isomerase (KSI) and scytalone dehydratase (STD) (Figure 9) (Woodford K et al. unpublished). The putative active site/binding pocket is shown in Figure 10. A catalytic mechanism has been proposed for the 7α-dehydration step based on the conservation in secondary structure and site directed mutagenesis of the key active site amino acids. Tyr30 acts as a general acid withdrawing electron density from the 3-oxo-group. This shift in electron density is hypothesized to make the 6β-hydrogen labile for removal by the general base His83 (assisted by Asp35). His83 is thought to donate its hydrogen to the 7α-hydroxy forming the water
Figure 9. Model of the bile acid 7α-dehydratase (baiE) based on structural data from scytalone dehydratase, nuclear transport factor 2, and steroid D5-isomerase. The α-helices are denoted by white strands, β-sheets are denoted by the yellow backbone, and the blue structure represents a steroid molecule modeled into the binding/active site pocket. Model kindly provided by Dr. Alexey G. Murzin (Cambridge University).
Figure 10. Putative binding/active site pocket of bile acid 7α-dehydratase. Putative active site amino acid residues are shown in relation to the bile acid substrate. See text for discussion of the proposed catalytic mechanism.
leaving group which is stabilized by Asp106. Site-directed mutagenesis based on the proposed mechanism supports the important role of the putative active site amino acids in enzyme catalysis. It is hypothesized that the baiI gene encodes a bile acid 7β-dehydratase due to amino acid sequence homologies between the baiE and baiI gene products.

Genes involved in the reductive arm of the 7α/β-dehydroxylation pathway have not been isolated. These genes should encode oxidoreductases catalyzing the reduction of 3-dehydro-4,6-deoxycholenoic acid → 3-dehydro-4-deoxycholenoic acid → 3-dehydro-deoxycholic acid → DCA as well as a bile acid exporter to remove secondary bile acid end-products from the bacterium (Figure 8). Genes encoding putative transcriptional regulators have been detected upstream of the bile acid inducible promoter region (Table 3) (Mallonee DH and Hylemon PB; unpublished data). Additional studies will be required to determine the mechanism of induction/repression of this pathway and to identify additional bai genes.

The benefits of 7α/β-dehydroxylation to the bacterium
### Table 3. Bile acid Inducible (bai) genes characterized from *Clostridium scindens* VPI 12708

<table>
<thead>
<tr>
<th>bai gene</th>
<th>MW (kDa)</th>
<th>Catalytic Activity/function</th>
<th>Gene Family</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>baiA</td>
<td>27</td>
<td>3α-HSDH</td>
<td>short-chain alcohol/acyl dehydrogenase</td>
<td>68</td>
</tr>
<tr>
<td>baiB</td>
<td>58</td>
<td>bile acid-CoA ligase</td>
<td>AMP-binding</td>
<td></td>
</tr>
<tr>
<td>baiCD</td>
<td>70</td>
<td>3-dehydro-4-CDCA/CA oxidoreductase</td>
<td>Pyridine nucleotide-dicarboxylate oxidoreductase; NADH: Flavin Oxidoreductase</td>
<td>127</td>
</tr>
<tr>
<td>baiH</td>
<td>72</td>
<td>3-dehydro-4-UDCA/epiCA oxidoreductase</td>
<td>Pyridine nucleotide-dicarboxylate oxidoreductase; NADH: Flavin Oxidoreductase</td>
<td>a</td>
</tr>
<tr>
<td>baiE</td>
<td>19.5</td>
<td>7β-dehydrogenase</td>
<td>COG4375</td>
<td>130</td>
</tr>
<tr>
<td>baiF</td>
<td>22</td>
<td>7β-dehydrogenase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>COG4375</td>
<td>166</td>
</tr>
<tr>
<td>baiF</td>
<td>47.5</td>
<td>bile acid-CoA hydrolase</td>
<td>Type III CoA transferase</td>
<td>132</td>
</tr>
<tr>
<td>baiF</td>
<td>50</td>
<td>H⁺ dependent bile acid transporter</td>
<td>major facilitator superfamily</td>
<td>128</td>
</tr>
<tr>
<td>baiA</td>
<td>46</td>
<td>transcriptional regulation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AraC/XylS</td>
<td>a</td>
</tr>
<tr>
<td>baiB</td>
<td>21</td>
<td>transcriptional regulation&lt;sup&gt;b&lt;/sup&gt;</td>
<td>RpoB; Permeases of the major facilitator superfamily</td>
<td>a</td>
</tr>
</tbody>
</table>

<sup>a</sup> unpublished data (Hylemon P. B., et al.)

<sup>b</sup> hypothesized function
The ability to utilize bile acids as electron acceptors is an important niche for 7α-dehydroxylating bacteria in the human colon. The 7α/β-dehydroxylation pathway requires multiple oxidative and reductive steps with a net 2 electron reduction (Figure 8). The hypothesized energy benefits of this pathway assume, however, that the baiF gene encodes a CoA-transferase and the toxic end-products, the secondary bile acid, are removed from the microenvironment in vivo (precipitation and binding to insoluble fiber). The generation of secondary bile acids may also function to exclude bacteria sensitive to these hydrophobic molecules.

**Secondary bile acids and disease**

In humans, DCA accumulates in the bile acid pool to high levels in some individuals. An increase in DCA in the bile acid pool is associated with a decrease in CDCA (Figure 11). Unlike rodents, the human liver can not 7α-hydroxylate DCA forming CA. Hence, under normal physiological conditions, there is no metabolic pathway for removing DCA from the bile acid pool in man. The amount of DCA in the bile acid pool is a function of at
Figure 11. Relationship between the percentage of CDCA and DCA in bile of patients at McGuire VA Hospital (Richmond, VA) (P. B. Hylemon et al., unpublished data).
least three variables: 1) rate of formation and absorption of DCA through the colon (input) (132); 2) colonic transit time (133) and 3) colonic pH (134).

High levels of DCA in blood, bile and feces have been correlated with an increased risk of cholesterol gallstone disease and colon cancer, two major diseases of “Western Society” (5, 135). High levels of CA 7α-dehydroxylating fecal bacteria have been correlated with increased amounts of DCA in bile of a subset of cholesterol gallstone patients (132). Treatment of these cholesterol gallstone patients (high DCA group) with antibiotics significantly decreased the levels of fecal CA 7α-dehydroxylating bacteria, DCA in bile and the cholesterol saturation index in bile (132). Early studies by Low-Beer and coworkers (1978) reported that treating control individuals with metronidazole, an antibiotic effective against anaerobic bacteria, significantly decreased the cholesterol saturation index of bile (135). Moreover, excess DCA in bile has been reported to decrease the nucleation time for cholesterol crystallization (136, 137). In total, these results suggest a possible link between intestinal
bacteria, DCA, and the risk of cholesterol gallstone disease in some patients.

DCA and LCA have been linked to colon carcinogenesis in a number of laboratory animal models and human epidemiological studies (For reviews see 5, 138). Most animal studies conclude that DCA is a promoter of the carcinogenesis process (139-142). However, some researchers argue that bile acids may cause DNA damage and act as carcinogens in humans (138). Higher levels of DCA are found in the blood of colon cancer patients as compared to control patients (98, 143). Moreover, DCA is a logical candidate for promoting colon carcinogenesis for the following reasons: 1) it is found in fecal water in high concentrations (> 100 μM) (138); 2) it can cross biological membranes via passive diffusion; 3) it can activate mammalian cell signaling pathways that are known to be involved in promoting the carcinogenesis process. In this regard, cell signaling pathways activated by DCA in mammalian epithelial cells include: protein kinase C (144); ERK 1/2 via the epidermal growth factor receptor (101, 145, 146); β-catenin (147) and JNK 1/2 (148). Secondary bile acids have been shown to cause apoptosis in
colonic epithelial cells, and high concentrations of DCA and LCA in stool may promote carcinogenesis by exerting selective pressure for emergence of epithelial cell mutants which are resistant to apoptosis (for example, via loss of p53) (138). LCA has been found to be an excellent activator of the vitamin D receptor (149, 150). Activation of this receptor in intestinal epithelial cells activates genes that metabolize LCA (150). This may be a protective mechanism that evolved to limit LCA toxicity to intestinal epithelial cells.

**Current trends and Future Issues**

Bile salt metabolism is a widespread and fundamental property of the gastrointestinal microflora encompassing the most commonly isolated species of intestinal bacteria including, but not limited to the genera *Bacteroides, Clostridium, Lactobacillus, Bifidobacterium, Eubacterium,* and *Escherichia.* However, our current understanding of the intestinal microbiology of bile salt modifications is limited to cultivated species. Discovering novel genes in the gut microbiome (collective genomes of the GI flora) encoding BSH, HSDH and enzymes involved in the 7α/β-dehydroxylation pathway through molecular techniques will
facilitate a much greater understanding of the diversity and complexity of these reactions in the human colon. Techniques such as PCR-denaturing gradient gel electrophoresis (PCR-DGGE) can be utilized to measure the diversity of organisms based on specific phylogenetic markers such as 16s rDNA as well as functional genes, potentially bile salt modifying enzymes (151, 152). Measuring the true diversity of bile salt modifying bacteria is crucial in studying the relationship between the levels and activity of these bacteria and disease risk.

Determining the conditions in which secondary bile acids are formed in significant quantities and retained in the enterohepatic circulation of certain individuals is suggested to be important in the etiology of cholesterol gallstone disease and colon cancer. Since secondary bile acids are formed exclusively through bacterial enzymatic reactions, the study of microbes capable of bile acid 7α/β-dehydroxylation is important in understanding these chronic gastrointestinal illnesses. The goal of such research is to find ways to block the source of secondary bile acid production. Long-term use of antibiotics to
prevent 7α-dehydroxylation of bile acids would be impractical. The design of pharmaceuticals to block the 7α-dehydroxylation pathway is a possibility. However, this approach requires targeting microbial enzymes and runs the risk of eventual drug resistance as with antibiotics. Alternatively, reducing secondary bile acid production may be achieved through administering specialized CA accumulating probiotic bacteria (153). Studies with bifidobacteria and lactobacilli isolated from human feces have shown their ability to assimilate CA spontaneously in vitro (153, 154). The mechanism for CA uptake in lactic acid bacteria appears to be diffusion of a hydrophobic weak acid through the membrane via the transmembrane proton motive force (153, 154). The higher intracellular pH causes the bile acids to become trapped due to ionization.

The use of specific bacteria as “drugs” to treat chronic gastrointestinal illnesses caused in part by other intestinal bacteria certainly has potential, though rigorous studies are needed to demonstrate the effectiveness of such therapies. Overall, clinical trials using probiotics to solve specific health disorders have
been met with mixed results. Discrepancies between studies are due in part to different methodologies, choice of probiotic strains, colony forming units (CFU) administered/day and patient characteristics. One particular variable that would have to be addressed when determining bile acid assimilation by lactic acid bacteria in clinical trials is determining viability in vivo after passage through gastric juice and bile. A bacterium must be alive in order to create a membrane potential capable of accumulating bile acids. Targeted delivery of probiotic bacteria to the intestine in microencapsulated form has shown promise in improving viability in the presence of gastric conditions and bile (155, 156). Another issue is whether the introduction of billions of probiotic bacteria to the small bowel will have an effect on bile acid input into the colon. Significant bile salt hydrolysis proximal to the terminal ileum reduces the efficiency of bile salt uptake through high affinity transport, allowing enhanced excretion of bile acids in feces. This principle is behind attempts at lowering serum cholesterol using probiotics with bile salt hydrolytic activity (157-159). Thus, the choice in
probiotic delivery mechanisms is important in addressing both issues of bacterial viability as well as preventing increased bile salt hydrolysis proximal to the terminal ileum. The potential impact of probiotic bacteria in reducing secondary bile acid formation in the human colon through sequestering bile acids would be greatly reduced if these same bacteria cause an increase in bile acid input into the large intestine greater than their capacity for sequestering bile acids.
STATEMENT OF RESEARCH PROBLEMS AND OBJECTIVES

Clostridium scindens is able to 7α- and 7β-dehydroxylate primary bile acids through an inducible multi-gene regulon. Several of these gene products have been identified, cloned and characterized. However, a number of remaining questions exist that will provide a more complete understanding of bile acid metabolism in the human colon. First, while it has been observed that Clostridium scindens is able to 7β-dehydroxylation ursodeoxycholic acid to lithocholic acid, genes involved in this process have yet to be identified. In addition, no physiological role has been given to the homologous baiCD and baiH gene products located together on the polycistronic oxidative operon of both Clostridium scindens and Clostridium hiranonis. Second, while the gene organization for the oxidative operons in the “high activity” CA 7α-dehydroxylating activity strains Clostridium scindens and Clostridium hiranonis have been
well characterized, the gene organization for the "low activity" CA 7α-dehydroxylating activity strain *Clostridium hylemonae* has yet to be determined. Finally, genes in the "reductive arm" of the bile acid 7α-dehydroxylating pathway have yet to be identified. These genes are predicted to encode flavoproteins which reduce the 3-oxo-Δ4,6-DCA intermediate to 3-oxo-Δ4-DCA and finally to 3-oxo-DCA. We predict the existence of a hydroxysteroid dehydrogenase which will reduce the 3-oxo-DCA intermediate to the secondary bile acid DCA. Finally, we predict an ABC permease which will act to remove DCA from the bacterial cell.

**The objectives of these studies include:**

**Objective 1: Characterization of the baiCD and baiH gene products**

1) Perform bioinformatic and secondary structural predictions of baiCD and baiH gene products with other proteins in the databases to predict the functions of these gene products
2) Subclone the baiCD and baiH genes from Clostridium scindens into E. coli for overexpression of recombinant protein.

3) Synthesize appropriate substrates for enzyme assay and determine reaction product identity by thin layer chromatography and mass spectrometry.

**Objective 2: Isolate and characterize bai genes from Clostridium hylemonae TN271**

1) Isolate a fragment of the bai oxidative operon from Clostridium hylemonae via polymerase chain reaction, clone and sequence this product.

2) Perform bi-directional genome-walking by PCR to obtain the remainder of the genes and cis-elements in this operon.

3) Characterize operon “in silico”

4) Determine mRNA initiation site(s).

**Objective 3: Identify novel bai genes in intestinal clostridia**

1) Locate reductive gene(s) in Clostridium scindens and Clostridium hylemonae
2) Clone, overexpress and purify recombinant protein of one reductive gene in *E. coli*

3) Synthesize bile acid substrate for characterization of reductive gene product

4) Determine mRNA initiation site(s).
MATERIALS AND METHODS

Bacterial strains, culture media, and conditions

*Clostridium scindens* VPI 12708 (formerly *Eubacterium* sp. strain VPI 12708) was routinely cultivated under anaerobic conditions in Brain Heart Infusion (BHI) broth containing sodium cholate (100 μM) and maintained as previously described (111). *Clostridium hylemonae* TN271 was a generous gift of Dr. Fusae Takimine, University Riken, Japan. *C. hylemonae* was cultivated under anaerobic conditions in BHI containing 0.02 % fructose. *Clostridium absonum* (ATCC 27555) was purchased from ATCC. *C. absonum* was initially grown in cooked meat broth for 24 hrs at 37°C and subsequently stored at 4°C and used for no more than 3 weeks as starter culture source. The cells were cultured in BHI supplemented with 0.2 % fructose (w/v). *E. coli* BL21-CodonPlus (DE3)-RIL and ArcticExpression Competent *E. coli* were purchased from Stratagene (La Jolla, CA) and used for expression of recombinant proteins. Recombinant *E. coli* cells were grown with shaking in modified TYGPN (tryptone 20 g, yeast extract 10
g, 0.8 % glycerol, 5 g Na₂HPO₄, 10 g KNO₃, and 3 μM hematin per liter; pH 7.0) containing 100 μg/ml ampicillin.

General PCR and recombinant DNA methods

Total genomic DNA from *C. scindens* VPI 12708 was purified as described previously (169) and used as a template for PCR amplification of the *baiH* gene using primers *baiHF*-PstI (5’-TAGCACTGCAGGCCTATGTT-3’) containing *PstI* restriction site and *baiHR*-SacI (5’-ATCAGAGCTCATTCCTCAG-3’) containing *SacI* restriction site (Figure 12). The expression vector for the *baiCD* gene from *Clostridium scindens* VPI 12708 was constructed in pSPORT1 (Invitrogen, Carlsbad, CA). The *baiCD* gene was cloned using primers *baiCDF*-SalI (5’-CCGTGACGGAGAGTAACAAAATGAGTTACG-3’) containing *SalI* restriction site and *baiCDR*-BglII (5’-CGTAAGCTTCTATGTTACG-3’) containing *BglII* restriction site. The *baiJ* gene was PCR amplified using primers 12708baiJ-F (5’-TGCATCTCCCTCGAGGGCAGATTAGATGTTACG-3’) containing *XhoI* restriction site (bold) and 12708baiJ-R (5’-TACAGGTACCATGACTGTCCCGATGCT-3’) containing *KpnI*
Figure 12. Schematic representation of cloning strategy for baiH gene from Clostridium scindens VPI 12708. Primers baiHF-PstI and baiHR-SacI (see text) with internally designed restriction sites were used to amplify a 2.2 kb stretch encoding the baiH gene. Following restriction digestion of both the insert and vector, and GENECLEAN purification of electrophoresed restriction products, the 2.2 kb baiH insert was ligated with T4 DNA ligase to the 4.1 kb pSport1 vector with PstI and SacI “sticky ends” generating the 6.3 kb vector pSport1-baiH.
Forward primer carrying PstI site

Reverse primer carrying SacI site

PstI / SacI digestion

PstI

SacI

Ligation

T7 Promoter

PstI

Sacl

LacI

 promoter

Ori

Amp

T7 Promoter

PstI

Sacl

LacI

 promoter

Ori

Amp
restriction site (bold). The \textit{baiL} gene was PCR amplified using forward primer \textit{baiLpETF} (5’-\textbf{GTCA}\textit{GAATTC}\textit{CAGTTTGCCATGGAGG}-3’) containing \textit{EcoRI} restriction site (bold) and reverse oligonucleotide \textit{baiLpETR} (5’ \textbf{ATTCTCGAG}\textit{CATATTCAGGAAGCCGGCGTCG}-3’) containing \textit{XhoI} site (bold) for \textit{pET-21a(+)} vector.

The PCR reaction mixture contained 100 ng of \textit{C. scindens} VPI 12708 genomic DNA, 100 pmol each primer, 0.3 mM dNTPs, Pfu polymerase buffer, and 2.5 U Pfu DNA polymerase in a total volume of 50 μl. Cycling parameters included an initial 3 min denaturation at 94°C followed by 30 cycles of amplification 94°C for 35 s, 52°C for 30 s and 68°C for 2 min followed by one additional 7 min elongation at 68°C. Amplification of the \textit{baiH} PCR product was performed on a DNA Thermal cycler (MJ research, Watertown, MA).

The \textit{baiJ} gene and all subsequent PCRs were performed on an Eppendorf Mastercycler Gradient PCR thermocycler (Eppendorf, Westbury, NY, USA). The \textit{baiH} PCR product and \textit{pSport1} (Invitrogen, Carlsbad, CA) vector were double-digested with \textit{PstI} and \textit{SacI}, gel purified (GENECLEAN spin kit) and ligated with T4 DNA ligase (New England Biolabs).
The *baiJ* PCR product and pT7-SBP-2 Expression vector (Sigma, St. Louis, MO) were double-digested with *XhoI* and *KpnI*, gel purified (GENECLEAN spin kit) and ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). Plasmids were transformed into *E. coli* DH5α (Bio-line, Randolph, MA) by electroporation using an Eppendorf 2510 electroporator at 2500V. The recombinant plasmids (pSport1-baiH, pT7SBP2baiJ, p12708baiL) were purified from *E. coli* DH5α and transformed into *E. coli* BL21-CodonPlus (DE3)-RIL or ArcticExpress *E. coli* for overexpression. The sequence was verified at the Virginia Commonwealth University Nucleic Acid Core Facility using standard primers.

**Polyacrylamide gel electrophoresis and immunoblot analysis**

Protein samples were separated and analyzed on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) (170). Protein samples were prepared for SDS-PAGE by heating for 5 min at 100°C in an equal volume of sample buffer (0.1 M Tris-HCl, 5 % SDS, 0.9 % 2-mercaptoethanol (2ME), 20 % glycerol, pH 6.8). Gels were stained with 0.2 % (w/v)
Coomassie brilliant blue R-250 (Sigma, St. Louis, MO) in ethanol: acetic acid: water (30:10:60, v/v/v). A broad-range protein weight marker (Bio-Rad, Hercules, CA) was used for protein size determinations. For western blot analysis, proteins were similarly separated by SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane by electroblotting using a Bio-Rad Immuno-Blot assay kit.

Antiserum against purified baiH gene product was raised in rabbits as described previously (121). Detection of the baiH gene product on nitrocellulose membranes was performed using a goat anti-rabbit alkaline phosphatase conjugate. Detection of the baiJ gene product was performed using anti-Strep-Tactin Horse radish peroxidase (HRP) conjugate (IBA, Gottingen, Germany). Detection of the baiL gene product was performed using Novagen HRP Anti-his tag monoclonal antibody (Novagen, Madison, WI, USA).

**Enzyme Assays**

NADH:FOR activity was determined at 25°C by monitoring the oxidation of NADH at 340 nm ($\epsilon_{340} = 6.22$ mM$^{-1}$
1 cm⁻¹) using a Beckman DU-640 spectrophotometer (Fullerton, CA). The standard assay mixture (1 ml) contained 100 mM sodium phosphate buffer (pH 6.8), 150 μM NADH, 150 μM FAD and an appropriate amount of enzyme added to initiate the reaction. Assays were performed under aerobic conditions in a 1 ml quartz cuvette with a 1 cm pathlength. One unit of NADH:FOR activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH min⁻¹mg⁻¹ of protein. Protein concentrations were determined according to Bradford (171) using the Bio-Rad protein assay kit, with BSA as standard for routine assays.

BaiH and BaiCD oxidoreductase activity assays were performed in reaction mixtures containing the following: 50 mM sodium phosphate buffer (pH 6.8), 1.5 mM NAD⁺, 225 μM [24⁻¹⁻¹⁴C]-3-dehydro-UDCA (3-oxo-7β-hydroxy) or [24⁻¹⁻¹⁴C]-3-dehydro-CDCA (3-oxo-7α-hydroxy) (specific activity of 0.01 μCi μmole⁻¹ substrate) and purified recombinant BaiH protein (100 μg) or 1 mg cell extract containing overexpressed BaiCD.

BaiJ oxidoreductase activity assay was performed in the following mixture: 100 mM sodium phosphate buffer (pH 6.8), 1.5 mM NAD⁺ (or NADP⁺), 90 μM [24⁻¹⁻¹⁴C] 3-dehydro-
DCA(~SCoA) (specific activity 550 DPM/nmole). The reaction mixture (500 μl) was incubated 37 °C for 30 min. BaiJ reaction was initially brought to pH 10 with 5N NaOH and boiled 30 min to hydrolyze CoA ester (125). The pH was then lowered to ~3 with 5 N HCl and bile acid metabolites were extracted three times with 1 vol ethyl acetate, dried under a stream of nitrogen gas, dissolved in 15 μl MeOH, spotted, separated by thin layer chromatography on Baker flex silica gel IB-2 plates (J.T Baker Chemical Co, Philipsburg, NJ) using a solvent system S1 (benzene:dioxane:glacial acetic acid 75:20:2 (v/v/v)) and radiolabeled bile acid metabolites were visualized by autoradiography (Kodak MS film, Rochester, N.Y) (172). Extracts prepared from E. coli containing pSport plasmids without baiH or baiCD gene inserts served as negative controls. Sections of TLC silica gel containing bile acid reaction products were scraped and radioactivity quantified by liquid scintillation spectrometry (Packard, Meriden, CT).
Expression and purification of recombinant baiH gene product

*E. coli* BL21-CodonPlus(DE3)-RIL harboring pSport1-baiH were grown in 5 ml of modified TYGPN medium containing 100 μg of ampicillin per ml at 37°C for 8 hrs. The culture was stored at 4°C overnight. The next day, cells were collected by centrifugation (3,000 × g, 10 min) and suspended in 5 ml of TYGPN medium containing 100 μg/ml ampicillin. This cell suspension was used to inoculate 2 liter flasks containing 600 ml TYGPN broth medium containing 100 μg/ml ampicillin. The cells were cultivated at 37°C with shaking at 250 rpm for about 3 hr to reach an A$_{600}$ of about 0.4 at which point the cultures were induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM final concentration. Cultures were grown at 25°C for an additional 4 hr at which time the cells were harvested by centrifugation at 5,500 × g, 15 min at 4°C. The cells were suspended in 400 ml of buffer A (50 mM sodium phosphate buffer; pH 6.8, 2% (w/v) glycerol, 15 mM of 2-mercaptoethanol (2ME)) cells were lysed by sonicating twice for 10 min intervals and cell debris removed by
centrifugation at 21,000 × g for 30 min. The supernatant fluid containing soluble recombinant BaiH was removed for further purification.

All purification methods were followed essentially as described previously with some modifications (129). Frozen cells were suspended in buffer A, disrupted by sonication on ice, and centrifuged at 21,000 × g for 30 min. Protein was concentrated and protein under 50 kDa removed using Centriprep YM50 (50 kDa cut-off) concentrators (Millipore, Billerica, MA). The protein solution was then applied to a 2 x 10 cm Protein–Pak DEAE 40HR column (Waters, Milford, MA) equilibrated with buffer A. After the column was washed with 60 ml of buffer A, NADH:FOR was eluted with a 500 ml linear gradient of NaCl (0 to 1.0 M) in buffer A at a flow rate of 2 ml min⁻¹. Fractions with a yellow color and NADH:FOR activity were pooled and dialyzed overnight against 2 L of the same buffer. The dialyzed solution was loaded onto a 1.0 × 5.5 cm Cibacron blue 3GA agarose column (Sigma, St. Louis, MO) equilibrated with buffer A. The column was then washed with 100 ml of buffer A, and protein partially eluted with 20 ml of buffer A containing 1 mM NADH, 1 mM FAD, and 20 mM dithiothreitol (DTT). The
remaining protein was eluted with buffer A containing 1M KCl. Active protein fractions eluted with NAD and FAD were pooled and loaded onto a 1.0 × 6.4 cm Bio-Scale CHT5-1 hydroxyapatite column (Bio-Rad, Hercules, CA) equilibrated with buffer C (5 mM potassium phosphate buffer pH 7.0, 2% glycerol, 15 mM 2-ME). After the column was washed with buffer C at 1ml min\(^{-1}\), active fractions were eluted with a linear gradient of 0 to 0.5 M potassium phosphate (pH 7.0). The purified protein from this step was dialyzed with buffer A, concentrated by a Centriprep YM50 concentrator and stored in buffer A containing 50% glycerol (w/v) at -20 °C. Protein remained active for 4 weeks under these conditions.

**Expression and purification of the baiJ and baiL gene product**

*E. coli* Arctic ExpressBL21-CodonPlus(DE3)-RIL harboring pT7SBP-baiJ (Figure 13) was grown in 5 ml of LB medium (tryptone 10 g; yeast extract 5g; NaCl 10g) with 100 μg of ampicillin per ml at 37°C for 8 hrs. The culture was transferred to 2 Liter flasks containing 500 ml
Cloning strategy for baiJ gene from C. scindens VPI 12708. The baiJ gene was PCR amplified using primers 12708baiJ-F (5’-TGCATCTCCCTCGAGGGCACATTATGTCCAGGC-3’) containing XhoI restriction site (bold) and 12708baiJ-R (5’-TAGGTACCCAGCATGTCTCCGATGCT-3’) containing KpnI restriction site (bold). Double digestion of PCR product and vector pT7SBP-2 with XhoI and KpnI, gel purification and ligation resulted in vector pT7SBP2baiJ.
The diagram illustrates the structure of a bacterial plasmid. The plasmid contains several restriction sites and genetic elements.

**Restriction Sites:**
- Xhol
- KpnI

**Genetic Elements:**
- **baiJ**
- **pT7SBP2baiJ** (6.6 kb)
- **T1T2**
- **lacI**
- **amp**
- **pBR322 origin**
- **F1 origin**
Modified TYGPN broth medium containing 100 μg/ml ampicillin. The cells were cultivated at 18°C with shaking at 250 rpm for about 18 hr to reach an A₆₀₀ of about 0.4 at which point the cultures were induced by addition of IPTG to 0.8 mM final concentration. Cultures were grown for an additional 12 hr at which time the cells were harvested by centrifugation at 5,500 × g, 15 min at 4°C. The cells were suspended in 40 ml of Strep buffer (50 mM sodium phosphate buffer; pH 7.9, 15 mM of 2-mercaptoethanol (2ME) and 1M NaCl) and the cells were lysed by sonicating twice for 15 min intervals. Cell debris was removed by centrifugation at 21,000 × g for 30 min. The supernatant fluid containing soluble recombinant BaiJ was removed for further purification.

All purification methods using IBA Strep-tag purification were followed according to the manufacturer (IBA, Gottingen, Germany). Eluents were concentrated by Centriprep YM10 (10 kDa cut-off) concentrators (Millipore, Billerica, MA).

The baiL gene was expressed in E. coli BL21 CodonPlus(DE3)-RIL in a similar manor to the baiJ gene, except at 25°C and 3 hour growth following induction with
IPTG. Protein concentration was determined by Bradford protein assay.

**Enzymatic and biological preparation of bile acid substrates**

[24-\textsuperscript{14}C]-CDCA was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Mo), and determined to be 99.9% pure by TLC. *C. absonum* expresses both 7\textalpha-HSDH and 7\textbeta-HSDH and can be used to efficiently epimerize CDCA to UDCA (76, 77). [24-\textsuperscript{14}C]-UDCA was biologically synthesized from [24-\textsuperscript{14}C]-CDCA using *Clostridium absonum* ATCC 27555 grown in BHI. *C. absonum* was induced at O.D\textsubscript{600nm} 0.4 by addition of 200 \textmu M sodium chenodeoxycholate (CDCA) plus 2 \textmu Ci of [24-\textsuperscript{14}C]-CDCA for production of [24-\textsuperscript{14}C]-UDCA (0.01 \textmu Ci \textmu mole\textsuperscript{-1}). After 24 hr growth following induction, the pH of the culture medium was lowered to \textsim pH 3 and bile acid metabolites were extracted twice with ethyl acetate, dried under a stream of nitrogen gas and dissolved in 100 \textmu l of absolute methanol (MeOH). [24-\textsuperscript{14}C] CDCA, [24-\textsuperscript{14}C] 7-oxo-LCA and [24-\textsuperscript{14}C] UDCA were separated by TLC as described above. Non-radiolabeled CDCA and UDCA standards
(Sigma, St. Louis, Mo, USA) were also chromatographed along with the radiolabeled reaction products and this portion of the plate stained with phosphomolybdic acid for detection. Radiolabeled bands co-migrating with UDCA standard (Sigma) were scraped and extracted with ethyl acetate, dried, and suspended in MeOH.

[24-\textsuperscript{14}C] UDCA was converted to [24-\textsuperscript{14}C] 3-dehydro-UDCA (3-oxo-\(7\beta\)-hydroxy) enzymatically using commercially available 3\(\alpha\)-HSDH (Sigma) in the following reaction mixture: 0.5 ml total volume 50 mM sodium phosphate buffer (pH 6.8), 1 U 3\(\alpha\)-hydroxysteroid dehydrogenase, 1.5 mM NAD\textsuperscript{+}, 100 \(\mu\)M unlabeled UDCA and 1 \(\mu\)Ci [24-\textsuperscript{14}C]-UDCA. The reaction mixture was allowed to incubate for 4 h at 37 \(^\circ\)C. Bile acids were separated by TLC as described above and radiolabeled bile acid reaction products were detected by autoradiography.

[24-\textsuperscript{14}C] 3-oxo-DCA was produced from commercially available [24-\textsuperscript{14}C] CA and non-labeled CA (Sigma). 1 \(\mu\)Ci [24-\textsuperscript{14}C] CA and 5\(\mu\)l 100 mM CA (500 nmoles) was added to 5 ml BHI and inoculated with \textit{C. scindens} VPI 12708 and grown 48 hrs at 37\(^\circ\)C. The reaction products were extracted in the usual manner. The concentrated reaction products were
separated by TLC in solvent system S1 (172). The band corresponding to DCA standard was scraped and [24-$^{14}$C] DCA was extracted with ethyl acetate and concentrated under nitrogen gas. The pellet was resuspended in 50 mM sodium phosphate buffer and reacted with commercially available 3α-HSDH in the presence of 100 μM NAD$^+$ as co-substrate. Substrate and product were separated by TLC and band corresponding to [24-$^{14}$C] 3-oxo-DCA ($R_f$=1.74; Solvent SI) was extracted (118).

**Chemical synthesis of bile acid substrates**

3-oxo-DCA-CoA is hypothesized to be a common substrate for both the baiJ and baiL gene products. Thus, 3-oxo-DCA was synthesized from commercially available DCA essentially as described by Jones et al. (1949) and Danielsson et al. (1962) with some modifications (173, 174). Toluene was substituted for benzene in the reflux step as well as the chromatography. Briefly, 5 grams of deoxycholic acid was methylated overnight in 250 ml
absolute methanol and 3 ml concentrated sulfuric acid. The solvent was evaporated under nitrogen gas and the crude methyl ester was resuspended in chloroform and chromatographed over 100 g silicic acid with a step-wise gradient of acetone in chloroform up to 25%. The DCA-methyl ester was then oxidized in 140 ml toluene and 55 ml acetone with 5.3 g aluminum tert.-butoxide under reflux for 18 hours. After cooling, the reaction mixture was poured into an equal volume 2N sulfuric acid and emulsified. The toluene layer was separated and washed once again with 2N sulfuric acid followed by equal volumes of 7% sodium hydrogen carbonate (twice), and water (once). The toluene layer was then dried under CaCl$_2$ and evaporated. The methyl ester was hydrolyzed overnight in 2 M methanolic potassium hydroxide. The crude acid was added slowly to 0.5 N HCl and filtered under vacuum. The crude acid was dissolved in ethyl acetate, dried with sodium sulfate, concentrated under nitrogen gas and chromatographed on 100 g silicic acid with an increasing gradient of acetone in toluene. Fractions eluted with 15% acetone in toluene yielded semi-pure 3-oxo-DCA as observed on thin layer chromatography. Identity of 3-oxo-DCA was
confirmed by comparison of $R_f$ value after reaction with 3α-HSDH in the presence of 150 μM NADH. Commercially available deoxycholic acid (Sigma) was used as a standard. Material used in enzyme reactions was scraped from TLC plate and purified by ethyl acetate extraction.

The sodium hydrogen carbonate fraction was acidified with dilute (10% v/v) sulfuric acid and allowed to precipitate overnight. The precipitate was vacuum filtered and worked up and purified as described above.

Pure 3-oxo-DCA was esterified to coenzyme A by the method of Killenberg and Dukes (1976) which was a slight modification of Shah and Staple (1968) (175, 176). Tetrahydofuran and methylene chloride were distilled prior to use and γ-collidine was dried over sodium sulfate. 3.5 mg [24-14C] 3-oxo-DCA (550 DPM/nmole) was added to 700 μl dry methylene chloride and incubated ten minutes at room temperature. 250 μl of methylene chloride containing 1.4 μl ethyl chloroformate was added and the solution was magnetically stirred on low for one hour. The solvent was then evaporated under nitrogen gas and the pellet was suspended in 600 μl tetrahydrofuran. The tetrahydrofuran solution was slowly added to 600 μl solution of 62.5 M CoA
in water (pH 8.0). After fifteen minute incubation, the pH was lowered to 5 with 1% perchloric acid and the organic solvent evaporated under nitrogen gas. The aqueous layer containing 3-oxo-deoxycholyl-CoA was washed three times with 2 vol diethyl ether. The aqueous solution was acidified with 170 ml 10% perchloric acid and the 3-oxo-deoxycholyl-CoA was collected by centrifugation at 15,000 x g for 30 minutes. The pellet was stored at -80°C overnight and used the next day.

Purification and mass spectrometry of BaiCD and BaiH reaction products

The analysis and identification of BaiH catalyzed bile acid reaction product was carried out on a Shimadzu HPLC (Kyoto, Japan) with a SIL-HTC Autosampler by reverse-phase HPLC using a Brownlee RP-300 (Perkin Elmer, Wellesley, MA) C8 7m 2.1 i.d. × 100 mm column. The mobile phase consisted of 10 mM ammonium acetate (solvent A) and 10 mM ammonium acetate in acetonitrile (solvent B). The column was equilibrated in solvent A. After injection of a 5 ml sample of the bile acid, solvent B was increased from 0-100% at 10% min⁻¹. The column was regenerated by
switching back to solvent A and re-equilibrating for 5 min. The flow rate was 0.2 ml min$^{-1}$ and the eluted material was passed into the electrospray ionization interface of a MDS Sciex/Applied Biosystems API 4000 Q Trap (Foster city, CA) operating in the negative ion mode. Mass spectra were recorded over a 350-700 m/z range.

**Genome-walking by polymerase chain reaction**

Genome-walking DNA libraries were prepared using the Clontech Universal Genome-Walker Kit according to the manufacturer. DNA was prepared essentially as described previously (177) with a few alterations. To produce high molecular weight DNA (~50 kb), pipetting steps during phenol/phenol:chloroform:iso-amyl alcohol were changed to pouring the aqueous phase, which prevents substantial DNA sheering. An 18 gauge needle was used to pierce the very bottom of 50 ml falcon conical tubes following centrifugation and the organic phase was emptied. DNA was then precipitated by addition of 10% 3N sodium acetate and 3 volumes of absolute ethanol, washed and resuspended in TE buffer, ph 7.6, overnight with agitation at 45 ºC.
DNA quality was determined on a 0.5% agarose gel containing 75 μg/ml ethidium bromide. Prior to electrophoresis, the DNA sample was heated to 65°C. The gel was run 18 hours at 25 V overnight along with a High Molecular Weight DNA Marker (Invitrogen). DNA >48 kb was restriction digested and adapter ligated using GenomeWalker kit. Genomic DNA libraries were stored at -20°C. Oligonucleotides were designed using Primer3 software with T_m values between 60°C and 70°C with ≥ 45% G+C and ≥ 25 mer in length.

PCR reactions were prepared using Advantage cDNA Polymerase Kit or Advantage 2 Polymerase Kit (Clontech) as follows: 40 μl ddH_2O, 5 μl 10x Advantage PCR buffer, 1 μl dNTP mix (10 mM each), 1 μl adaptor primer AP1 (10 μM), 1 μl gene-specific primer (10 μM), 1 μl Advantage Polymerase mix (50X) and 1 μl adaptor-ligated DNA restriction library template. Reaction mixtures were mixed briefly and tubes quick spun. “Touchdown” PCR amplification was performed on Eppendorf Mastercycler Gradient PCR machine using the following parameters: 95°C for 25 s, 72°C for 3 min (7 cycles); 94°C for 25 s, 67°C for 3 min (32 cycles); 67°C for 7 min, hold 4°C. Products (5 μl) were separated by
electrophoresis on a 1% agarose/EtBr gel and visualized on a Fotodyne analyzer. PCR products of interest were GENECLEAN purified from the gel and ligated into a pCR8 GW TOPO TA Cloning vector (Invitrogen) and transformed into TOP 10 Chemically Competant E. coli according to the manufacturer (Invitrogen). Colonies were cultivated in LB broth containing 100 μg/ml spectinomycin. Plasmids were purified using Qiagen Miniprep Spin Kit according to the manufacturer. Sequencing was performed at the Nucleic Acid Research Facility, School of Medicine, Virginia Commonwealth University on ABI 3700 Sequencer using oligonucleotides synthesized by Integrated DNA Technologies (Coralville, IA, USA).

**SMART-RACE Polymerase Chain Reaction**

*C. scindens* VPI 12708 as well as *C. hylemonae* were cultivated in 400 ml BHI containing 0.02% fructose to an O.D.₆₀₀nm 0.3 (*C. scindens*) or 0.2 (*C. hylemonae*) at which point the cultures were induced 3 times at hourly intervals with 50 μM CA. One hour after the third induction, 1 ml culture was transferred to a serum bottle containing [24⁻¹⁴C] CA (0.5 μCi/500 nmoles) and incubated
37°C 30 minutes. A non-induced control was also tested for 7α-dehydroxylation activity. Cells were centrifuged 13,000 x g for 30 min at 4°C. The cell pellet was suspended in 1 ml TE buffer and cells transferred to 1.5 ml microcentrifuge tube and spun 1 min at 13,600 x g. The supernatant was decanted and bacterial cells were stored in RNAlater solution (Ambion, Austin, TX, USA) and stored at -70°C until further processing.

Once 7α-dehydroxylating activity, and thus induction, was confirmed by presence of [24-14C] DCA in [24-14C] activity assay, total RNA was extracted using the Ribopure Bacteria RNA extraction kit (Ambion) essentially according to the manufacturer with the exception that cells were broken on a Mini-bead beater (Biospec Products Inc, Bartlesville, OK, USA) at 3,000 rpm for two cycles of 1 min. Optional DNase treatment was performed for 1 hour at 37°C. Some of the RNA was poly-A tailed prior to cDNA synthesis using the A-Plus Poly(A) Polymerase Tailing Kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer with a 15 minute extension time. cDNA was generated using the SMART-RACE cDNA synthesis kit (Clontech) according to the manufacturer starting with 1
μg total RNA. 5’CDS primer and 3’CDS primer (Clontech) were used to make 5’ RACE ready cDNA and 3’ RACE ready cDNA, respectively, when poly-A RNA was used as the template; whereas gene-specific primers replaced 5’CDS primer and 3’ CDS primer when total bacterial RNA was used as template. Reaction was carried out for 1.5 hrs at 42°C and terminated by addition of 50 μl TE buffer pH 7.6 and heating at 70°C for 7 minutes. SMART RACE PCR of 5’ ends was performed using the Advantage-GC 2 PCR Kit (Clontech) according to the manufacturer’s instruction. 0.5 μl GC-Melt was added to each 50 μl reaction volume along with 2.5 μl cDNA sample. A nested PCR was performed when product concentration was low or to reduce non-specific products. PCR products were excised from agarose gel and purified using the GENECLEAN Spin Kit (MP Biomedicals, Solon, Ohio). The purified PCR product was then cloned into a pCR8 GW TOPO TA cloning vector (Invitrogen), transformed into TOP 10 Chemically Competent E. coli (Invitrogen) and selected on LB agar containing 100 μg/ml spectinomycin. Plasmids containing insert were sequenced.

**Sequence analysis and Assembly**
Sequence ABI files were edited and assembled using Vector NTI software (Invitrogen, Carlsbad, CA, USA), as well as CAP3 Sequence Assembly Program
http://pbil.univ-lyon.fr/cap3.php. Pairwise alignments were performed to determine percent identity/similarity using EMBOSS Needle program
http://www.ebi.ac.uk/emboss/align/. Homology searches were made against the UniProt database. Multiple sequence alignments were prepared using T-COFFEE
http://tcoffee.vital-it.ch/cgi-bin/Tcoffeecgi/index.cgi (178) or CLUSTALW2
http://www.edi.ac.uk/Tools/clustalw2/index.html with some manual editing based on structural data. Sequence conservation within multiple alignments was emphasized using the BOXSHADE utility
http://www.ch.embnet.org/software/BOX_form.html. Protein Mr calculations were performed using the Compute pI/MW tool on the Expasy Proteomics Server
http://www.expasy.ch/tools/pitool.html. Open reading frames (ORF) were detected using the ORF Finder Program

*Clostridium scindens* VPI 12708 *baiH* (AAC45417) and *Clostridium hiranonis* *baiCD* (AAF22846) genes were sequenced previously (59). Secondary structural predictions were carried out with the PSIPRED program (http://bioinf.cs.ucl.ac.uk/psipred/) (180, 181). Secondary structural predictions of the BaiH gene product were compared with the known secondary structure from the crystal structure of 2, 4-dienoyl CoA reductase (*dcr*) from *Escherichia coli* (182).

**Nucleotide sequence accession number**

The *baiC* and *baiD* genes from *C. scindens* were originally reported as separate genes, however, after discovery that the *baiCD* is a single gene product in *C. hiranonis*, the *baiC* and *baiD* gene region was resequenced from *C. scindens* and found to encode a single gene (D.H. Mallonee and P.B. Hylemon, unpublished data). Also, a *baiH* gene has been located downstream of the *bai* operon in *C. hiranonis* and has not been reported previously (J.E.
Wells and P.B. Hylemon, unpublished data). GenBank accession numbers for the baiCD gene from *C. scindens* VPI 12708 is U57489 and the baiH gene from *C. hiranonis* TO931 is AF210152. Genbank accession numbers for the baiBCDEFGHI operon from *C. hylemonae* TN271 is (EU675332). The baiA gene from *C. hylemonae* TN271 can be found using accession number (EU675329). Genbank accession numbers for the baiJKL genes from *C. scindens* VPI 12708 and *C. hylemonae* TN721 are (Eu675330) and (EU675331), respectively.
RESULTS

Characterization of the BaiCD and BaiH

Amino acid sequence alignment and secondary structural prediction of the BaiH.

Protein-protein BLAST searches of the baiH gene product suggests homology with a family of pyridine-nucleotide dependent flavoproteins (Table 4) including NADH oxidase from Thermoanaerobacter brockii (CAA47660) (183), 2-enoate reductase from Clostridium tyrobutyricum (CAA71086) (184) and Moorella thermoacetica (CAA76082) (184), 2, 4-dienoyl-CoA reductase from several bacterial species, and Old yellow enzyme (OYE) from Thermoanaerobacter tengcongensis (NP_623692) (185). E. coli 2, 4-dienoyl CoA reductase (dcr) (AAC76116) is a 73 kDa protein which uses NADPH as an electron donor and contains a 4Fe-4S cluster, 1 mol FMN, and 1 mol FAD per mole subunit (182). The dcr gene product from E. coli shares 32 % identity and 49 % similarity at the amino acid sequence level with the baiH gene product from C. scindens
Table 4. Characteristics of selected BaiH homologues

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Organism</th>
<th>Subunit M (kDa)</th>
<th>Quaternary Structure</th>
<th>Catalyst (redox)</th>
<th>Electron Donor</th>
<th>Electron Acceptor</th>
<th>Percent Identity/Similarity to BaiH</th>
<th>Accession Number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaiH</td>
<td>Clostridium solvans</td>
<td>72</td>
<td>homotrimer</td>
<td>1 FAD, Fe, S, NADH</td>
<td>3-dehydro-UDCA</td>
<td>NAD+</td>
<td>400/100</td>
<td>AAC45417</td>
<td>This study</td>
</tr>
<tr>
<td>BaiCD</td>
<td>Clostridium solvans</td>
<td>70</td>
<td>ND</td>
<td>ND</td>
<td>3-dehydro-UDCA</td>
<td>NAD+</td>
<td>32/40</td>
<td>EF539240</td>
<td>This study</td>
</tr>
<tr>
<td>Enolate reductase</td>
<td>Clostridium tyrobutyricum</td>
<td>73</td>
<td>homodimer</td>
<td>1 FAD, 1 FMN, 1 4Fe-4S</td>
<td>NADH</td>
<td>c, β-unsaturated carboxyl</td>
<td>30/48</td>
<td>CA710818</td>
<td></td>
</tr>
<tr>
<td>2,4-dienyl CoA</td>
<td>Escherichia coli</td>
<td>73</td>
<td>monomer</td>
<td>1 FAD, 1 FMN, 1 4Fe-4S</td>
<td>NADPH</td>
<td>unsaturated fatty acyl-CoA</td>
<td>26/49</td>
<td>1FS9A</td>
<td>21</td>
</tr>
<tr>
<td>Old Yellow Enzyme</td>
<td>Saccharomyces cerevisiae</td>
<td>49.9</td>
<td>homotrimer</td>
<td>1 FMN</td>
<td>NADPH</td>
<td>c, β unsaturated carboxyl</td>
<td>23/42</td>
<td>CO0358</td>
<td>57, 58</td>
</tr>
<tr>
<td>YglM(CYE2)</td>
<td>Eaeillus subtilis</td>
<td>37.4</td>
<td>homotetramer</td>
<td>1 FMN</td>
<td>NADPH</td>
<td>c, β unsaturated carboxyl and threonine</td>
<td>29/49</td>
<td>BAA12619</td>
<td>14, 26</td>
</tr>
<tr>
<td>NoxB1</td>
<td>Archaeoglobus fulgidus</td>
<td>66</td>
<td>monomer</td>
<td>FAD</td>
<td>NADH</td>
<td>unknown</td>
<td>35/55</td>
<td>NP069231</td>
<td></td>
</tr>
</tbody>
</table>

* Data inconclusive (2)
† alignment between full length CYE2 and amino acids 1-67 of BaiH
‡ alignment between full length YgNH and amino acids 1-961 of BaiH
VPI 12708. A 2.4 Å structure has been solved for the E. coli dcr gene product allowing a basis of comparison for secondary structure prediction of the baiH gene product (Figure 14). The predicted secondary structure of the BaiH is very similar to that of the dcr gene product suggesting a similar TIM-barrel fold. It is interesting to note the conserved cysteine consensus pattern of C-(2X)-C-(2-3X)-C-(11-12X)-C between the BaiH, E. coli Dcr and other homologues suggesting that the BaiH and BaiCD contain 4Fe-4S centers.

Several baiCD and baiH homologues introduce carbon-carbon double bonds. This suggests that the baiCD and baiH gene products encode flavoproteins which may introduce carbon-carbon double bonds into the bile acid structure. Previously, we identified a 3-oxo-Δ⁴-cholic acid intermediate in the bile acid 7α-dehydroxylation pathway (117). Because there are two homologous genes and two stereochemistries differing about the 7-hydroxyl between CA, CDCA (7α-hydroxy) and UDCA (7β-hydroxy), we hypothesize that the baiCD and baiH introduce the Δ⁴-bond in bile acids, recognizing one stereochemistry about the 7-hydroxyl or the other.
Amino acid sequence alignment of the BaiH (AAC45417) from *C. scindens* VPI 12708 with protein homologues: 2,4-dienoyl-CoA reductase (dcr) (AAC76116) from *E. coli*, 2-enoate reductase (CAA71086) from *C. tyrobutyricum*, the baiCD gene product from *C. scindens* (EF539210) and *C. hiranonas* (AAF22846) and the baiH gene product from *C. hiranonis* (EF539211) Secondary structural elements based on the crystal structure of DCR (182) are shown (below alignment). Predicted secondary structural elements of the BaiH protein are also shown (above alignment). Arrows correspond to β-sheets and loops to α helices. Residues corresponding to FMN binding (■), FAD binding (*), and cysteine residues involved in a 4Fe-4S center (♦) from the crystal structure of DCR are also shown. The alignment was generated with the T-COFFEE program (178) and sequence similarities/identities highlighted with Boxshade 3.1.
Purification of recombinant BaiH

Initially, the baiH gene was cloned into a pET24a (+) vector with C-terminal hexa-histidine tag to make a BaiH-His recombinant. The BaiH-His showed NADH:FOR activity in crude extract prior to nickel affinity chromatography. However, following nickel affinity chromatography, the purified enzyme lost detectable NADH:FOR activity. Problems with His-tag use has also been reported for the YqjM protein from Bacillus subtilis; a baiH homologue (186). The quarternary structure of recombinant His-tagged YqjM was reported to be altered by the His-tag resulting in expression of an inactive enzyme. This is not the case with the baiH gene product as the enzyme showed NADH:FOR activity in crude extract similar to previously reported in recombinant BaiH lacking affinity tag (129) before His-binding chromatography (data not shown). The BaiH is known to contain 1 mol copper per subunit (129). While the mechanism for this loss of activity is not known, we speculate that the nitriloacetic
acid, which chelates Ni$^{2+}$, Cu$^{2+}$, and Co$^{2+}$, and in turn binds the hexa-histidine tail, may have chelated copper from the enzyme causing inactivation (187, 188). Addition of various concentrations of Cu$^{2+}$ to the pure enzyme did not restore activity. Therefore, we opted to clone the gene into the pSport1 vector without an affinity tag.

SDS-PAGE analysis of the active fractions after CHT5-1 hydroxyapatite chromatography revealed a highly enriched protein with a subunit molecular mass of 72 kDa that was reactive against anti-BaiH antiserum (Figure 15). The specific activity and purification fold were dramatically increased by Cibacron Blue 3GA and hydroxyapatite chromatography (Table 5). The baiH gene product was purified 23-fold over the crude extract. The final enzyme preparation had a NADH:FOR specific activity of 16.4 U mg$^{-1}$. NADH:FOR enzyme activity existed in bright yellow fractions as observed previously (129).

**Stereospecific NAD(H)-dependent 3-oxo-Δ$^4$-cholenoic acid oxidoreductase activity.**

The hypothesis that the baiH gene product encodes an NAD(H)-dependent 3-oxo-Δ$^4$-cholenoic acid oxidoreductase was
Figure 15. SDS-PAGE and immunoblot analysis of baiH gene product (NADH:FOR) overexpression and purification  
(a.) Proteins were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue R-250. 
Lane 1, cell free extract after IPTG induction (20 μg loaded); Lane 2, flow-through from DEAE 40HR (20 μg loaded); 
Lane 3, flow-through from Cibacron Blue 3GA agarose (20 μg loaded); lane 4, flow-through from Hydroxyapatite 
CHT5-1 column (5 μg of purified enzyme loaded). (b.) Immunoblot of SDS-PAGE using anti-BaiH antiserum.
Table 5. Purification scheme of baiH gene protein in *E. coli* BL21(DE3)-RIL(pSportI-baiH)

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Volume (mL)</th>
<th>Total Protein (mg/mL)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free extract</td>
<td>35</td>
<td>410</td>
<td>93.3</td>
<td>0.7</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Centriprep treatment</td>
<td>20</td>
<td>175</td>
<td>80</td>
<td>1.2</td>
<td>1.7</td>
<td>86</td>
</tr>
<tr>
<td>DEAE 40 HR Cellulose</td>
<td>11</td>
<td>92</td>
<td>66</td>
<td>3</td>
<td>4.3</td>
<td>71</td>
</tr>
<tr>
<td>Cibacron blue 3GA agarose</td>
<td>5.2</td>
<td>63</td>
<td>55.5</td>
<td>8</td>
<td>11.4</td>
<td>60</td>
</tr>
<tr>
<td>Hydroxyapatite CHT</td>
<td>2.3</td>
<td>28</td>
<td>35.1</td>
<td>16.4</td>
<td>23.4</td>
<td>38</td>
</tr>
</tbody>
</table>
tested by addition of substrates [24-14C] 3-dehydro-UDCA or [24-14C] 3-dehydro-CDCA with NAD\(^+\) and purified BaiH. A single reaction product of more hydrophilic character was detected by autoradiography following TLC when [24-14C] 3-dehydro-UDCA and NAD\(^+\) were substrates (Figure 16). Previous studies have shown the specificity of this enzyme for NAD(H) (129) and the BaiH did not show 3-oxo-\(\Delta^4\)-cholenoic acid oxidoreductase activity with NADP(H) (data not shown). The bile acid metabolite generated following BaiH catalyzed reaction with NAD\(^+\) was scraped from TLC plates, pooled, further purified by ion exchange chromatography and identified by electrospray mass spectrometry (Figure 17). The deprotonated molecular ion given by the product was 2 Da lighter than that given by the 3-dehydro-CDCA substrate, consistent with a loss of 2 hydrogens from the substrate and potentially indicating a 3-dehydro-\(\Delta^4\)-CDCA structure. Previous observations showed loss of the 5\(\beta\) hydrogen during incubation of [5\(\beta\)-\(^3\)H] [24-14C] CA to CA induced whole cells and cell extracts of C. scindens VPI 12708 (118). Taken together, these observations strongly suggest the formation of a 3-dehydro-\(\Delta^4\)-UDCA structure.
Figure 16. TLC autoradiograph of BaiCD and BaiH catalyzed NAD(H)-dependent 3-oxo-Δ⁴-cholenoic acid oxidoreductase activity assays. The reactions were carried out in a 500 μl volume consisting of 50 mM sodium phosphate buffer (pH 6.8) containing 225 μM of each respective substrate with a specific activity of 0.01 μCi μmole⁻¹. A: [24-¹⁴C] 3-dehydro-CDCA standard, B: [24-¹⁴C] 3-dehydro-UDCA, C: [24-¹⁴C] 3-dehydro-CDCA + 100 μg cell-free extract of E. coli (plasmid, no insert) D: [24-¹⁴C] 3-dehydro-CDCA + 1.6 U purified BaiH, E: [24-¹⁴C] 3-dehydro-CDCA + 100 μg E.coli BL21(DE3) expressing baiCD. F: [24-¹⁴C] 3-dehydro-UDCA + 100 μg E. coli BL21(DE3) (plasmid, no insert), G: [24-¹⁴C] 3-dehydro-UDCA + 100 μg cell-free extract of E.coli BL21(DE3) expressing baiCD, H: [24-¹⁴C] 3-dehydro-UDCA + 1.6 U purified BaiH. All reactions were performed with 1.5 mM NAD+ as co-substrate.
HPLC and mass spectrometry analysis of BaiH catalyzed bile acid reaction products. The bile acid metabolites produced by purified baiH gene protein were separated by TLC, scraped and further purified by reverse-phase HPLC-ESI-MS. The initial mobile phase was 10 mM NH$_4$OAc; after injection bound materials were eluted by a 0-100 % linear gradient of acetonitrile in 10 mM NH$_4$AOc at 10 % min$^{-1}$. The eluate was passed into the electrospray interface operating in the negative ion mode. Ions eluting from m/z 350-700 were recorded and analyzed. The upper trace is a selected ion chromatogram for m/z 389. The mass spectra for the peak at 11.52 min were summed and presented in the bottom trace. The m/z 389.2 ion corresponds to the expected [24-$^{14}$C] 3-dehydro-4-UDCA product.
In addition, the baiCD gene was overexpressed in *E. coli* BL21 (DE3) and tested for 3-oxo-Δ⁴-cholenoic acid oxidoreductase activity. The baiCD gene product was detected by SDS-PAGE at the expected molecular mass of 70 kDa (Figure 18). Addition of baiCD overexpressed *E. coli* cell extract to reaction mixture containing 1.5 mM NAD⁺ resulted in a product of greater hydrophilicity than the starting substrate [24-¹⁴C] 3-dehydro-CDCA (Figure 16). This reaction product was not obtained using cell-extract of *E. coli* BL21(DE3) containing pSport1 vector without baiCD gene insert. BaiCD did not recognize the substrate [24-¹⁴C] 3-dehydro-UDCA. The product derived from reaction with [24-¹⁴C] 3-dehydro-CDCA was scraped from TLC plates, pooled and subjected to mass-spectrometry (Figure 19). The major mass ion was 2 amu less than the 3-dehydro-CDCA substrate and thus consistent with the loss of 2 hydrogens from the substrate bile acid and potentially a 3-dehydro-Δ⁴-CDCA structure. Figure 20 illustrates the reactions catalyzed by the baiCD and baiH gene products.
Figure 18. SDS-PAGE of cell-extract of recombinant *E. coli* BL21(DE3) overexpressing *baiCD* gene. (M) Marker (UI) uninduced, (I) induced 1 mM IPTG. 30 μg protein loaded and gel stained with Coomassie brilliant blue R-250. A large band at ~ 70 kDa was detected in IPTG induced cell-extracts indicating overexpression of *baiCD* gene.
Figure 19. HPLC and mass spectrometry analysis of BaiCD catalyzed bile acid reaction products. The bile acid metabolites produced by cell-extract of *E. coli* overexpressing *baiCD* gene were separated by TLC, scraped and further purified by reverse-phase HPLC-ESI-MS. The initial mobile phase was 10 mM NH$_4$OAc; after injection bound materials were eluted by a 0-100 % linear gradient of acetonitrile in 10 mM NH$_4$AOc at 10 % min$^{-1}$. The eluate was passed into the electrospray interface operating in the negative ion mode. Ions eluting from m/z 350-700 were recorded and analyzed. The upper trace is a selected ion chromatogram for m/z 389. The mass spectra for the peak at 11.59 min were summed and presented in the bottom trace. The m/z 389.3 ion corresponds to the expected [24-$^{14}$C] 3-dehydro-4-CDDA product.
Figure 20. Proposed reactions catalyzed by the *baiCD* and *baiH* gene product. R group may be the carboxylic acid or the coenzyme A conjugate.
Isolation of bai genes from Clostridium hylemonae

TN271

Identification and Characterization of the baiBCDEFHGI operon

Apart from basic biochemical characteristics and 16s rDNA sequence, little is known about C. hylemonae. Previously, our laboratory performed activity assays on cultured 7α-dehydroxylating bacteria and found that these organisms clustered into two groups based on relative rate of conversion of [24-14C] CA to [24-14C] DCA (189). Much effort has been focused on the “high activity strains” C. scindens and C. hiranonas, however, organisms such as C. hylemonae, described as a “low activity strain” has received little characterization (See Intro). Recent data indicates that C. hylemonae may play a significant role in bile acid metabolism in the human colon based on in vivo studies where this species was found to be a numerically predominant 7α-dehydroxylating bacterium (190). Therefore, characterization of the bile acid 7-dehydroxylating pathway in this organism is important. Genes involved in the bile acid 7α-dehydroxylating pathway
have yet to be isolated and sequenced from any “low activity strain”. We were thus interested in elucidating the gene organization, gene and promoter sequence, as well as upstream regulatory genes and comparing these elements to “high activity strains” to see whether there are obvious differences that might underly the ten fold differences in relative rate of bile acid 7α-dehydroxylation (189).

Wells and Hylemon (2000) designed redundant PCR primers targeting the baiCD gene which are aimed at detecting 7α-dehydroxylating bacteria in the human colon (59). Wells and Hylemon (2000) reported detection of the baiCD gene of C. hylemonae TN271 using these oligonucleotides. Therefore, these primers were used to amplify a 1.2 kB segment of the baiCD gene from C. hylemonae which was subsequently cloned into a pCR8 GW TOPO TA vector and sequenced (Figure 21). BLAST analysis of the sequence data and CLUSTALW alignment of the nucleotide and translated amino acid sequence verified the identity of a baiCD gene in C. hylemonae. We hypothesized that the bai genes involved in the oxidative arm of the bile acid 7α-dehydroxylation pathway (baiA – baiI) would
Figure 21. Region of partial baiCD gene from C. hylemonae TN271 used to design upstream and downstream genome-walking PCR primers. Sequence represents sense-strand of baiCD gene, arrows denote direction of amplification and grey font represents regions of primer design.
be located on a polycistronic operon flanking the baiCD gene. Therefore, a bidirectional genome-walking by PCR strategy was used to isolate the remaining bai genes.

A slight modification of the method of Ridlon et al. (2005) was utilized to prepare genomic DNA (>50 kb) from both C. scindens VPI 12708 and C. hylemonae TN271 for genome-walking by PCR (see Materials and Methods) (Figure 22) (177). Restriction libraries derived from this DNA sample yielded PCR products averaging 2 kb with a range of 0.7 kb to 4 kb. The limit of this technique, according to the manufacturer (Clontech GenomeWalker Universal Kit) is around 6 kb. Upstream genome-walking PCR using primer WALKUP1 and subsequent nested amplification using primer WALKUP1Nest resulted in 2,139 bp of sequence data which overlapped the baiCD gene data by 673 bp resulting in a 2,690 bp contig (Figure 23). Analysis of this data revealed the 5’ end of the baiCD gene. A second open reading frame (ORF) was detected 19 bp upstream of the baiCD gene. BLAST results suggest this ORF encodes an AMP-dependent coenzyme A ligase with highest identity to the baiB gene from C. hiranonis. Continued upstream genome-walking resulted in a PCR product of 1,964 bp
Figure 22. Preparation of genomic DNA for genome-walking by PCR restriction libraries. Representative 0.4% agarose/EtBr gel showing 1, 2, and 3 μg genomic DNA from *C. scindens* VPI 12708. Size comparisons made with Invitrogen’s High Molecular Weight DNA ladder. Electrophoresis was performed at 25 volts for 18 hours.
Figure 23. Genome-walking by PCR Upstream of partial baiCD gene in Clostridium hylemonae TN271. PCR products amplified using WALKUP1 and WALKUP1NEST PCR primers (Table 6). “M” is marker “D”, “E”, “P”, “S” and “EN” represent genome-walking restriction library templates digested with DraI, EcoRV, PvuII, and StuI, respectively. “EN” represents nested PCR amplification of EcoRV primary PCR product diluted 1:50. The PCR product generated from nested amplification was purified by GENE CLEAN and ligated into a pCR8 GW TA TOPO cloning vector (2.8 kb) to yield vector p271CDUP1 (4.9 kb).
a. 

b. 

pUC ori 

TA cloning site 

attL1 

attL2 

Spec' 

p271ENC Dup1 

4.9 kb 

2.1 kb 

EN CDUP1
(Figure 24). Assembly of this data resulted in a 4,586 bp contig containing the complete baiB gene. The 1.5_kb baiB gene is predicted to encode a 57.7 kDa polypeptide similar in size to the reported 58 kDa subunit recombinant BaiB polypeptide from _C. scindens_ VPI 12708 (125). Two additional interesting features were identified within this sequence data. First, a conserved bile acid inducible promoter was located on the sense strand 49 bp upstream of the baiB gene (Figure 25) (59, 191). SMART-RACE PCR analysis of the cDNA from a CA induced culture of _C. hylemonae_ led to the identification of the mRNA start site which appears in a short purine rich stretch corresponding to the ribosome binding site (Figure 26). Interestingly, the distance between the mRNA start site in the baiB polycistronic operon in _C. hylemonae_ is only 11 bp from the initiation codon while in the “high activity strains” there is a distance of 108 bp in _C. hiranonis_ (59) and 68 bp in _C. scindens_ (191). As proof of principle, SMART RACE PCR was also performed on the baiB gene of _C. scindens_ VPI 12708, whose mRNA initiation site was previously determined by primer extension (191). The results obtained in the present study agree with those of
Second upstream genome-walking by PCR from partial \textit{baiCD} gene in \textit{Clostridium hylemonae} TN271. a.) PCR products amplified using WALKUP2 and WALKUP2NEST PCR primers (Table 6). Nested PCR product (DN=DraI template, nested primer amplification product) purified from 1% agarose gel by GENE CLEAN Spin Kit and ligated into pCR8 GW TOPO TA cloning vector to make vector p271CDUP2 (b.).
a. 

b. 

M  DN
3.0 kb
2.0 kb
1.0 kb

pUC ori
T2
attL1
TA cloning site

1.9 kb

p271DNCDUP2
4.7 kb

Spec"
Figure 25. Boxshade of CLUSTALW alignment of *bai* promoters upstream of *baiB* gene between *C. hylemonae* TN271, *C. scindens* VPI 12708 and *C. hiranonis* sp. strain TO931. Putative -10 element is indicated below alignment. Transcription initiation sites (TIS) highlighted in yellow (See Fig 26 for TIS data for *C. hylemonae*).
Figure 26. SMART RACE PCR analysis to determine TIS for the \( baiB \) transcript in \textit{Clostridium hylemonae} TN271. (a.) RT-PCR analysis of \textit{C. hylemonae} TN271 cDNA generated from CA induced (I) and uninduced (UI) cultures. Primers against \( baiB \) gene (above) and \( gyrA \) gene (below) were used to determine whether \( baiB \) transcript was expressed. Below RT-PCR is the corresponding [24-\(^{14}\)C] CA activity assay performed prior to RNA isolation of I and UI cultures of \textit{C. hylemonae}. [24-\(^{14}\)C] DCA is detected only in CA induced (50 \( \mu \)M) culture. (b.) 5' SMART RACE PCR of \( baiB \) transcript using RACEB325 oligonucleotide against cDNA from induced culture of \textit{C. hylemonae} TN271. PCR product was purified by GENECLEAN Spin Kit and cloned into a pCR8 GW TOPO TA cloning vector and sequenced. (c.) TIS for \( baiB \) transcript (underlined; direction of transcription designated by arrow). "*" designates conserved \( bai \) promoter elements (See Figure 25), putative ribosome binding site in bold, "†" designates mRNA start site determined for \( baiB \) in \textit{C. scindens}, "‡" designates mRNA start site for \( baiB \) in \textit{C. hiranonis} sp. strain TO931, italics indicates anticodons with corresponding amino acids (below).
a. UI  I
   
   DCA
   CA

b. M  B

C.

ATATTATCCATGCTAGAGTTGTAAAGTTATATCAGGGCGAAATATGGACTTG
  **  *  **        ** ******    * *****
             M  D  L
Mallonee et al. (1990) providing proof of principle and additional evidence for the TIS of the \textit{baiB} operon in \textit{C. scindens} VPI 12708 (Figure 27) (191).

The second notable feature is a conserved 47 kDa AraC/XylS transcription factor located on the anti-sense strand upstream of the \textit{bai} promoter. Figure 28 illustrates the conserved helix-turn-helix motifs identified in the amino acid sequence of the “bile acid regulatory A” gene (\textit{barA}). Additional genome-walking upstream of the \textit{barA} did not result in ORFs predicted to be involved in bile acid metabolism.

Downstream genome-walking from the \textit{baiCD} fragment yielded a PCR product of 1,131 bp which increased contig length to 5,625 bp (Figure 29). This stretch of sequence resulted in completion of the 1.9 kb \textit{baiCD} gene predicted to encode a 70 kDa polypeptide. In the first section, overexpression of the recombinant \textit{baiCD} gene from \textit{C. scindens} VPI 12708 in \textit{E. coli} resulted in a 70 kDa polypeptide as estimated by SDS-PAGE (Figure 18). In addition, 46 bp downstream of the \textit{baiCD} gene we located a 510 bp ORF predicted to encode the 19.8 kDa bile acid 7α-dehydratase (\textit{baiE} gene). Dawson et al (1996) reported
Determination of transcription initiation site for *baiB* gene from *Clostridium scindens* VPI 12708 based on SMART RACE PCR analysis. Alignment shows determination of transcription initiation site (TIS) (arrow, underline) based on alignment of SMART RACE sequence data (SR7, SR8) with *baiB* gene and upstream region from *Clostridium scindens* VPI 12708 (12708baiB). TIS is determined based on the first base following 3' end of SMART II A oligo sequence (boxed region) (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'). This nucleotide (underlined, arrow) must also correspond to the known sequence of the upstream region of the gene. The TIS determined through SMART RACE analysis agreed with primer extension analysis performed by Mallonee et al (1990) (191).
Figure 28: ClustalW alignment of bile acid regulatory A gene (barA) between *C. hylemonae* TN271, *C. scindens* VPI 12708 and *C. hiranonis* sp. strain TO931. Regions highlighted in red are predicted helix-turn-helix motifs shared between members of the Arac/XylS family of transcription factors.
Figure 29. Downstream genome-walk (DS1) from baiCD gene from Clostridium hylemonae TN271. a.) Agarose gel of DS1 products using DraI (D), EcoRV (E), PvuII (P), StuI (S) restriction libraries of C. hylemonae TN271 genomic DNA as template with adaptor primer AP1 and gene-specific primer WALKDS-1 (See Table 6). b.) “DN” and “EN” represent nested PCR amplification using nested adaptor primer AP2 and gene-specific primer WALKDSNEST1 (See Table 6). C.) Schematic representation of TA cloning of GENECLEAN purified “DN” product (arrow) into pCR8/GW/TOPO TA vector (2.8 kb) yielding 3.9 kb vector p271DNCDDS1.
a. M D E P S M DN EN

p271DNCDDS1 3.9 kb

b. p271DNCDDS1 3.9 kb

1.1 kb

Spec"
overexpression of the 19 kDa baiE gene from C. scindens VPI 12708 (128). Multiple sequence alignment of this ORF with the baiE genes from C. scindens VPI 12708 and C. hiranonis sp. strain TO931 revealed >80% amino acid sequence identity and conservation of active site amino acids predicted through computer modeling and site-directed mutagenesis data (Figure 30) (Hylemon et al unpublished data). Continued downstream GW-PCR resulted in a 3,856 bp PCR product which when added to the previous alignment results in a contig of 9,291 bp (Figure 31). An ORF was located 25 bp downstream of the baiE gene 1,275 bp in length predicted to encode a 47 kDa polypeptide in the Type III CoA transferase family of proteins. BLAST analysis suggests this gene encodes the baiF gene product. Ye et al (1999) previously determined the M_r of the recombinant baiF from C. scindens VPI 12708 to be 47.5 kDa (130). A second ORF, 1.4 kb in length, was located 53 bp downstream of the baiF gene whose deduced amino acid sequence suggests this gene encodes a 49.5 kDa AraJ-like efflux permease. BLAST analysis suggests this ORF encodes the baiG gene product. Mallonee et al (1996) overexpressed the 50 kDa baiG gene product in E. coli and showed that it
Figure 30. Boxshade of CLUSTALW alignment of 7α-dehydratase gene (baiE) between C. hylemonae TN271, C. scindens VPI 12708, and C. hiranonis sp. strain TO931. Asterisks denote conserved active site amino acids predicted by computer modeling and site-directed mutagenesis (Hylemon et al. unpublished data).
12708baiE  MTLEERVEALNEEGGKLDEALNE/DAKLYRFR/bCLDGRWRDELETTLSPNIbTVSbNSGKLV  61
T0S31baiE  MTLEERVEALNEEGGKLDEALNE/DAKLYRFR/bCLDGRWRDELETTLSPNIbTVSbNSGKLV  61
TN271baiE  MTLEERVEALNEEGGKLDEALNE/DAKLYRFR/bCLDGRWRDELETTLSPNIbTVSbNSGKLV  61

12708baiE  FHSPKbEVE/DYbLbKb3bSHFbK/EfISbMBHGPETbIDSETTATbGRbYbLEDALbIPTDC  KYbCVC  119
T0S31baiE  FHSPKbEVE/DYbLbKb3bSHFbK/EfISbMBHGPETbIDSETTATbGRbYbLEDALbIPTDC  KYbCVC  119
TN271baiE  FHSPKbEVE/DYbLbKb3bSHFbK/EfISbMBHGPETbIDSETTATbGRbYbLEDALbIPTDC  KYbCVC  120

12708baiE  INNGGTYbDKbYEKIDGQy*/YLTGYbRiYEEHFMbRbDPKIHbITMNbKH--  166
T0S31baiE  INNGGTYbDKbYEKIDGQy*/YLTGYbRiYEEHFMbRbDPKIHbITMNbKHOP  168
TN271baiE  INNGGTYbDKbYEKIDGQy*/YLTGYbRiYEEHFMbRbDPKIHbITMNbKHOP  169
Second downstream genome-walk (DS2) from \textit{baiCD} gene from \textit{Clostridium hylemonae} TN271. 

a.) Agarose gel of DS2 products using \textit{DraI} (D), \textit{EcoRV} (E), \textit{PvuII} (P), \textit{StuI} (S) restriction libraries of \textit{C. hylemonae} TN271 genomic DNA as template with adaptor primer AP1 and gene-specific primer WALKDS-2 (See Table 6). b.) "EN" and "PN" represent nested PCR amplification using nested adaptor primer AP2 and gene-specific primer WALKDSNEST2 (See Table 6). C.) Schematic representation of TA cloning of GENECLEAN purified "PN" product (arrow) into pCR8/GW/TOPO TA vector (2.8 kb) yielding 6.6 kb vector p271PNCDDS2.
a. M D E P S
   3.0 kb
   2.0 kb
   1.0 kb

b. M EN PN
   3.0 kb
   2.0 kb
   1.0 kb

C.

- p271PNCDDS2
  - 3.8 kb
  - 6.6 kb
  - pUC ori
  - Spec'
  - attL1
  - attL2
  - TA cloning site

+ PN CDGS2

<table>
<thead>
<tr>
<th>3.0 kB</th>
<th>2.0 kB</th>
<th>1.0 kB</th>
<th>3.0 kB</th>
<th>2.0 kB</th>
<th>1.0 kB</th>
<th>3.0 kB</th>
<th>2.0 kB</th>
<th>1.0 kB</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. b.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.52</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
is involved in transporting primary bile acids across the bacterial membrane (126). Further analysis revealed a partial ORF 19 bp downstream of the \textit{baiG} gene encoding an “Old Yellow Enzyme” like FMN oxidoreductase with the \textit{baiH} gene from \textit{C. hiranonis} sharing the highest amino acid identity. The third downstream GW-PCR resulted in completion of the \textit{bai} oxidative operon (Figure 32). The 2,019 bp \textit{baiH} gene was completed and the deduced amino acid sequence suggested this gene encodes a 71.7 kDa polypeptide. The \textit{baiH} gene from \textit{C. scindens} VPI 12708 has been shown to encode a 72 kDa polypeptide (Figure 5)(121, 129). Finally, the 549 bp \textit{baiI} gene was located 38 bp downstream of the \textit{baiH} gene and predicted to encode a 21.8 kDa polypeptide. The complete \textit{bai} oxidative operon from \textit{C. hylemonae} is represented schematically in Figure 33 and compared to the gene organization in \textit{C. scindens} and \textit{C. hiranonis}. Table 6 lists oligonucleotides used in isolation and sequencing of the \textit{baiBCDEFGHI} operon in \textit{C. hylemonae}. Table 7 compares amino acid sequence identity/similarity between \textit{C. hylemonae} and \textit{C. scindens}. Clearly, there is a high degree of conservation both between the genes and several of the intergenic regions;
Figure 32. Third downstream genome-walk (DS3) from the baiCD gene of Clostridium hylemonae TN271. 
a.) Agarose gel of DS3 products using Dral (D), EcoRV (E), PvuII (P), StuI (S) 
restriction libraries of C. hylemonae TN271 genomic DNA as template with adaptor primer 
AP1 and gene-specific primer WALKDS-2 (See Table 6). b.) “DN” represents nested PCR 
amplification using nested adaptor primer 
AP2 and gene-specific primer WALKDSNEST2 (See Table 6). C.) Schematic 
representation of TA cloning of GENE CLEAN 
purified “DN” product (arrow) into 
pCR8/GW/TOPO TA vector (2.8 kb) yielding 5.9 
kb vector p271DNCDDS3.
Figure 33. Schematic representation and comparison of the bai oxidative operons of C. hylemonae TN721 and C. scindens VPI 12708. See Table 7 for comparison of amino acid sequence identity and other parameters. Notice the high degree of conservation in gene organization, with the exception of the baiA gene, present in both C. scindens and C. hiranonis though conspicuously missing from the bai oxidative operon of C. hylemonae TN271.
C. hylemonae TN271

AraC/XylS → baiB (CoA ligase) → baiCD (3-dehydro-4-7α oxidoreductase) → baiE → baiF → baiG → baiH (3-dehydro-4-7β oxidoreductase) → baiA2 → baiF → baiG → baiH → baiI

C. scindens VPI 12708

barA → baiB → baiCD → baiE → baiA2 → baiF → baiG → baiH → baiI

C. hylemonae TN271

1, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 11,000, 12,000
Table 6. Oligonucleotides Used in Isolating *baiBCDEFGHI* Operon from *Clostridium hylemonae* TN271

<table>
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<th>Primer name</th>
<th>Sequence (5'→3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td><strong>Redundant oligonucleotides</strong></td>
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</tr>
<tr>
<td>baiC-D-F</td>
<td>5'GTTATAGCCCTTTAATTGCCCTTGG</td>
<td>GenBank</td>
</tr>
<tr>
<td>baiC-D-R</td>
<td>5'AAATCTCCTGAGTTACATGATTTTGG</td>
<td>GenBank</td>
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<tr>
<td><strong>Genome-walking oligonucleotides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>API</td>
<td>CTGATCACGATCATCTAGACG</td>
<td>Clontech</td>
</tr>
<tr>
<td>A1</td>
<td>ATTTAGGGCTACACTAC</td>
<td>Clontech</td>
</tr>
<tr>
<td>08LX10257-1</td>
<td>GACATCGTCTCTCCATGCAAG</td>
<td>This study</td>
</tr>
<tr>
<td>08LX10257-A</td>
<td>GACATCGTCTCTCCATGCAAG</td>
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</tr>
<tr>
<td>08LX10257-B</td>
<td>GACATCGTCTCTCCATGCAAG</td>
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</tr>
<tr>
<td>08LX10257-C</td>
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</tr>
<tr>
<td>08LX10257-E</td>
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</tr>
<tr>
<td>08LX10257-F</td>
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</tr>
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<td>08LX10257-G</td>
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</tr>
<tr>
<td>08LX10257-H</td>
<td>GACATCGTCTCTCCATGCAAG</td>
<td>This study</td>
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<tr>
<td><strong>Primer-walking oligonucleotides</strong></td>
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<td>08LX10257-C</td>
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<tr>
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Table 7. Comparison of *Clostridium hylemonae* TN271 and *Clostridium scindens* VPI 12708 bai operon DNA sequences

<table>
<thead>
<tr>
<th>Gene or region</th>
<th>Length (bp)</th>
<th>Identity %</th>
<th>Enzyme Function</th>
<th>Identity %</th>
<th>Similarity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>baiB</td>
<td>1,563 vs. 1,545</td>
<td>65.7</td>
<td>bile acid-CoA ligase</td>
<td>84.4</td>
<td>78.9</td>
</tr>
<tr>
<td>baiB-CD (Intergene)</td>
<td>45 vs. 44</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baiCD</td>
<td>1,920 vs. 1,923</td>
<td>75</td>
<td>3-dehydro-4-steroid deshydrogenase</td>
<td>80.5</td>
<td>89.2</td>
</tr>
<tr>
<td>baiCD-baiE (Intergene)</td>
<td>43 vs. 46</td>
<td>51.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baiE</td>
<td>501 vs. 510</td>
<td>76.6</td>
<td>Bile acid 7-alpha-dehydratase</td>
<td>82.8</td>
<td>92.3</td>
</tr>
<tr>
<td>baiA</td>
<td>747 vs. 750</td>
<td>76.2</td>
<td>3 alpha-hydroxy steroid dehydrogenase</td>
<td>86.5</td>
<td>92.4</td>
</tr>
<tr>
<td>baiF</td>
<td>1,281 vs. 1,275</td>
<td>78.7</td>
<td>Bile acid-CoA hydrolase/transferase?</td>
<td>89</td>
<td>94.8</td>
</tr>
<tr>
<td>baiF-baiG (Intergene)</td>
<td>5 vs. 25</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>baiG</td>
<td>1,434 vs. 1,428</td>
<td>71.1</td>
<td>Bile acid transporter</td>
<td>80.3</td>
<td>88.9</td>
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<tr>
<td>baiG-baiH (Intergene)</td>
<td>26 vs. 19</td>
<td>55.6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>baiH</td>
<td>1,986 vs. 2,019</td>
<td>74.2</td>
<td>3-dehydro-4-steroid deshydrogenase</td>
<td>80.8</td>
<td>90.0</td>
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<tr>
<td>baiH-baiI (Intergene)</td>
<td>17 vs. 38</td>
<td>41.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baiI</td>
<td>555 vs. 549</td>
<td>68.2</td>
<td>Bile acid 7beta-dehydratase</td>
<td>51.4</td>
<td>76.6</td>
</tr>
</tbody>
</table>
not surprising given the close phylogenetic relation between these species as determined by neighbor-joining of 16s rDNA sequences of 7α-dehydroxylating bacteria and other gut microbes (106). One of the most striking observations is the fact that the baiA gene is not found within this operon in C. hylemonae as it is in C. scindens and C. hiranonis. We predict that the baiA gene will be found as a single cistron with a bai promoter region as observed in C. scindens with the baiA1 and baiA3 genes (67). Taken together, these data indicate that genes located on the 9.5 kb bai oxidative operon of the “low activity strain” C. hylemonae TN271 are highly conserved both in organization as well as amino acid sequence with the homologous bai polycistronic operons identified in the “high activity” species C. scindens and C. hiranonis with the exception of the baiA gene.

The ability of C. hylemonae TN271 to 7β-dehydroxylate UDCA has not been reported in the literature. Therefore, C. hylemonae and C. scindens were induced with 50 μM CA for 2 hrs and tested for the ability to 7α-dehydroxylate [24-14C] CA and [24-14C] CDCA as well as 7β-dehydroxylate [24-14C] UDCA for 30 minutes (Figure 34) at 37°C as well as
Figure 34. Autoradiographs of TLC of 7α-dehydroxylation and 7β-dehydroxylation activity assay reaction products. (a.) C. scindens VPI 12708 induced with 50 μM CA during mid-log phase in BHI. 1 ml culture inoculated in 1 ml activity assay consisting of BHI containing 0.1 μCi/2 μmole bile acid incubated 37°C for 30 min. Lane 1: [24-14C] CA activity assay of CA induced culture of C. scindens. Lane 2: [24-14C] CA activity assay of uninduced culture of C. scindens. Lane 3: [24-14C] UDCA activity assay of CA induced culture of C. scindens. Lane 4: [24-14C] UDCA activity assay of uninduced culture of C. scindens. Lane 5: [24-14C] chenodeoxycholic activity assay of uninduced activity assay. Lane 6 represents [24-14C] CDCA activity assay of cholic acid induced culture of C. scindens. The same order applies in (b.) for cultures of C. hylemonae. Arrows depict positions of non-radiolabelled bile acid standards. Induced cultures of C. scindens and C. hylemonae were able to 7α-dehydroxylate both CA and CDCA (lanes 1 and 6) while only C. scindens was able to 7β-dehydroxylate UDCA (lane 3). Uninduced cultures showed no activity against bile acid substrates. Solvent system was S1 according to Eneroth (172).
a. LCA →
   DCA →
   CDCA →
   UDCA →
   CA →

b. LCA →
   DCA →
   CDCA →
   UDCA →
   CA →
overnight incubation (Data not shown). Both *C. scindens* and *C. hylemonae* were able to 7α-dehydroxylate both CA and CDCA resulting in [24-\(^{14}\)C] DCA and [24-\(^{14}\)C] LCA, respectively. However, while *C. scindens* was able to 7β-dehydroxylate UDCA, we were unable to detect 7β-dehydroxylation activity by *C. hylemonae* despite sequence data that indicated the presence of genes encoding enzymes suggested to be involved in 7β-dehydroxylation. It is currently unclear why this activity is not present.

**Isolation and characterization of a baiA gene in**

*Clostridium hylemonae* TN271

The 27 kDa baiA gene product from *C. scindens* VPI 12708 catalyzes the first oxidative step in the bile acid 7α-dehydroxylating pathway following ATP-dependent CoA ligation (125). As noted in the previous section, and illustrated in Figure 33, the baiA gene was not found in the oxidative polycistronic bai operon in *C. hylemonae*. Therefore, redundant primers were designed based on a multiple sequence alignment of bai genes from *C. scindens* and *C. hiranonis* (Figure 35). A PCR product of 722 bp was
Figure 35. Design of degenerate PCR oligonucleotides for isolation of the baiA gene in C. hylemonae TN271. Clustal alignment of baiA1, baiA2 and baiA3 genes from C. scindens VPI 12708 and the baiA gene from C. hiranonis sp. strain TO931. Arrows indicate direction of PCR primers, the sequence of sense and antisense primer is shown below alignment.
baiA1: ATGAAACTTGTACAGGACAAAATTACAATTATCACAGGCGGAACCCGTGGAATCGGATTC 60
baiA3: ATGAAACTTGTACAGGACAAAATTACAATTATCACAGGCGGAACCCGTGGAATCGGATTC 60
baiA2: ATGAATCTCGTACAAGACAAAGTTACGATCATCACAGGCGGCACAAGAGGTATTGGATTC 60
baiATO931: ATGAACTTAGTACAGGACAAAATAGTTATAATAACAGGTGGAACAAGTGGTATAGGTCTT 60

*****  * ***** ****** *    ** ** ***** ** **  * ** ** **  *

baiA1: GCAGCAGCAAAACTCTTTATTGAGAATGGAGCAAAAGTCTCCATATTTGGCGAGACCCAG 120
baiA3: GCAGCAGCAAAACTCTTTATTGAGAATGGAGCAAAAGTCTCCATATTTGGCGAGACCCAG 120
baiA2: GCCGCTGCCAAAATATTTATCGACAATGGCGCAAAAGTATCCATCTTCGGAGAGACGCAG 120
baiATO931: TGCGCAGCAAAAATATTTATCGACAATGGCGCAAAAGTATCCATCTTCGGAGAGACGCAG 120

*****  * ***** ****** *    ** ** ***** ** **  * ** ** **  *

baiA1: GATCGCGAATGTATATCTGTTCCTTGCATCCGACCTGGCTAGCGGCATCACGGCTACGAC 719
baiA3: GATCGCGAATGTATATCTGTTCCTTGCATCCGACCTGGCTAGCGGCATCACGGCTACGAC 719
baiA2: GATCGCTAATGTATACCTGTTCCTGGCATCTTGGCAAGCGGCATTACGGCTACTAC 719
baiATO931: AATAGCTAACACTTACTTATTCTTAGCTTCTGACTTAGCTAGTGGTATAACAGCTACAAC 719

*****  * ***** ****** *    ** ** ***** ** **  * ** ** **  *

baiA1: GATCAGCGTAGATGGGGCTTACAGGCCATAG--- 750
baiA3: GATCAGCGTAGATGGGGCTTACAGGCCATAG--- 750
baiA2: GGTCAGCGTAGACGGGGCTTACAGACCATAA--- 750
baiATO931: TGTAAGCGTTGACGGTGCTTATAGACCATCATAA 753

baiA271F: 5’-CAG GYG GMA CMM GWG GWA THG G-3’
baiA271R: 5’-GGR CTR TAA GCM CCR TCW TCG C-3’
obtained, sequenced and predicted to encode the baiA gene by BLAST search (Figure 36). The complete 789 bp gene encoding a predicted 27.9 kDa protein was obtained following bidirectional genome-walking from the initial sequence data (Figure 37). Sequence analysis upstream of the baiA gene revealed a conserved bai promoter and RBS (Figure 37). Regions identified as highly conserved within bai promoters showed 100% identity between the upstream baiB promoter and that of the baiA promoter. 5′ SMART RACE analysis suggests the mRNA initiation site is 56 bp upstream of the baiA gene (Figure 37). Figure 38 displays an alignment of the baiA gene from C. hylemonae with baiA genes from C. scindens and C. hiranonis.

**Identification and characterization of novel genes suggested to be involved in bile acid metabolism in Clostridium hylemonae and Clostridium scindens**

The 3.3 kb contig containing the baiA and upstream bai promoter was further analyzed for additional ORF potentially involved in the reductive arm of the bile acid 7α/β-dehydroxylation pathway (Figure 39; Figure 40) (Table
Figure 36. Isolation of partial baiA gene from *C. hylemonae* TN271 using degenerate PCR oligonucleotides designed from conserved regions of *C. scindens* and *C. hiranonis* baiA genes. Arrow indicates PCR product corresponding to partial baiA gene as shown in nucleotide sequence alignment. PCR product was cloned into a pCR8 GW TOPO TA cloning vector resulting in vector p271OXA.
b.

1. **TN271OXA**
   - 0.72 kb
   - TA cloning site
   - attL1
   - attL2
   - Specr

2. **p271OXA**
   - 3.52 kb

3. **TA Cloning Site**

4. **pUC ori**

---

c.

1. **1670Ch a.a**
   - TRU0071b a.a
   - UP003a a.a

2. **650 bp**

3. **1000 bp**

4. **168 bp**

---

**Image a.**

- Gel electrophoresis result

**Image b.**

- Circular diagram of TN271OXA and p271OXA

**Image c.**

- Table of sequences
Figure 37. Isolation of baiA gene from C. hylemonae TN271 and characterization of upstream promoter elements. (a.) Schematic representation of sequence alignment and location of baiA gene relative to PCR product alignment. (b.) CLUSTALW alignment of C. hylemonae TN271 oxidative baiB promoter (TN271Oprom) with baiA promoter (TN271Aprom). Boxed regions correspond to conserved bai promoter elements. (c.) Results of 5’ SMART RACE PCR analysis of baiA cDNA from CA induced culture (See Figure 20). mRNA initiation site (bold, underlined) indicated by arrow which points in direction of transcription. Italicized letters indicate 5’ end of baiA gene with corresponding amino acids (below).
a. 

b. TN2710prom  TATCCTAGTCAAGTATGTAAGTGGTTAAAGTTATATCAA 42
TN271Aprom  TATCCTAGTCAAGTATGTAAGTGGTTAAAGTTATATCAA 41

**************  ***  **********************

RBS

M  K

C. ATGTCATAGTCAAGTATGTAAGTGGTTAAAGTTATATCAAACGGAAATATTAGCAATATAGAAAGCTAAAACCGCAGGAAAAGGAAATCATGAAA

********  ********  ********

Figure 38. CLUSTALW alignment of amino acid sequence of baiA genes from *C. hylemonae* TN271, *C. scindens* VPI 12708 and *C. hiranonis* sp. strain TO931. The baiA gene encodes a 3α-hydroxysteroid dehydrogenase specific for cholyl-CoA (58).
12708 MNLVQDKVTIITGGTRGIGFAAAKIFIDNGAKVSIFGETQEEVDTAQLKELYPEEEVL 60
TN271 MKLVQDKITVITGGTRGIGFTAAKIFIENGAKVSIFGETKEEVDTALAEKELYPEEEVL 60
TO931 MNLVQDKIVITGGTSGIACLAAKIFMDNGATVSIFGKTQEEVDAAKAELKETHPDKEVL 60

12708 GFAPDLTSRDAVMAAVGQVAQKYGRDLVMINNAGITSNVFSRVSEEKFHIMDINTVG 120
TN271 GFAPDLTSRDAVMAAVRAVAEKYGRLDVMINNAGITMNTVFSRVEAFKHMMDINTVG 120
TO931 GFAPDLTNRDENVMAAVGAVAEEKYGRDLVMINNAGVTSSNVFSRVPEEFTYLMINTVG 120

12708 FNGAWCAYQCMDAKKGVIINTASVTGYFGLSLGVSFAQKAVIGLTHGREGIIRKNI 180
TN271 FNGAWAYCQCMKGAQQGVTINTASVTGYGSLLGSGFPAKAVIGLTHGREGIIRKNI 180
TO931 FNGAWAAYHCLKGKEKIIINTASVTGYHSLLGSGFYPSKASEAVGFTQALGREGIIRKNI 179

12708 RVVGVAPGVVNTDNGNPPEIMEGYLKALPMKRMLEPEEIANYVFLASDGIATATT 240
TN271 RVVGVAPGVVTDNTPMFPSEILELYKSLFPMKRMKAEIANYVFLASDGIATATT 240
TO931 RVVGVAPGVVNTMPNPIDEILGYSFPMKRMLEPEEIANYVFLASDGIATATT 240

12708 VSVDGAYRP---------- 249
TN271 VSDDGAYSPRANSTQLFVQGI 262
TO931 VSVDGAYRFS---------- 249

** **** *
Figure 39. Genome-walking PCR upstream of partial baiA gene of Clostridium hylemonae TN271. A nested PCR product derived from PvuII restriction library template (PN) ~2.5 kb was purified by GENECLEAN and ligated into pCR8 GW TOPO TA vector to produce vector p271PNUP1A.
Figure 40. Downstream genome-walking PCR (DS1) from baiA gene of Clostridium hylemonae TN271. A nested PCR product derived from EcoRV (EN) restriction library template ~2.3 kb was purified by GENE CLEAN and ligated into pCR8 GW TOPO TA vector to produce vector p271ENDS1A.
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8). An ORF was located 235 bp upstream of the *baiA* gene. The putative 732 bp gene was predicted to encode a 26 kDa short chain dehydrogenase/reductase. Members of this family include the *baiA* gene which encodes a 3α-HSDH. However, these two gene products shared only 19% amino acid sequence identity and 35% similarity. This ORF is of interest because we expect the final reductive step in the pathway to encode a 3α-HSDH recognizing 3-dehydro-DCA and/or 3-dehydro-LCA. A second partial ORF was located 67 bp upstream of the putative short chain dehydrogenase. Intriguingly, this ORF was suggested to encode a type III CoA transferase whose highest sequence identity was that of the *baiF* gene from *C. scindens* VPI 12708. A second upstream genome-walk resulted in a PCR product 2,107 bp in length which increased the current contig length to 4,949 bp (Figure 41). The *baiF* homologue sequence was completed and found to be 1,332 bp in length and predicted to encode a 49.5 kDa polypeptide. The *baiF* gene product from *C. scindens* VPI 12708 was previously overexpressed in *E. coli* and reported to encode a 47.5 kDa CoA hydrolase (130); however, recent data suggests this gene product belongs to the Type III family of CoA transferases (131). The *baiF*
Figure 41. Second genome-walking PCR upstream of baiA from C. hylemonae TN271. a.) Nested PCR obtained from PvuII restriction digest (PN) of C. hylemonae genomic DNA template. b.) 2 kb product GENE CLEAN purified from agarose gel and ligated into pCR8 GW TOPO TA vector resulting in vector p271UP2A.
**a.**

M    PN

3.0 kb

2.0 kb

1.6 kb

**b.**

**p271UP2A**

4.9 kb

2.1 kb

TA cloning site

---

A    PN AUP2    A

T1

attL1

T2

attL2

pUC ori

Specf
and this second ORF share 60% amino acid sequence identity, 75% amino acid similarity. Figure 42 shows an amino acid sequence alignment between the baiF gene and the putative CoA transferase.

Directly upstream, 52 bp from the hypothetical CoA transferase gene was another partial ORF with a flavin binding domain. Further genome-walking resulted in 760 bp of sequence data and completion of this 1,722 bp ORF encoding a putative 62 kDa flavoprotein (Figure 43). BLAST analysis suggested this gene product encodes a flavoprotein similar to fumarate reductases and 3-ketosteroid-Δ¹-dehydrogenases. The former can likely be ruled out due to the lack of four conserved N-terminal heme binding sites (CXXCH) found in fumarate reductases such as that of Shewanella putrefaciens MR-1, whose structure has been solved (192). The latter possibility was of particular interest because we expect one of the reductive genes to encode a 3-oxo-Δ⁴-steroid oxidoreductase. A fourth upstream genome-walk resulted in a 2,765 bp of additional sequence data which increased contig length to 7,993 (Figure 44). This additional sequence data allowed analysis upstream of the putative 3-
Figure 42. Boxshade of CLUSTALW amino acid sequence alignment of baiF gene product of C. hylemonae and a predicted Type III CoA transferase which we have named the baiK gene (See text).
Figure 43. Third upstream genome-walking PCR from baiA gene of C. hylemonae TN271. a). Agarose gel of DS2 products using DraI (D), EcoRV (E), PvuII (P), StuI (S) restriction libraries of C. hylemonae TN271 genomic DNA as template with adaptor primer AP1 and gene-specific primer WALKDS-2 (See Table 6). b.) “EN” and “PN” represent nested PCR amplification using nested adaptor primer AP2 and gene-specific primer WALKDSNEST2 (See Table 6). “M” represents 1 kb DNA ladder. C.) Schematic representation of TA cloning of GENECLEAN purified “DN” product (arrow) into pCR8/GW/TOPO TA vector (2.8 kb) yielding 3.56 kb vector p271DNCUDP3.
a. 

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c. 

```
DN AUP3

0.76 kb

p271UP3A
3.56 kb

pUC ori

A

Spec'
```

```
T1

attL1

TA cloning site

T2

attL2
```
Figure 44. Fourth upstream genome-walking PCR from baiA gene of Clostridium hylemonae TN271. a.) Nested PCR obtained from EcoRV (EN) restriction digest of C. hylemonae genomic DNA template. b.) 2.7 kb product GENECLEAN purified from agarose gel and ligated into pCR8 GW TOPO TA vector resulting in vector p271UP4A (5.5 kb)
a.

M  EN

3.0 kb
2.0 kb
1.6 kb

b.

p271UP4A 5.5 kb

2.7 kb

DN AUP4

T1

attL1

TA cloning site

T2

attL2

pUC ori

Specr

A

A
oxo-Δ⁴-steroid oxidoreductase gene for a bai promoter sequence. Indeed, we located a putative bai promoter 24 bp upstream of this ORF (Figure 45). Additional conserved bai promoter regions were not found in any other portions of this contig. 5’ SMART RACE PCR was performed on the putative 3-oxo-Δ⁴-steroid oxidoreductase gene and the mRNA start site was determined at the very 5’ end of the conserved bai region suggesting the promoter is located upstream of this conserved region (Figure 46). It is quite possible that this conserved region is a promoter region for both the baiA and baiB operons; however, this region does not appear to be a promoter for the baiJKL genes. The functional significance of this conserved region remains to be elucidated.

For the sake of simplicity in description we will provide a preliminary name to these gene products following the sequence of nomenclature ending with the baiI gene on the oxidative operon. This first ORF encoding the putative 3-oxo-Δ⁴-steroid oxidoreductase will be called the baiJ gene. The baiF homologue will be named the baiK gene and the putative short chain dehydrogenase will be referred to as the baiL gene.
Figure 45. Identification of conserved bai promoter elements upstream of the putative baiJ gene of *Clostridium hylemonae* TN271.
Figure 46. Determination of transcription initiation site for \textit{baiJ} gene from \textit{Clostridium hylemonae} TN271 based on SMART RACE PCR analysis. Alignment above shows determination of transcription initiation site (TIS) based on alignment of SMART RACE sequence data (SR3, SR4) with \textit{baiJ} gene and upstream region from \textit{Clostridium hylemonae} TN271 (TN271D4). TIS is determined based on the first base following 3' end of SMART II A oligo sequence (italics) (5'\textit{AAGCAGTGGTATCAACGCAGAGTACGCGGG}3'). This nucleotide (underlined, arrow) must also correspond to the known sequence of the upstream region of the gene. Therefore, the TIS is 47 bp upstream of the ATG start site (underlined). RBS=ribosome binding site.
Isolation of the baiJ gene from C. scindens VPI 12708 and elucidation of conserved baiJKL genes.

A necessary question immediately arose: are these genes conserved in other 7α-dehydroxylating bacteria? To answer this question, the baiJ gene from C. hylemonae TN271 was analyzed for short 7-8 amino acid stretches with regions of low codon degeneracy. Amino acids 183-189 (EEMCYNY) and 394-401 (NEWSYQYH) were selected as sites of lowest degeneracy, a section whose nucleotide sequence would result in an expected PCR product of 654 bp (Figure 47). A codon usage table was prepared using sequence data from bai genes from C. scindens VPI 12708 using the codonW program (Figure 48). This table allowed design of least degenerate oligonucleotides for an attempt at isolation of the baiJ gene from C. scindens VPI 12708 (Table 9). Amplification using forward primer Delta4F1 and each of the reverse primers Delta4R1, Delta4R2 and Delta4R3 each resulted in a product around 650 bp (Figure 49) (See Table 9 for primer sequences). The 650 bp PCR product amplified using Delta4F1 and Delta4R2 primers was excised from the
Figure 47. Amino acid sequence of \textit{baiJ} gene from \textit{Clostridium hylemonae} TN271. Residues highlighted in green have one or two possible codons. Bold regions correspond to amino acids 183-189 (EEMCNY) and 394-401 (NEWSYQYH). These regions were selected based on low codon degeneracy which will be used to design oligonucleotides to potentially isolate the \textit{baiJ} gene from \textit{Clostridium scindens} VPI 12708.
Figure 48. Codon usage table for *Clostridium scindens* VPI 12708 and design of redundant oligonucleotides for isolation of the *baiJ* gene from *C. scindens* VPI 12708. a). Codon usage table generated using *bai* genes from *Clostridium scindens* as input (U57489). b). Design of forward and reverse degenerate oligonucleotides based on regions identified in Figure 45.
a.

Phc UUU 50.78 Ser UCU 25.095 Tyr UAU 70.135 Cys UGU 15.045

UUC 78.123 UCC 51.194 Ala UAC 34.063 UGG 52.155

Leu UUA 15 0.46 GCA 17 0.55 Ter UAA 5 1.00 Ter UGA 2 0.75

UGU 15 0.33 CCG 6 0.30 Val 1 0.36 Trp UGG 33 1.60

CUU 71 1.80 Pro CUC 33 0.92 His CAU 38 1.29 Arg CGU 29 1.27

CUC 11 0.28 CCC 6 0.17 Cac 21 0.71 Cgc 33 1.45

CUA 1 0.03 CCA 35 0.95 Gin CAA 1 0.02 Cga 4 0.16

CUG 123 3.11 CCG 69 1.93 Cag 72 1.97 Cgg 8 0.35

Ile AUU 41 0.54 Thr ACU 30 0.45 Asn AAU 61 1.07 Ser AGU 7 0.27

AUC 164 2.17 ACC 55 1.33 Ala AAC 53 0.95 Aac 50 1.80

AUA 22 0.29 ACA 49 1.18 Lys AAA 61 0.56 Arg AGA 44 1.53

Met AUG 124 1.00 AGC 42 1.01 Arg AGG 124 1.34 Arg 19 0.33

Val GUA 44 0.74 Lys GCU 44 0.54 Asp GAA 68 0.89 Glu GGU 12 0.16

GUC 42 0.70 GCC 51 0.74 Gin GAC 64 1.11 Gcc 127 1.73

GUA 102 1.71 GCA 114 1.95 Glu GAA 128 1.12 Gaa 136 1.86

GUG 51 0.88 GCG 68 0.92 Gag 100 0.88 Ccg 18 0.26

3152 codons in 12708 (used Universal Genetic code)

b.

Forward Primer

TN271 sequence: GAG GAG ATG TGC TAC AAC TAC CTG ATG GAA
Amino Acid Seq: E E M C Y N Y L M E

Reverse Primer

TN271 sequence: AAC TAA TGG AGC TAC CAG TAT CAC
Amino Acid Seq: E E M C Y N Y L M E

Isolation of partial $baiJ$ gene from *Clostridium scindens* VPI 12708.  a). PCR products generated from amplification with primers Delta4F1 and Delta4R2 from both *Clostridium hylemonae* TN271 and *Clostridium scindens* VPI 12708 genomic DNA templates.  b). 0.65 kb PCR product from *Clostridium scindens* VPI 12708 was GENE CLEAN purified and cloned into a pCR8/GW/TOPO TA vector generating a 3.45 kb vector p12708D4deg.  c.) CLUSTALW alignment of sequence data obtained from p12708D4deg insert against $baiJ$ gene from *Clostridium hylemonae* TN271.
b. 0.65 kb

TA cloning site

attL1

attL2

p12708D4deg

3.45 kb

pUC ori

Specf

Specr

T1

T2

M 12708

TN271 12708

2.0 kb
1.6 kb
1.0 kb
0.85 kb
0.65 kb
0.5 kb

0.65 kb

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12708

TN271

12708 A

2.0 kb
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T2

T1

attL1

attL2

p12708D4deg

3.45 kb

pUC ori

Specf

Specr

T1

T2

M 12708

TN271 12708

2.0 kb
1.6 kb
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2.0 kb
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0.5 kb

12708

TN271

12708 A
Table 9. Oligonucleotides used in isolation of bea/KL operon and flanking sequence from Clostridium scindens VPI 12708

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| ARS | ACCGCTAGTCGACCGCTTGTTCG | Clontech |
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| 1170O9531R1 | CTCTATGCACTTTCTGGGAACCA | This study |
| 1170O9531L981 | CTCTATGCACTTTCTGGGAACCA | This study |
| 1170O9531R981 | CTCTATGCACTTTCTGGGAACCA | This study |
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| 1170O9531R13481 | CTCTATGCACTTTCTGGGAACCA | This study |
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| 1170O96031L1 | CTCTATGCACTTTCTGGGAACCA | This study |
| 55O1270SL9 | AAGAAATCGGCTCTTATGGGCG | This study |
| 55O1270SR9 | AAGAAATCGGCTCTTATGGGCG | This study |

| Genome walking oligonucleotides | | |
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| 1170O9533R1 | CTCTATGCACTTTCTGGGAACCA | This study |
| 1170O96071L1 | CTCTATGCACTTTCTGGGAACCA | This study |
| 1170O96071R1 | CTCTATGCACTTTCTGGGAACCA | This study |

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agarose gel, GENECLEAN purified, cloned into a pCR8 TOPO TA cloning vector and both strands sequenced. An alignment of the partial baiJ sequence obtained from C. scindens against the baiJ gene from C. hylemonae TN271 shows the high degree of amino acid sequence conservation (Figure 49). The partial baiJ gene from C. scindens was used to design bidirectional genome-walking PCR oligonucleotides to both complete the gene sequence and obtain information about any flanking ORF.

The first upstream genome-walk from the partial baiJ gene resulted in a 1,330 bp product (Figure 50). Addition of this sequence data to the partial baiJ sequence resulted in a contig of 1.8 kb. This sequence data contained the 5′ end of the baiJ gene. A conserved “bai promoter region” was located directly upstream of the baiJ gene and shared a high degree of sequence identity with the putative bai promoter region found upstream of the baiJ gene of C. hylemonae (Figure 51). SMART RACE analysis of the 5′ end of the baiJ gene revealed the TIS upstream of the putative conserved bai “promoter” region (Figure 52). These results were similar, but not identical to the TIS of the baiJ gene from C. hylemonae
Figure 50. Genome-walking PCR upstream of partial *baiJ* gene of *Clostridium scindens* VPI 12708. a.) Nested PCR products obtained from *DraI* (DN) and *EcoRV* (EN) restriction digest of *C. scindens* genomic DNA template. Due to larger size, product “EN” was isolated, cloned and sequenced. b.) 1.3 kb product GENECLEAN purified from agarose gel and ligated into pCR8 GW TOPO TA vector resulting in vector p12708D4ENUP1 (1.3 kb).
Figure 51. BOXSHADE of CLUSTALW alignment of upstream conserved bai promoter elements located upstream of baiJ genes of Clostridium scindens VPI 12708 and Clostridium hylemonae TN271.
Figure 52. Verification of bile acid induction and 5’ SMART RACE PCR analysis of baiJ transcript from C. scindens VPI 12708. (a). TLC autoradiograph (Top) between CA induced (I) and CA uninduced (UI)cultures of C. scindens VPI 12708. RT-PCR (Below) of baiB gene comparing cDNA produced from RNA isolated from CA I and CA UI cultures of C. scindens VPI 12708 using primers baiB12708RTF (5’-GAT GAG CAG GGC TTA TCT GTA CTT T-3’), and baiB12708RTR (5’-GTA TCT CTT TCC CTG TCT CAA TGA C-3’) and compared to gyrA gene. (b). 1% Agarose gel stained with ethidium bromide showing 5’ RACE PCR products using gene-specific primer SR12708D1 (5’-TTC TAT GTC CTC ATC CGT TGC CCC TG-3’). Lane “M” is a 1 kB DNA marker, lanes 1, 2, and 3 represent PCR products obtained at various annealing temperatures (62°C, 64°C, 66°C, respectively). Arrow indicates band that was isolated, and sequenced. (c). Schematic representation of TIS obtained from 5’RACE sequence data. “✝” = TIS position obtained from 5’RACE of C. hylemonae TN271 baiJ gene (Figure 46). “‡” = TIS position determined by primer extension (Mallonee et al. 2000) and 5’RACE (Figure 27) for the baiB gene from C. scindens VPI 12708. “●” represents TIS position determined by primer extension analysis of the baiB gene from C. hiranonis sp. strain TO931. “◊” represents TIS position determined by 5’RACE PCR of baiB gene from C. hylemonae TN271 (Figure 26).
a. $\text{TATTGCGGGG}$

b. $\text{GATTAATTTTAAATTATACAGCCTGAATTATAAG TGTTAGAATGA}$

[Image: Diagram showing bands for baiB and gyrA]
TN271. The TIS, located upstream of this conserved region, effectively rules this region out as the baiJ promoter.

In addition, a partial ORF was found 128 bp upstream of the baiJ gene on the antisense strand. A BLAST search of the ORF suggested this gene belonged to the LysR family of transcription factors. Due to the proximity of the TIS of the baiJ gene to the LysR like gene on the opposite strand, it is very likely that the baiJ promoter overlaps the LysR-like gene. A second upstream genome-walk resulted in a 4.0 kb PCR product which increased the contig to 5.7 kb (Figure 53). This sequence data completed the remainder of a 960 bp gene encoding a deduced 36.2 kDa polypeptide predicted to encode a transcriptional regulator in the LysR family. A second ORF located on the anti-sense strand relative to the baiJ gene was found 73 bp upstream of the putative transcription factor. This 465 bp ORF was predicted to encode a 17.8 kDa polypeptide in the Tryptophan-rich sensory protein (TspO)/ mitochondrial benzodiazepine receptor (MBR) homolog family of signal transduction integral membrane proteins. Further upstream, and on the
Figure 53. Second upstream genome-walking PCR (UP2) from partial baiJ gene of Clostridium scindens VPI 12708. a). Agarose gel of UP2 products using DraI (D), EcoRV (E), PvuII (P), StuI (S) restriction libraries of C. scindens VPI 12708 genomic DNA as template with adaptor primer AP1 and gene-specific primer 12708WalkUP2 (See Table 6). b.) “DN” and “SN” represent nested PCR amplification using nested adaptor primer AP2 and gene-specific primer 12708WalkUP2N (See Table 6). A positive clone was obtained for product “SN”, so this was used for sequencing. “M” represents 1 kb DNA ladder. c.) Schematic representation of TA cloning of GENECLEAN purified “SN” product (arrow) into pCR8/GW/TOPO TA vector (2.8 kb) yielding 6.8 kb vector p12708D4SNUP2.
same strand relative to the baiJ gene, we located the baiA1 gene and promoter region which has been previously reported (122). This gene was located directly upstream of the TspO homologue. It is interesting to note that the baiA gene and baiA promoter located in C. hylemonae was found in proximity to this putative reductive bai operon.

The initial genome-walk downstream of the partial baiJ gene resulted in a PCR product 1,929 bp in length (Figure 54). Alignment with the previous sequence data resulted in a contig of 7,114 bp. This additional sequence data resulted in completion of the 1,722 bp baiJ gene predicted to encode a 61.2 kDa polypeptide. Amino acid sequence identity of the baiJ gene is highly conserved between C. scindens and C. hylemonae (89%) (Figure 55). In addition, a partial baiK gene was located 37 bp downstream of the baiJ gene in C. scindens. A second downstream genome-walk resulted in a fragment 3,366 bp in length increasing the contig length to 10.13 kb. The 1,314 bp baiK was completed and predicted to encode a 49.1 kDa polypeptide. The baiL gene, 732 bp in length, was located 79 bp downstream of the baiK gene. The
Figure 54. Downstream genome-walking PCR (DS1) from partial \textit{baiJ} gene of \textit{Clostridium scindens} VPI 12708. a.) Nested PCR products obtained from \textit{DraI} (DN) and \textit{EcoRV} (EN) and \textit{StuI} (SN) adaptor ligated restriction libraries of \textit{C. scindens} VPI 12708 genomic DNA templates. Due to larger size, product "EN" was cloned. b.) 1.9 kb product GENECLEAN purified from agarose gel and ligated into pCR8 GW TOPO TA vector resulting in vector p12708D4ENDS1 (4.7 kb).
a. Image of a gel with bands at 3.0 kb, 2.0 kb, and 1.6 kb.

b. Diagram of a plasmid with labels:
- p12708D4ENDS1
- 1.9 kb
- Spec'
- pUC ori
- T1
- T2
- attL1
- TA cloning site
- attL2
- 4.7 kb
Boxshade representation of CLUSTALW amino acid sequence alignment of putative 3-oxo-Δ^4-reductases (baiJ) between Clostridium scindens VPI 12708 and Clostridium hylemonae TN271.
deduced amino acid sequence suggests this gene encodes a 26.4 kDa polypeptide. In addition, a partial ABC transport protein was located 85 bp downstream of the baiL gene in C. scindens. This short intergenic distance suggests this gene may be found on the same operon. Indeed, we hypothesize that an additional ABC transporter will be involved in translocating DCA and LCA into the extracellular environment (Figure 8). A short 850 bp genome-walk (Figure 56), and a fourth genome-walk of 3 kb (Figure 57) resulted in completion of the ABC transport gene 1854 bp in length, encoding a deduced 70.3 kDa polypeptide. In addition, two ORF were located directly downstream of the ABC transport protein. BLAST analysis suggests this ORF encodes a flavoprotein with the highest similarity to guanine deaminases. The second ORF is predicted to encode a flavoprotein with greatest similarity to xanthine dehydrogenases and 4-hydroxybenzoyl-CoA reductases.

Figure 58 is a schematic representation of the putative reductive bai operons in both C. scindens VPI 12708 and C. hylemonae TN271. Some notable features include the similar organization of the baiJKL genes,
Figure 56. Third genome-walking PCR downstream (DS3) of \textit{baiJ} gene product in \textit{Clostridium scindens} VPI 12708. a). Agarose gel of DS3 products using \textit{DraI} (D), \textit{EcoRV} (E), \textit{PvuII} (P), \textit{StuI} (S) restriction libraries of \textit{C. scindens} VPI 12708 genomic DNA as template with adaptor primer AP1 and gene-specific primer 12708WDS3 (See Table 6). b.) “DN” and “SN” represent nested PCR amplification using nested adaptor primer AP2 and gene-specific primer 12708WDS3N (See Table 6). Attempts to clone product “SN” were unsuccessful. While “EN” was smaller in size, the specificity was high and attempted cloning yielded several positive clones. “M” represents 1 kb DNA ladder. b.) Schematic representation of TA cloning of GENE CLEAN purified “EN” product (arrow) into pCR8/GW/TOPO TA vector (2.8 kb) yielding 3.65 kb vector p12708D4ENDS3.
a.

- DN2
- DN
- E
- EN
- P
- PN
- S
- SN

3.0 kb
2.0 kb
1.6 kb
1.0 kb
0.85 kb

b.

- p12708D4ENDS3
- 3.65 kb
- pUC ori
- attL1
- attL2
- TA cloning site
- Spec^r

- 0.85 kb
Figure 57. Genome-walking PCR downstream (DS4) of partial *baiJ* gene of *Clostridium scindens* VPI 12708. a.) Nested PCR products obtained from *DraI* (DN) restriction digest of *C. scindens* VPI 12708 genomic DNA template. b.) 3.0 kb product was GENECLEAN purified from agarose gel and ligated into pCR8 GW TOPO TA vector resulting in vector p12708D4DNDS4 (5.8 kb).
Figure 58. Schematic representation of putative reductive genes from Clostridium scindens VPI 12708 and Clostridium hylemonae TN271. The baiA genes encode 27 kDa 3α-hydroxysteroid dehydrogenases. The baiJ genes encode 62 kDa flavoproteins. The baiK genes encode a predicted 49 kDa Type III CoA transferase similar to the baiF. The baiL genes encode 27 kDa proteins of the short chain reductase family. Finally, the baiM encodes a predicted ABC transport protein hypothesized to function as a secondary bile acid efflux protein. “P” represents conserved bai “promoter” region.
C. scindens

C. hylemonae

Xanthine dehydrogenase
4-hydroxybenzoyl-CoA reductase

Guanine deaminase
(TIM barrel)

TspO/MBR

ABC transporter

LysR

baiA

baiJ baiK baiL

ABC transporter

ArsR ferrodoxin

1 kb
upstream conserved elements with the oxidative bai upstream region, and proximity of TIS. Interestingly, the upstream ORFs are not conserved between C. scindens and C. hylemonae. Each region includes an ORF predicted to encode a transcription factor of a different family of proteins on opposite strands. In addition, the ORF immediately upstream of the baiJ gene from C. hylemonae TN271 is predicted to encode an 18 kDa 4Fe-4S ferrodoxin presumably involved in shuttling electrons. This ORF was not located adjacent to the baiJKL genes from C. scindens. Finally, the baiA gene and conserved upstream “promoter” appear to cluster around the baiJKL genes.

Isolation of a partial baiJ gene in Clostridium hiranonis sp. strain TO931

The baiJ gene from C. hylemonae TN271 and C. scindens VPI 12708 are very similar at the amino acid sequence level. Designing oligonucleotides for isolation of the same gene from more distantly related species can be difficult with such high sequence identities. Attempts were made to isolate the baiJ gene from C. hiranonis using
redundant primers in Table 9 without success. Therefore, a multiple sequence alignment was prepared with the amino acid sequence of the *baiJ* gene product against several homologues in the databases. This way, regions could be detected that would normally be highly conserved within this family of flavoproteins. Two gene products were selected for the alignment: 1). ZP_01362869.1, a fumarate reductase flavoprotein subunit precursor from *Clostridium* sp. OhILAs (2) ZP_00802728.1, a Flavocytochrome C from *Alkaliphilus metalliredigeneres* QYMF. These protein sequences were then aligned with the 12708 *baiJ* using ClustalW (Figure 59). Two regions of high amino acid sequence identity with low codon degeneracy spanning a 282 bp stretch within the *baiJ* gene were identified (Figure 59). A codon usage table was prepared using *bai* gene sequences from *C. hiranonis* sp. strain TO931 (accession no. AF210152) (Figure 60). These primers were used to isolate a 657 bp gene product corresponding to what appears to be a partial *baiJ* gene of *C. hiranonis* sp. strain TO931 (Figure 61). A genome-walking library will have to be constructed for *C. hiranonis* sp. strain TO931 to elucidate the remaining gene and flanking regions;
Figure 59. Boxshade of CLUSTALW alignment between the baiJ gene product from Clostridium scindens VPI 12708 and flavoprotein homologues. ZP_01362869.1 represents the GenBank accession number for the fumarate reductase flavoprotein subunit precursor from Clostridium sp. OhILAs and ZP_00802728.1 represents the GenBank accession number for the Flavocytochrome c from Alkaliphilus metalliredigenes QYMF. Arrows represent regions of high amino acid conservation and low codon degeneracy which will be used to design oligonucleotides to attempt to isolate the baiJ gene from more distantly related 7α-dehydroxylating clostridia.
Figure 60. Codon Usage Table for *Clostridium hiranonis* sp. strain TO931. The CodonW program was used with *bai* gene sequences from *C. hiranonis* sp. strain TO931 as input (GenBank accession no. AF210152).
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Figure 61. Isolation of putative baiJ gene from Clostridium hiranonis sp. strain TO931. a.) Agarose gel of PCR products derived from amplification with primers TO931D4F (5’-GCW GCW GAY TCW ATA GAA GAA TTA GC-3’), and TO931D4R (5’-TTC WCC WGC WGC TAA WCC WGG TAT-3’) against (-) no DNA, (12708) Clostridium scindens VPI 12708 genomic DNA, (TO931) Clostridium hiranonis sp. strain TO931. A single PCR product was obtained from C. hiranonis sp. strain TO931. b.) PCR product (arrow) was cloned into a pCR8/GW/TOPO TA vector to obtain a 3.05 kb vector pD4TO931. c.) Boxshade alignment of multiple sequence alignment of C. hylemonae TN271 baiJ (TN271baiJ), C. scindens VPI 12708 baiJ (12708baiJ) and partial sequence of C. hiranonis sp. strain TO931 baiJ. Asterisks signify amino acids corresponding to gene region where primers anneal.
however, this data represents a significant step in possibly recovering these putative reductive genes from a third 7α-dehydroxylating clostridial species present in the human colon.

Cloning and Overexpression of baiL gene in Escherichia coli.

The baiL gene is predicted to encode a 26 kDa polypeptide in the 3-ketoacyl-acyl carrier protein reductase (fabG) family (Figure 62). We predict this protein will convert 3-oxo-DCA(~SCoA) and/or 3-oxo-LCA(~SCoA) to the secondary bile acids DCA(~SCoA) and LCA(~SCoA), respectively. Therefore, the strategy for characterizing this gene product was to clone the baiL gene from C. hylemonae TN271 into a pET21(a+) vector, overexpress the recombinant protein in E. coli codon plus BL21(DE3) and test 3α-hydroxysteroid oxidoreductase activity with substrates NAD(P)H and [24-14C] 3-oxo-DCA or NAD(P) and [24-14C] DCA (See Material and Methods).

After cloning (Figure 63) and sequencing of the vector insert to verify proper reading frame, the BaiL was overexpressed in E. coli (Figure 64a). The BaiL protein
Figure 62. Boxshade of CLUSTALW amino acid sequence alignment of \textit{baiL} genes from \textit{Clostridium scindens} VPI 12708 and \textit{Clostridium hylemonae} TN271 with similar short chain dehydrogenases/3-keto acyl reductases.
| ZF_01696223.1 | -----HCQHKVAVTCAAGSCKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| ZF_02668472.1 | -----HCQHKVAVTCAAGSCKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| ZF_01373200.1 | -----HCQHKVAVTCAAGSCKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| ZF_01722298.1 | -----HCQHKVAVTCAAGSCKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| ZF_02463417.1 | -----HCQHKVAVTCAAGSCKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| ZF_02629629.2 | MSKIDGTSBAYPTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| TM271bail | -----HCQHKVAVTCAAGSCKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| 1L720bail | -----HCQHKVAVTCAAGSCKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |

| ZF_01696223.1 | QLHTSCTGSTDILNRAHHSKSPQSGTLDLAKSCLQKCL---CITAYLRLPLH---CHIKIANSCTYISAVCTYGS |
| ZF_02668472.1 | QLHTSCTGSTDILNRAHHSKSPQSGTLDLAKSCLQKCL---CITAYLRLPLH---CHIKIANSCTYISAVCTYGS |
| ZF_01373200.1 | QLHTSCTGSTDILNRAHHSKSPQSGTLDLAKSCLQKCL---CITAYLRLPLH---CHIKIANSCTYISAVCTYGS |
| ZF_01722298.1 | QLHTSCTGSTDILNRAHHSKSPQSGTLDLAKSCLQKCL---CITAYLRLPLH---CHIKIANSCTYISAVCTYGS |
| ZF_02463417.1 | QLHTSCTGSTDILNRAHHSKSPQSGTLDLAKSCLQKCL---CITAYLRLPLH---CHIKIANSCTYISAVCTYGS |
| ZF_02629629.2 | QLHTSCTGSTDILNRAHHSKSPQSGTLDLAKSCLQKCL---CITAYLRLPLH---CHIKIANSCTYISAVCTYGS |
| TM271bail | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| 1L720bail | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |

| ZF_01696223.1 | GNGCPE66AAYTASWAAGCTHGCSCCHLLGIECRAIAFGRPTIVPDITICSEPP-----AVLERSAAMTCT--- |
| ZF_02668472.1 | GNGCPE66AAYTASWAAGCTHGCSCCHLLGIECRAIAFGRPTIVPDITICSEPP-----AVLERSAAMTCT--- |
| ZF_01373200.1 | GNGCPE66AAYTASWAAGCTHGCSCCHLLGIECRAIAFGRPTIVPDITICSEPP-----AVLERSAAMTCT--- |
| ZF_01722298.1 | GNGCPE66AAYTASWAAGCTHGCSCCHLLGIECRAIAFGRPTIVPDITICSEPP-----AVLERSAAMTCT--- |
| ZF_02463417.1 | GNGCPE66AAYTASWAAGCTHGCSCCHLLGIECRAIAFGRPTIVPDITICSEPP-----AVLERSAAMTCT--- |
| ZF_02629629.2 | GNGCPE66AAYTASWAAGCTHGCSCCHLLGIECRAIAFGRPTIVPDITICSEPP-----AVLERSAAMTCT--- |
| TM271bail | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| 1L720bail | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |

| ZF_01696223.1 | -----GCGQGQSAKSL استراوس تعرض يحظى به لمسات الأسمات --- ايلات GSM flotation |
| ZF_02668472.1 | -----GCGQGQSAKSL استراوس تعرض يحظى به لمسات الأسمات --- ايلات GSM flotation |
| ZF_01373200.1 | -----GCGQGQSAKSL استراوس تعرض يحظى به لمسات الأسمات --- ايلات GSM flotation |
| ZF_01722298.1 | -----GCGQGQSAKSL استراوس تعرض يحظى به لمسات الأسمات --- ايلات GSM flotation |
| ZF_02463417.1 | -----GCGQGQSAKSL استراوس تعرض يحظى به لمسات الأسمات --- ايلات GSM flotation |
| ZF_02629629.2 | -----GCGQGQSAKSL استراوس تعرض يحظى به لمسات الأسمات --- ايلات GSM flotation |
| TM271bail | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| 1L720bail | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |

| ZF_01696223.1 | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| ZF_02668472.1 | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| ZF_01373200.1 | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| ZF_01722298.1 | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| ZF_02463417.1 | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| ZF_02629629.2 | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| TM271bail | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
| 1L720bail | -----HCQHKVAVTCAAGGSKGKSANACTGGAASSIVKNSVQGVTACDCLAGGTPLAKHGDVD---QTLLKANHASSPTDY |
Figure 63. Cloning strategy of baiL gene from *C. scindens* VPI 12708.
Overexpression of recombinant baiL gene from *Clostridium hylemonae* TN271 in *E. coli* BL21 (DE3). “M” stands for Kaleidograph protein standard, Lane 1 = 30 μg soluble protein from IPTG uninduced *E. coli*, Lane 2 = 25 μg insoluble protein from IPTG uninduced *E. coli*, Lane 4 = 30 μg soluble protein from 1 mM IPTG induced *E. coli*, Lane 5 = 10 μg insoluble fraction 1 mM IPTG induced *E. coli*, Lane 6 = 15 μg insoluble fraction IPTG induced *E. coli*, Lane 7 = 30 μg insoluble fraction 1 mM IPTG induced *E. coli*. a). Cultivation in LB broth b). Cultivation in LBS broth (See Text).
was highly expressed, though found in inclusion bodies. Several attempts were made with different colonies, ranges of IPTG concentration (0.1-1mM), temperatures (18°C to 37°C) and media (LB, TYGPN). Indeed, we attempted the sorbitol/betaine method (medium LBS) of Blackwell and Horgan (1991) although results were similar between LB and LBS (Figure 64b) (193). We verified expression of His-tag using anti-hexahistidine antibodies in the insoluble, but were unable to detect any soluble recombinant BaiL-His (Figure 65).

The baiL gene was also cloned in a pET24(a+) vector as well as a pSport1 vector from both the SP6 and T7 promoters and overexpressed. In each case, the BaiL was located in the insoluble fraction. Attempts at refolding by solublization in 8M urea followed by step-down buffer exchanges with urea in the presence of DTT and arginine were unsuccessful in producing soluble protein.

Cloning and overexpression of the baiJ gene product in Escherichia coli.
Figure 65. Western blot analysis of baiL expression in soluble and insoluble fractions of IPTG induced E. coli cell extracts. Protein samples were electrophoresed on an SDS-PAGE gel (12%) and electroblotted onto nitrocellulose. The blot was probed with anti-hexahistidine antibody. Lane S=soluble fraction from 1 mM IPTG induced E. coli whole cells; Lane IS= insoluble fraction solubilized in 8.5 M urea. Each lane contains 20 μg protein. Expected size of recombinant baiL gene product is 27 kDa. Position of molecular weight markers shown to left of blot.
The \textit{baiJ} gene is predicted to encode a $3\text{-oxo-}\Delta^4\text{-DCA(}-\text{CoA})$ oxidoreductase. To test this hypothesis, the \textit{baiJ} gene was PCR amplified with primers 12708baiJ-F (5’-TGCATCTCC\textsc{ctcgag}GGCACATTATGGCTCCAGG-3’) and 12708baiJ-R (5’-TACAGGTAC\textsc{aagct}TACTGACTGTCCCGATGCT-3’). Double digestion of the pT7-SBP-2 expression vector and PCR product with \textit{XhoI} and \textit{KpnI} restriction endonucleases and ligation resulted in the pT7SB2baiJ vector (Figure 13). The vector was initially transformed into \textit{E. coli DH5$\alpha$} and subsequently cloned into expression host \textit{E. coli ArcticExpress BL21(DE3)}. After verifying the sequence and reading frame of the insert, the BaiJ gene was overexpressed by IPTG induction (0.8 mM). The ArcticExpress strain was chosen so that growth and thus expression could be slowed to allow for less inclusion body formation which was observed for the \textit{baiL} gene. Figure 66 shows the purification and immunoblot detection of the recombinant BaiJ gene product. The strep-tag was chosen based on results with the BaiH protein which lost activity following Ni$^+$ affinity chromatography. The strep-tag recognizes biotin, which should not result in chelation of metals or flavins. The BaiJ polypeptide was
Figure 66. SDS-PAGE and Western immunoblot of elution fraction after biotin binding chromatography of overexpression of recombinant BaiJ-SBP fusion protein. 10 μg protein was loaded onto a 12% polyacrylamide gel. MW (kDa) marker represented to left of SDS-PAGE (a) and right of immunoblot (b). Expected size of recombinant fusion protein: ~66 kDa.
purified to about 95% based on visual inspection of SDS-PAGE of eluent fraction. The eluent was concentrated by centrifugation on Centriprep YM10 (10 kDa cut-off) concentrators. The concentrated material was yellow in color; indicative of bound flavins such as observed for the BaiCD and BaiH proteins (121).

Synthesis of bile acid CoA thioester was tested by differential solubility. CoA esters of bile acids are water soluble at low pH, while free bile acids precipitate and can be extracted into organic solvents; particularly relatively hydrophobic bile acids such as 3-oxo-DCA. 30 μl CoA conjugate sample (90 μM; 25,000 DPM) was added to 500 μl reaction buffer (100 mM sodium phosphate, pH 6.8) and the pH was lowered to 3 with 1N HCl. The sample was extracted three times with two volumes ethyl acetate. The ethyl acetate fraction and 10μl aqueous phase were counted by liquid scintillation. The aqueous phase was then brought to pH 10 with 5N NaOH and boiled 30 min. The pH was then lowered to 3 with 10 N HCl and extracted three times with two volumes ethyl acetate. Again, the ethyl acetate fraction and the aqueous phase were counted.

Figure 67 shows that radioactivity is extracted only after
Figure 67. Measurement of radioactivity in both aqueous and organic phase before and after sodium hydroxide treatment. This assay was used to provide evidence for the presence of a CoA conjugate based on relative solubility. Ethyl acetate (ETOAC) extraction “before NaOH” represents organic extraction of the aqueous phase containing bile acid CoA conjugates. The pH was lowered to 3 with 1N HCl. Free bile acids are expected to partition to the organic phase, while conjugates are expected to remain in the aqueous phase. “After NaOH” represents the DPM value in the organic and aqueous phase once the aqueous phase from the first extraction was increased to pH 10 with 5 N NaOH, boiled for 30 min, and lowered to pH 3 with 5 N HCl. Extraction of the aqueous phase resulted in the majority of radioactivity partitioning in the organic phase, suggesting hydrolysis of CoA thioester.
A bar chart showing the DPM (Disintegrations Per Minute) levels before and after NaOH treatment. The chart compares ETOAC and Aqueous samples before and after treatment.

- Before NaOH: ETOAC has a very low DPM level, Aqueous has a much higher level.
- After NaOH: ETOAC has a significant increase in DPM, Aqueous shows a slight decrease.

The chart indicates a noticeable change in DPM levels due to the NaOH treatment, particularly for the ETOAC samples.
boiling at pH 10, suggesting that the CoA thioester is being hydrolyzed.

Initial enzyme assays were run for 30 min at 37°C in a 500 μl volume of 100 mM sodium phosphate buffer containing 90 μM [24-14C] 3-oxo-deoxycholyl-CoA (or the free acid) (550 DPM/nmole), 150 μM NAD(P)(H) and 30 μg purified BaiJ. When 3-oxo-DCA and NADP were substrates, a product of greater hydrophilicity than the substrate was obtained whose $R_f$ value was even lower than the commercial DCA standard (Figure 68). This strongly suggests the 3α-hydroxyl group is reduced in addition to an oxidation reaction. Additional characterization will be required to determine the function of this enzyme. In summary, the 62 kDa BaiJ gene was expressed and purified and initial enzyme assays suggest this polypeptide metabolizes bile acids; however, this enzyme does not appear to have 3-oxo-$\Delta^4$-DCA oxidoreductase activity.

Figure 68. Autoradiograph of a thin layer chromatography of BaiJ reaction products. Standards (not shown) are represented by arrows. 3-oxo-DCA and 3-oxo-DCA~SCoA (90 μM; 25,000 DPM) were used as substrates in a 500 μl reaction volume. The reaction buffer, 100 μM sodium phosphate (pH 6.8), containing 150 μM pyridine nucleotide (labeled) and 30 μg purified recombinant BaiJ-SBP.
Future characterization and studies of bai gene expression by RT and qRT PCR requires normalization using “housekeeping genes”. Several studies have used the gyrA subunit in this capacity (194, 195). At the time of study there was no genomic data for any 7α-dehydroxylating bacterium in the databases, which has since changed (currently Clostridium scindens VPI 12708 and Clostridium scindens ATCC 35704 by our lab and Jeffrey Gordon’s lab, respectively). The strategy for isolating the gyrA subunit from Clostridium hylemonae TN271 was to generate a multiple sequence alignment of several gyrA genes from various species of Clostridia in addition to two gyrA genes from Alkaliphilus oremlandii OhILAs. After a multiple alignment was generated and searched for regions of low codon degeneracy based on a codon usage table generated for C. hylemonae TN271 (Figure 69) two regions of high sequence identity and low codon degeneracy were identified. Figure 70 illustrates these regions in Logo form along with redundant oligonucleotide variations based on these sequence regions. PCR amplification using sixteen combinations of forward and reverse redundant oligonucleotides resulted in five positive reactions.
Figure 69. Codon usage table for *Clostridium hylemonae* TN271. The CodonW program was used to generate table using *baiJ*, *baiK* and *baiL* gene sequences as input.
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<th>Amino Acid</th>
<th>Frequency</th>
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</thead>
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<tr>
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<td>Tyr</td>
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<tr>
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1567 codons in baiAP2delta43HSD (used Universal Genetic code)
Design of degenerate oligonucleotides for isolation of the gyrA gene from *C. hylemonae* TN271. A CLUSTALW alignment was prepared from the gyrA gene from the following organisms: *Alkaliphilus oremlandii* OhILAs (ABW17577.1; EAT31502.1), *C. perfringens* str. 13 (NP_560923.1), *C. beijerinckii* NCIMB 8052 (YP_001307153.1), *C. tetani* E88 (NP_780806.1), *C. acetobutylicum* ATCC 824 (AAK77994.1). Short stretches of conserved amino acids were examined manually within this alignment for low codon degeneracy. Two regions were found spanning 235 amino acids which would give a PCR product of 705 bp. a) Logo representation of conserved residues 77-84 (*C. tetani* gyrA) identified in multiple sequence alignment of gyrA genes as highly conserved and low in codon degeneracy. Lowest degenerate forward primers (gyrAF1, gyrAF2) are listed. b) Logo representation of residues 299-307 (*C. tetani* gyrA) identified as highly conserved and containing residues with low codon degeneracy. The sense strand reverse translated is shown below amino acid residues (bold) with wobble based listed as “N”. “Rev Comp” is the reverse complementary strand for reverse oligonucleotides (gyrAR-gyrAR8).
### a. Primer Name                    Sequence

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<th>Sequence</th>
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</tr>
<tr>
<td>gyrAF2</td>
<td>GGV AAR TAY CAY CCH CAY GGV GAY</td>
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</tbody>
</table>

### b. Primer Name                    Sequence

<table>
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<th>Sequence</th>
</tr>
</thead>
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</tr>
<tr>
<td>gyrAR2</td>
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<tr>
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<td>TCK ATR CAK ATS CGC ATK CCY TCT CT</td>
</tr>
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<td>TCK ATS ACC ATT CTC ATK CCY TCT CT</td>
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<tr>
<td>gyrAR7</td>
<td>TCK ATS ACC ATT CTC ATK CCY TCS CG</td>
</tr>
<tr>
<td>gyrAR8</td>
<td>TCK ATS ACC ATS CGC ATK CCY TCT CT</td>
</tr>
</tbody>
</table>
Cloning of PCR product derived from primer pair gyrAF1/gyrAR4, sequencing of this product and alignment of the deduced amino acid sequence suggests the \textit{gyrA} gene has been isolated. Primers gyrAF1 and gyrAR8 were used to isolate the partial \textit{gyrA} gene from \textit{C. scindens} VPI 12708 (data not shown) which was cloned into a pCR8/GW/TOPO, sequenced and aligned with section of \textit{gyrA} gene from \textit{C. hylemonae} TN271. Following this data, the entire \textit{gyrA} gene from \textit{C. scindens} VPI 12708 was detected by local BLAST search of the 13X coverage of the \textit{C. scindens} VPI 12708 genome (Ridlon JM, Alves J, Kang D, Hylemon PB and Buck G unpublished data). A multiple sequence alignment of the \textit{gyrA} genes from \textit{C. hylemonae} TN271, \textit{C. scindens} VPI 12708 and \textit{gyrA} genes from clostridial genome data available from GenBank (Figure 72). A set of RT-PCR primers were designed to detect the \textit{gyrA} gene of \textit{C. hylemonae} TN271 (gyrATN271F 5’-ACTTTGACGACACGACACACGTAAC-3’; gyrATN271R 5’-CAGTATATGTTCGATCGTCGT) (primers used in Figure 26) and \textit{C. scindens} VPI 12708 (gyrA12708F 5’-ATCTATGGAGCACTGGTAAATATGG-3’; gyrA12708R 5’-AGTTAGGAGCAAAATCAACCATTAC-3’) which showed high
Figure 71. PCR amplification of gyrA gene from *Clostridium hylemonae* TN271. Combinations of oligonucleotides listed in Figure 65 were used against 50 ng *C. hylemonae* TN271 genomic DNA template. Combinations of primers used in PCR reaction are as follows: Lane M= 1kb DNA ladder; Lane 1= gyrAF1, gyrAR; Lane 2= gyrAF1, gyrAR2; Lane 3= gyrAF1, gyrAR3; Lane 4= gyrAF1, gyrAR4; Lane 5= gyrAF1, gyrAR5; Lane 6= gyrAF1, gyrAR6; Lane 7= gyrAF1, gyrAR7; Lane 8= gyrAF1, gyrAR8; Lane 9= gyrAF2, gyrAR; Lane 10= gyrAF2, gyrAR2; Lane 11= gyrAF2, gyrAR3; Lane 12= gyrAF2, gyrAR4; Lane 13= gyrAF2, gyrAR5; Lane 14= gyrAF2, gyrAR5; Lane 13= gyrAF2, gyrAR5; Lane 14= gyrAF2, gyrAR6; Lane 15= gyrAF2, gyrAR7; Lane 16= gyrAF2, gyrAR8. 750 bp PCR product from Lane 4 was cloned into a pCR8/GW/TOPO TA vector generating a ~3.5 kb vector p271gyrA.
Figure 72. Boxshade alignment of gyrA gene product from C. scindens VPI 12708 with other clostridial and bacterial gyrA genes. Alkaliphilus oremlandii OhILAs (ABW17577.1; EAT31502.1), C. perfringens str. 13 (NP_560923.1), C. beijerinckii NCIMB 8052 (YP_001307153.1), C. tetani E88 (NP_780806.1), C. acetobutylicum ATCC 824 (AAK77994.1).
specificity against genomic DNA from their respective target species. While preliminary studies suggest gyrA gene expression is not affected by bile acid induction, additional work must be done to verify the gyrA gene as an appropriate internal control for qRT-PCR. This represents an important step toward development of a quantitative assay to measure bai gene expression in C. scindens VPI 12708 and C. hylemonae TN271. In addition, these probes have yet to be tested on C. hiranonis sp. strain TO931 genomic DNA and may result in isolation of this gene from this organism as well.
Discussion

The first life on earth evolved in an anoxic environment. For several billion years, microorganisms have successfully exploited niches devoid of oxygen (196). In fact, it is interesting to note that one of the most densely inhabited environment on earth is the mammalian large intestine (>10^{11} bacteria/gram wet weight feces) (197, 198). To put this in perspective, the adult human body contains an order of magnitude more prokaryotic cells (10^{14}) than mammalian cells (10^{13}). The collective bacterial genomes (termed “microbiome”) of the human microflora encode an estimated 2-4 million genes, surpassing the human genome by a staggering 100-fold (199). Science continues to change the way we view ourselves, and our place in nature. Advances in genomics and GI microbiology suggest that the human body should be regarded as a superorganism—a complex ecosystem composed of all three domains of life (eukaryote, prokaryote and archaea) balanced by selective pressures from both the top-down and the bottom-up (200).
Intestinal bacteria and archaea can carry out hundreds, if not thousands, of enzymatic reactions not carried out by host cells. Relman and Falkow (2001) have called for the task of sequencing this microbiome, which they have called ‘the second human genome project’ (201). Intestinal microbes actively metabolize molecules of endogenous and exogenous origin. The products of this metabolism then interact with host cells with both beneficial and harmful consequences. Bile acid metabolism represents a particularly important aspect of microbial metabolism of endogenous substrates due to enterohepatic circulation, which allows accumulation of bacterial products of bile acid metabolism. Bile acids also represent a strong top-down selection pressure on potential colonizers (95). A microbe must be prepared to cope with mmol concentrations of bile salts and bile acids. Bile resistance is a major factor used to screen potential “probiotic” species (202). In addition, numerous studies have shown that bile acids induce expression of virulence genes in intestinal pathogens (95). Host-microbe co-evolution has resulted in widespread acquisition of
bile-salt metabolizing enzymes in all major gut colonizing microbial species (See introduction) (95).

Interestingly, it appears that genes involved in bile acid $7\alpha/\beta$-dehydroxylation are found in the tiniest fraction of *Firmicutes* classified within various clusters of the heterogenous genus *Clostridium* (189). $7\alpha$-dehydration of bile acids represents a microbial pathway which may significantly alter host physiology. Bile acids, once regarded as simple detergents involved in lipid digestion, are now recognized as potent signaling molecules (203). A full understanding of the biology and biochemistry of bile acid metabolism by intestinal bacteria represents an important and challenging area of research. The current work represents a significant step in understanding the biochemistry of bile acid $7\alpha/\beta$-dehydroxylation by intestinal clostridia.

The current work provides strong evidence that the *baiCD* and *baiH* gene products encode NAD$^+$-dependent 3-oxo-$\Delta^4$-cholenoic acid oxidoreductases recognizing $7\alpha$-hydroxy and $7\beta$-hydroxy bile acids, respectively (Figure 5). Previously, we demonstrated loss of the $5\beta$-hydrogen during $7\alpha$-dehydroxylation of $[5\beta^{-3}H] + [24^{-14}C]$-CA both *in vivo*
and from cell extracts of CA induced C. scindens whole cells suggesting introduction of a C₄-C₅ double-bond (118). Addition of [24⁻¹⁴C] 3-oxo-Δ⁴-cholenic acid to CA induced whole cells and cell-extracts of C. scindens VPI 12708 resulted in conversion to [24⁻¹⁴C] DCA (117). Subsequent studies elucidated the first two steps of the bile acid 7α/β-dehydroxylation pathway; CoA ligation (125) and oxidation of the 3α-hydroxy group producing a 3-dehydro-CA-CoA intermediate (58). Collectively, these data suggested the presence of an enzyme catalyzing the region-selective oxidation at C₄-C₅ yielding a 3-dehydro-Δ⁴-CA-CoA intermediate. Others have speculated that the baiH and baiCD genes were involved in C-C double bond oxidation/reduction based on homology to enzymes with similar function (204, 205). The fact that there are two homologous flavin oxidoreductases of similar M₀ (70-72 kDa) with 32% identity and 46% similarity that are co-expressed from a bai operon suggests a possible similar function.

This hypothesis was tested by incubation of a crude cell extract of E. coli overexpressing the baiCD gene with [24⁻¹⁴C] 3-dehydro-UDCA or [24⁻¹⁴C] 3-dehydro-CDCA, and
indeed the reaction yielded a 3-dehydro-Δ⁴-CDCA metabolite as suggested by mass spectrometry (Figure 19). 3-dehydro-UDCA did not appear to be a substrate of the baiCD gene product. Bile acid 7α-hydroxy groups are axial while 7β-hydroxy groups are equatorial. These data suggest that the key to substrate recognition between the baiCD and baiH is the stereochemistry of the 7-hydroxy group, whose orientation apparently requires a different architecture in the substrate binding pocket. Taken together these results suggest these homologous enzymes act at an analogous step in the 7-dehydroxylation pathway, and therefore, two distinct branches of the oxidative arm of the pathway exist in C. scindens and C. hiranonis.

The BaiH is a homotrimer composed of 72 kDa subunits, each subunit containing 1 mol of FMN and 1 mol of FAD per subunit. BaiH is homologous to a class of flavoenzymes involved in reduction of unsaturated fatty acids and aldehydes (Table 2) (129). Enoate reductase (enr) from Clostridium tyrobutyricum catalyzes the reduction of C-C double bonds of several α/β-unsaturated aldehydes as well as cyclic ketones and methylketones (184). Enr is a dodecamer with subunit Mr of 73 kDa each containing a 4Fe-
4S cluster and 1 mol of FMN and 1 mol of FAD (206). 2, 4-
dienoyl-CoA reductase from E. coli is a 73 kDa monomeric
enzyme that reduces C-C double bonds with NADPH and
contains FMN, FAD as well as a 4Fe-4S cluster (182, 207).
The noxB gene from Archaeoglobus fulgidus encodes an NADH
oxidase of unknown physiological function (204). The noxB
gene product is a 69 kDa monomer containing 1 mol FAD and
shares the same conserved ferredoxin domain (C-2X-C-2-3X-
C-11-12X-C) with each of these proteins including the baiH
and baiCD gene products (204). The noxB-2 gene shares 98.9
% identity with the noxB-1 gene from A. fulgidus and is
found upstream of medium-chain acyl-CoA ligase in this
bacterium suggesting these genes are also involved in
fatty acid metabolism (204). The more distantly related
Old Yellow Enzyme (OYE) family homologues are found both
in eukaryotes such as Saccaromyces cerevisiae (OYE1, OYE2)
(205) and S. carlsbergensis (OYE1) (208), as well as the
prokaryotes including Bacillus subtilis (YqjM) (209), and
Shewanella oneidensis (SYE1-SYE4) (210). OYE genes have
been extensively characterized both biochemically and
structurally (206, 209, 211). Numerous compounds act as
electron acceptors including various quinones, as well as
\(\alpha, \beta\)-unsaturated aldehydes and ketones which are reduced at the olefinic bond (211); however, only recently have physiologically relevant electron acceptors been identified. The oye2 and oye3 gene from Sac. cerevisiae, sye1 from She. oneidensis, and the yqjM gene from B. subtilis were shown to be inducible by small \(\alpha/\beta\)-unsaturated aldehydes during oxidative stress responses to lipid peroxidation (186, 210, 212) and actin cytoskeletal oxidative damage in yeast (213). Detoxification does not appear to be the function of the bile acid 7\(\alpha/\beta\)-dehydroxylating pathways, but rather the substrates appear to serve as electron sinks to enhance fermentation (95).

Electron flow in the E. coli DCR as determined by redox titrations (214), and crystallography (182) show that two reducing equivalents are supplied to FAD by direct hydride transfer and follow an electron flow from FAD to a 4Fe-4S cluster to FMN and finally to the oxidized substrate. OYE(s) reduce C=C double bonds through a ping-pong bi-bi mechanism in which FMN is reduced by NADPH, followed by substrate binding and hydride transfer from FMNH\(_2\) to the C\(_\beta\) carbon of the substrate (215). Additional
studies will be required to determine the mechanism of the reaction catalyzed by the \textit{baiCD} and \textit{baiH} gene products.

We observed low NAD(H)-dependent 3-oxo-\(\Delta^4\)-cholenoic acid oxidoreductase activity based on the amount of products formed, as observed by autoradiography of TLC (Figure 16). However, we would predict that the physiological substrates for these enzymes are the CoA conjugates of 3-oxo-cholanoic acids. This is based on the observations that the \textit{baiA} gene product encodes a 3\(\alpha\)-hydroxysteroid dehydrogenase that has high specificity for coenzyme A conjugated bile acids (58). However, large quantities of the CoA derivatives of 3-dehydro-CDCA and 3-dehydro-UDCA will have to be chemically synthesized to test this hypothesis. In this regard, we have detected NAD(H)-dependent 3-oxo-\(\Delta^4\)-cholenoic acid oxidoreductase activity using small quantities of enzymatically generated radiolabeled CoA conjugates of 3-dehydro-UDCA and 3-dehydro-CDCA (D. Kang, J.M. Ridlon, and P.B. Hylemon, unpublished data). Moreover, other members of this gene family recognize substrates linked to CoA (Table II). Future mechanistic and structural studies will require
chemical synthesis of CoA conjugated bile acid intermediates.

This is the first report of a gene product specifically involved in 7β-dehydroxylation of UDCA. We predict that the *bai*I gene encodes a bile acid 7β-dehydratase, based on its amino acid sequence identity (20%) and similarity (40%) with the *bai*E gene product as well as co-expression of this gene product during CA induction in *C. scindens* VPI. We have previously demonstrated that the *bai*E gene encodes a bile acid 7α-dehydratase (128). Determining the function of the *bai*I as well as identifying genes involved in reducing the 3-oxo-4, 6-choladienoic structure to LCA will be the focus of future of research.

In summary, these data indicate that the *bai*CD and *bai*H genes encode NAD(H)-dependent 3-oxo-Δ4-cholenoic acid oxidoreductases. These results can account for the ability of this organism to use UDCA as a substrate for 7β-dehydroxylation, and provide additional support for formation of a 3-oxo-Δ4-cholenoic acid intermediate during bile acid 7-dehydroxylation.
Doerner et al (1997) demonstrated that 7α/β-dehydroxylating bacteria cluster into two groups based on relative rates of conversion of CA to DCA (189). Our current understanding of the molecular biology and enzymology of bile acid 7α/β-dehydroxylation come from study of *C. scindens* VPI 12708 and *C. hiranonis* sp. strain TO931 which are “high activity strains” (1-7 nmol mg⁻¹ h⁻¹). Genes involved in bile acid 7α/β-dehydroxylation have not been identified in a “low activity” strain (0.05-0.16 nmol mg⁻¹ h⁻¹). Wells et al. (2003) detected the baiCD gene from the low activity strain *C. hylemonae* TN271 which facilitated design of bidirectional genome-walking PCR primers (106). The genome-walking by PCR technique is useful for amplification of unknown stretches of DNA provided at least a small amount of adjacent sequence data is available for design of gene specific primers. Adaptor sequences are engineered and ligated to restriction libraries of total genomic DNA. These adaptor molecules provide the second stretch of known sequence, allowing PCR amplification between adaptor-specific and gene-specific primers (216). PCR products up to ~4 kb were obtained in
this study, with an average of 2 kb using a starting genomic template >48 kb (Figure 21).

Two upstream genome-walking PCR products (Figures 22, 23) and three downstream genome-walking PCR products (Figures 28, 30, 31) were cloned, sequenced and assembled, along with the partial baiCD gene into a single contiguous sequence of ~9.5 kb. ORFs were identified by the ORF Finder program and BLAST searches were used to determine the putative function of these ORFs. The gene organization of the baiBCDEFGHI operon from C. hylemonae TN271 was almost identical to the baiBCDEAFGHI operon from C. scindens VPI 12708 (Figure 32). Table 7 compares both nucleotide and amino acid sequence identity/similarity between bai genes from C. hylemonae TN271 and C. scindens VPI 12708. Nucleotide sequence identity was >65% and amino acid sequence identities ranging from 61.4 to 88% with similarity ranging from 76.6% to 94.8%. The upstream putative “promoter” regions were also found to be highly conserved along with relative location of TIS (Figures 24, 25). Finally, a conserved transcriptional regulator of the AraC/XylS family (barA) was located upstream of the C. hylemonae TN271 baiB gene and
“promoter” region on the antisense strand (Figures 27, 32). It is unclear at present the reason for the 10 fold difference in levels of bile acid 7α-dehydroxylating activity between C. hylemonae TN271 and “high activity” strains. However, it is very likely that this regulation is at the transcriptional level. Indeed, design of qRT-PCR oligonucleotides against genes in the baiBCDE(A)FGHI operons from C. hylemonae and C. scindens VPI 12708 and quantitation of message following bile acid induction should provide the relevant information. Isolation and sequencing of the gyrA gene in the current study and current genomic data for Clostridium scindens will allow normalization of qRT-PCR results. Finally, it is interesting to note that C. hylemonae TN271, despite having a baiH and baiI gene, were unable to 7β-dehydroxylate UDCA over a 30 min or overnight incubation following induction by CA (Figure 33). It is unclear at present the reason for this loss-of-function. Importantly, this data represents the first characterization of bai genes from a “low activity” 7α-dehydroxylating bacterium.
The **baiA** gene from *C. hylemonae* TN271 was isolated by PCR amplification using redundant oligonucleotides whose design was based on a nucleotide multiple sequence alignment of **baiA** genes isolated from *C. scindens* VPI 12708 and *C. hiranonis* sp. strain TO931 (Figures 34, 35, 37). 5’ SMART RACE PCR analysis of the **baiA** transcript revealed a TIS 56 bp upstream of the **baiA** gene and downstream of the putative **bai** promoter region (Figure 36). Gopal-Srivastava et al (1990) identified multiple copies of the **baiA** gene in *C. scindens* VPI 12708, two of which were monocistrons with conserved **bai** “promoters” (67).

Completion of the **baiA** gene sequence, facilitated by bidirectional genome-walking from the partial sequence obtained with primers **baiA271F** and **baiA271R** (Table 8) resulted in isolation of genes with predicted function suggested to be involved in bile acid 7α/β-dehydroxylation. *In silico* (ORF Finder, BLAST) analysis upstream of the **baiA** gene revealed a deduced 26 kDa short-chain dehydrogenase/reductase (**baiL**), a 49.5 kDa **baiF** homologue (**baiK**) and a putative 62 kDa 3-keto-steroid Δ^1^-dehydrogenase (**baiJ**). Upstream of the **baiJ** gene was a
conserved bai “promoter” region (Figure 44). 5’ SMART RACE analysis suggests the TIS is at the very 5’ end of this conserved region while the TIS is downstream of this conserved region in the baiB and baiA operons of both C. scindens VPI 12708 and C. hylemonae TN271 (Figure 45). This would suggest that the baiJ promoter is upstream of this conserved region.

In order to determine whether these novel genes were conserved in C. scindens VPI 12708, the baiJKL genes from C. hylemonae were analysed for 7-8 amino acid stretches of low codon degeneracy (Figures 46, 47). A region within the baiJ gene allowed design of redundant oligonucleotides which allowed isolation of a partial baiJ gene from C. scindens VPI 12708. Several upstream and downstream genome-walks resulted in identification of the baiJKL genes in C. scindens VPI 12708 which were highly conserved both in sequence and organization to the baiJKL genes from C. hylemonae TN271 (Figure 56). Elucidation of the TIS for the baiJ gene provided similar results to those obtained for TIS of the baiJ gene from C. hylemonae in respect to the upstream position relative to the conserved “promoter” region identified previously for the baiB and
baiA genes from *C. scindens* VPI 12708 (124, 191), *Clostridium hiranonis* sp. strain TO931 (59) and here for the baiB gene of *Clostridium hylemonae* TN271 (Figure 26). This brings into question whether this conserved region is indeed a bile acid inducible promoter rather than a repressor or operator sequence. Figure 73 is a Weblogo representation of a CLUSTALW alignment of these conserved upstream regions between *C. scindens* VPI 12708, *C. hiranonas* sp. strain TO931 and *C. hylemonae* TN271. Further experimental work is necessary to determine the function of these regions.

A particularly interesting find in this study is a predicted Type III CoA transferase which appears to share a common evolutionary history with the baiF gene (Figure 42). This gene has been given the preliminary name baiK (Figure 58). Alignment of characterized type III CoA transferases such as the crotonoyl-CoA (*caib*) and *yfdw* gene products from *E. coli* as well as the formyl CoA transferase from *Oxalobacter formigenes* with the baiF and baiK gene products from *C. scindens* VPI 12708 and *C. hylemonae* TN271 demonstrates a remarkable degree of conservation (Figure 74). Numerous authors have
Figure 73. Logo representation of bai promoter regions from *Clostridium scindens* VPI 12708, *Clostridium hiranonis* sp. strain TO931 and *Clostridium hylemonae* TN271. Promoter sequences from Figure 24 and Figure 49 were aligned using T-COFFEE and FASTA format was run in the Weblogo program (http://weblogo.berkeley.edu/). Large letters represent based of potential biological relevance to promoter function.
Figure 74. Boxshade alignment of baiF and baiK genes from *C. scindens* VPI 12708 and *C. hylemonae* TN271 with homologues in the Type III CoA transferase family. Proteins used in alignment include: *E. coli* Crotonobetainyl-CoA:carnitine CoA-transferase (CaiB) (P31572), *E. coli* Formyl-CoA transferase (YfdWA) (P69902), *Oxalobacter formigenes* formyl-CoA transferase (fCTOx) (AAC45298).
speculated that CoA transfer proceeds through Asp^{169} acylation (217-219). Mutagenesis of Asp^{169} (D169A, D169S, D169E) in the formyl-CoA transferase (FRC) from O.formigenes resulted in abolition of, or appreciable decrease in specific activity despite no significant structural alterations (217). The proposed catalytic mechanism for the FCR suggests that formyl-CoA forms an acid anhydride with Asp^{169} resulting in elimination of \( -SCoA \). The second substrate, oxalate, then nucleophilically attacks the Asp^{169}-formate anhydride resulting in release of formate and formation of Asp^{169}-oxalate anhydride. The anion \( -SCoA \) then attacks the carbonyl carbon of oxalate resulting in release of oxalyl-CoA and regeneration of Asp^{169} (217). Asp^{169} is conserved in the \( baiF \) and \( baiK \) genes of C. scindens VPI 12708 and C. hylemonae TN271 suggesting that these proteins act through a similar mechanism (Figure 74). Crystallization with CaiB yielded crystals with only CoA bound suggesting hydrolysis occurs in the absence of the CoA accepting substrates (220). This would provide a plausible explanation for observed CoA hydrolase activity shown for the \( baiF \) gene product in the absence of co-substrate
We speculate that a similar mechanism is involved in bile acid CoA transfer by the \textit{baiF} and possibly the \textit{baiK} genes to that proposed for the FRC. Isolation of a 3-oxo-Δ⁴-DCA~CoA intermediate from cell extracts of cholic acid induced cultures of \textit{C. scindens} VPI 12708 would suggest that CoA transfer would occur at this step or possibly following formation of 3-oxo-DCA or DCA. Therefore, these three metabolites would substitute for formate in the FRC model and cholyl-CoA would substitute for oxalate in the FRC model.

There are several gene homologues in the oxidative arm of the 7α/β-dehydroxylation pathway performing a similar function but recognizing a distinct stereoisomer. Indeed, in the present work, the \textit{baiCD} and \textit{baiH} gene products were shown to act as 3-oxo-Δ⁴-steroid oxidoreductases recognizing 7α-hydroxy and 7β-hydroxy bile acids, respectively. Additionally, the \textit{baiE} was shown to 7α-dehydrate bile acids while the \textit{baiI} is predicted to 7β-dehydrate bile acids. In each case, stereospecificity is directed at the orientation of the 7-hydroxyl group. However, in the reductive arm of the 7α/β-dehydroxylation the only differences include hydroxylation at C12, and
stereochemical differences about the A/B ring. Assuming the \textit{baiK} gene is involved in bile acid CoA transfer, there are a few possibilities: (1) one transferase recognizes LCA (not hydroxylated at C12) while the other recognizes DCA (hydroxylated at C12) (2) one transferase recognizes DCA and LCA (A/B ring in “cis” orientation) while the other transferase recognizes alloDCA and alloLCA (A/B ring in “trans” orientation) (3) both transferases recognize the same substrate(s); similar to the multiple copies of the \textit{baiA} genes (124). Testing these possibilities will require acquisition of alloDCA (not available commercially; highly concentrated in rabbit gallstones (221)) and synthesis of 3-oxo-alloDCA(\textasciitilde\text{SCoA}).

The \textit{baiL} gene is predicted to encode a 3-keto-acyl reductase, similar in function to the \textit{baiA} gene. The predicted function in terms of bile acid 7\(\alpha\)-dehydroxylation is NAD(P)H dependent 3\(\alpha\)-hydroxysteroid dehydrogenation resulting in DCA and LCA. An attempt was made to overexpress the \textit{baiL} gene in \textit{E. coli} as a hexa-histidine tagged fusion protein. Unfortunately, and often, the production of biologically active recombinant proteins is prevented by the formation of insoluble
inclusion bodies. Numerous variables were modified to improve solubility including decreased temperature, IPTG concentration, different media, various host strains, and vectors. However, while the protein was highly expressed, I was unable to detect soluble BaiL by Western immunoblot or NAD(P)-dependent 3α-HSDH activity against DCA. It may be necessary to experiment with additional fusion tags, further decrease in temperature, or attempt expression in a different host. Shuttle vectors have been developed between *E. coli* and gram-positive bacterial genera including *Clostridium* (222).

The *baiJ* gene is predicted to encode a 62 kDa flavoprotein similar to fumarate reductase and 3-ketosteroid-Δ^1^-dehydrogenases. Alignment of the *baiJ* gene product with the fumarate reductase from *Shewanella putrefaciens* MR-1, whose structure has been elucidated, suggests the similarity between these proteins is in the Rossman fold of the FAD-binding motif. The BaiJ lacks the cytochrome domain containing four conserved heme-binding sites common to fumarate reductases (CXXCH) and there was no conservation of active-site amino acids (data not shown). The *baiJ* gene was cloned into a pT7-SBP-2 vector
containing an N-terminal Streptavidin Binding Peptide (SBP) fusion tag. IPTG induced overexpression of the BaiJ polypeptide resulted in a soluble protein ~64 kDa which was detected by anti-SBP antibody (Figure 66). Initial characterization of the enzyme with radiolabelled 3-oxo-DCA and 3-oxo-DCA-CoA resulted in three products formed when NADP was used as co-substrate. The two hydrophobic products were also detected when NAD\(^{+}\) was co-substrate. A substrate of greater hydrophilicity than DCA was detected in the presence of NADP. This class of enzyme is predicted to metabolize C=C double bonds. This hydrophilic product is puzzling because the migration suggests the 3-oxo group has been reduced. Further, the reduced migration may indicate oxidation of the bile acid structure as there is no other source of reducing equivalents. It is possible that a double bond is being introduced in the side-chain of the steroid rings. Indeed, \(\Delta^{22}\)-bile acids have been identified in rats (223). Additional studies will be required to determine the identity of these bile acid metabolites. These findings may indicate a novel bile acid metabolic activity in C. scindens VPI 12708.
Future studies may include determining which genes are co-transcribed in both the $baiBCDEFGHI$ and $baiJKL$ operons from $C.\ hylemonae$ TN271 and the $baiJKLM$ operon from $C.\ scindens$ VPI 12708. A sensitive method for determining this would be PCR amplification of cDNA fragments containing intergenic regions between adjacent ORF’s. Additionally, attempts to isolate $bai$ genes from $C.\ leptum$ and $C.\ sordellii$ using degenerate oligonucleotides based on conserved regions in the oxidative $bai$ genes from $C.\ scindens$ VPI 12708, $C.\ hylemonae$ TN271 and $C.\ hiranonis$ sp. strain TO931 have failed (Ridlon JM and Hylemon PB, unpublished data). It would be interesting to try and design degenerate oligonucleotides to isolate the $baiJKL$ genes, if present, in $C.\ leptum$. This group of “low activity” organisms was not detected by Southern blot (189) or by PCR using primers against the $baiCD$ gene (106). Isolation of $bai$ genes from this organism could allow design of gene probes which could be used to quantify levels of a wide group of known 7α-dehydroxylating bacteria. Additionally, large-scale metagenomic sequencing will provide a wealth of information which will, in the future, allow in silico
mining of putative bai genes. Another study of interest would be to determine whether the baiF and baiK genes encode bile acid CoA transferases, and determine physiological substrate(s) through kinetic analysis. The limiting factor in such a study is chemical synthesis of numerous bile acid intermediates as both free acids and CoA conjugates. In addition, comparison of wild-type proteins with site-directed mutagenesis of Asp\(^{169}\) would provide additional evidence for a common mechanism of the baiF and baiK with that of the FRC protein.

Finally, the major aim of research into bile acid 7α-dehydroxylation is to identify targets for development of specific inhibitors aimed at lowering 7α-dehydroxylation, and thus secondary bile acids in individuals with high levels (Figure 11). A particularly interesting gene was located upstream of the baiJKL genes and directly downstream, and on the opposite strand relative to the baiA gene in C. scindens VPI 12708 (Figure 58). The ORF encodes a protein similar to the TspO/MBR family of cell surface sensors. TspO, or Tryptophan Rich Sensory protein, is a negative regulator of photosynthesis in response to light/oxygen. The tryptophan rich region acts
as part of a hydrophobic pore that acts in pumping porphyrin across the cell wall. Could it be that this protein, in 7α-dehydroxylating bacteria, may be involved in regulation of 7α-dehydroxylating gene expression and efflux of hydrophobic secondary bile acids from the cell? MBR, or mitochondrial peripheral benzodiazepine receptor, has been shown to substitute for bacterial TspO (224) and is thought to function in cholesterol transport across the mitochondrial membrane. If this gene product could be shown to regulate bile acid repression/efflux, this could serve as potential drug target; particularly because this polypeptide is predicted to be located in the outer membrane. Also, an ABC transport protein was located directly downstream of the baIL gene; possibly co-transcribed. This suggests that this polypeptide may be involved in efflux of secondary bile acids. ABC transporters have been targeted for chemotherapy due to their ability to transport antibiotics and other cytotoxins from the cell (225). This protein, if involved in bile acid efflux, may serve as a potential drug target. A possible approach for studying the function of these genes is insertional inactivation. Recently, a new vector
has been developed specifically for insertional inactivation of clostridial genes, which has been named ClosTron (226). Phenotypic tests could be performed in the presence and absence of bile acids. Ultimately, a goal for future research will be to identify a target for drug development to lower secondary bile acid production in patients with high levels of these cytotoxic, and co-carcinogenic metabolites produced by the human microbiome.
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