

Identification and characterization of two bile acid coenzyme A transferases from *Clostridium scindens*, a bile acid 7 α -dehydroxylating intestinal bacterium

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Abstract The human bile acid pool composition is composed of both primary bile acids (cholic acid and chenodeoxycholic acid) and secondary bile acids (deoxycholic acid and lithocholic acid). Secondary bile acids are formed by the 7 α -dehydroxylation of primary bile acids carried out by intestinal anaerobic bacteria. We have previously described a multistep biochemical pathway in *Clostridium scindens* that is responsible for bile acid 7 α -dehydroxylation. We have identified a large (12 kb) bile acid inducible (bai) operon in this bacterium that encodes eight genes involved in bile acid 7 α -dehydroxylation. However, the function of the baiF gene product in this operon has not been elucidated. In the current study, we cloned and expressed the baiF gene in *E. coli* and discovered it has bile acid CoA transferase activity. In addition, we discovered a second bai operon encoding three genes. The baiK gene in this operon was expressed in *E. coli* and found to encode a second bile acid CoA transferase. Both bile acid CoA transferases were determined to be members of the type III family by amino acid sequence comparisons. Both bile acid CoA transferases had broad substrate specificity, except the baiK gene product, which failed to use lithocholyl-CoA as a CoA donor. Primary bile acids are ligated to CoA via an ATP-dependent mechanism during the initial steps of 7 α -dehydroxylation. The bile acid CoA transferases conserve the thioester bond energy, saving the cell ATP molecules during bile acid 7 α -dehydroxylation. ATP-dependent CoA ligation is likely quickly supplanted by ATP-independent CoA transfer.—Ridlon, J. M., and P. B. Hylemon. Identification and characterization of two bile acid coenzyme A transferases from *Clostridium scindens*, a bile acid 7 α -dehydroxylating intestinal bacterium. *J. Lipid Res.* 2012. 53: 66–76.

Supplementary key words secondary bile acids • deoxycholic acid • HPLC • protein expression

The human liver synthesizes two primary bile acids from cholesterol, chenodeoxycholic acid (CDCA; 3 α , 7 α -dihydro-5 β -cholan-24-oic acid) and cholic acid (CA; 3 α , 7 α , 12 α -trihydroxy-5 β -cholan-24-oic acid). Bile acids are conjugated to either taurine or glycine before active secretion into bile and in this form are termed bile salts. Bile salts function to solubilize lipids and lipid soluble vitamins from the duodenum through the jejunum of the small intestine. Upon reaching the terminal ileum, bile salts are actively transported across the intestinal epithelium into the portal blood and returned to the liver. This process is termed enterohepatic circulation and is ~95% efficient. However, roughly 400-600 mg of bile acids enter the large intestine every day, where they are metabolized by a diversity of microorganisms (1). Bile salts are rapidly deconjugated and converted to the secondary bile acids, deoxycholic acid (3 α ,12 α -trihydroxy-5 β -cholanoic acid) and lithocholic acid (3 α -hydroxy-5 β -cholan-24-oic acid) from CA and CDCA, respectively.

Decades of research strongly suggest that secondary bile acids are involved in human disease processes, including cancers of the colon, esophagus, and biliary track (2), as well as cholesterol gallstone disease in some patients with high levels of deoxycholic acid in bile (3). The level of deoxycholic acid in bile is believed to be controlled by two major factors: *i*) levels and activities of bile acid 7 α -dehydroxylating gut bacteria (3) and *ii*) colonic transit time (4). Colonic pH may also be a minor factor. Therefore, an understanding of the genetics and enzymology of bile acid 7 α -dehydroxylation is an important first step in finding ways to decrease secondary bile acids with potentially beneficial outcomes to the host.

Several bacterial species in the genus *Clostridium* have been isolated and shown to convert primary bile acids into secondary bile acids, a process termed bile acid

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Abbreviations: ADCA, allodeoxycholic acid; β -MCA, β -muricholic acid; bai, bile acid inducible; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FRC, formyl-CoA transferase; HSDH, hydroxysteroid dehydrogenase; LCA, lithocholic acid; ORF, open reading frame; UDCA, ursodeoxycholic acid.

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7 α -dehydroxylation (1). We previously proposed a multi-step biochemical pathway for bile acid 7 α -dehydroxylation (Fig. 1). In addition, we characterized the bile acid-inducible (*bai*) operons from *C. scindens* VPI 12708 (5), *C. hiranonis* DSM13275 (6), and *C. hylemonae* DSM 15053 (7) that encode enzymes involved in the initial steps of the bile acid 7 α -dehydroxylation pathway (Fig. 2). The current model of bile acid 7 α -dehydroxylation suggests that free primary bile acids are actively transported into the bacterial cell by a proton-dependent bile acid transporter encoded by the *baiG* gene (8). Once inside, the primary bile acid is ligated to CoA in an ATP-dependent manner by the *baiB* gene product (9). The *baiA* gene encodes a bile acid, 3 α -hydroxysteroid dehydrogenase, specific for primary bile acid CoA conjugates (10). We recently reported that the *baiCD* and *baiH* gene products encode stereospecific 3-dehydro-4-bile acid oxidoreductases recognizing 7 α -hydroxy bile acids (CA, CDCA) and 7 β -hydroxy bile acids [ursodeoxycholic acid (UDCA),; 3 α , 7 α -dihydro-5 β -cholan-24-oic acid], respectively (11). The rate-limiting and irreversible step in this pathway is 7 α -dehydration. This reaction is catalyzed by bile acid 7 α -dehydratase, which is encoded by the *baiE* gene (12). The 3-dehydro-4,6-bile acid intermediate is then sequentially reduced and exported from the cell; however, genes in the “reductive arm” of the pathway have yet to be identified. Previously, we showed that the *baiF* gene product hydrolyzes bile acid CoA conjugates (13). However, others and we have hypothesized, based on amino acid sequence comparisons with known CoA transferases, that this gene product may be a bile acid CoA transferase (1, 14). Here, we present strong evidence that the *baiF* gene encodes a bile acid CoA transferase with broad bile acid substrate specificity. In addition, we report the discovery and characterization of a second bile acid CoA transferase encoded on what appears to be second multigene operon involved in bile acid metabolism in *C. scindens*.

Chemicals and bacterial strains

Clostridium scindens ATCC 35704 was purchased from American Type Culture Collection. *C. scindens* VPI 12708 was stored as a 30% glycerol stock at -80°C prior to this study, and working stocks were cultivated in chopped meat broth. *E. coli* BL21 (DE3) RIL was purchased from Agilent Stratagene (Santa Clara, CA). *E. coli* was cultivated in Luria-Bertani medium containing appropriate antibiotics. *C. scindens* strains were cultivated in brain/heart infusion broth under nitrogen. Bile acids were purchased from Sigma-Aldrich (St. Louis, MO) and Steraloids (Newport, RI). ADCA was a generous gift from Dr. James P. Coleman, East Carolina University. CoA was purchased from Sigma-Aldrich.

Isolation of genomic DNA and genome-walking by PCR

Genomic DNA was isolated from *C. hylemonae*, *C. scindens* VPI 12708, and *C. scindens* ATCC 35704 by enzymatic treatment, followed by bead beating as described previously (7). Genome-walking DNA libraries were prepared using the Universal GenomeWalker Kit (Clontech) according to the manufacturer. The DNA extraction method used in this application and PCR conditions have been described previously (7).

RNA isolation and transcriptional analysis

Total RNA was isolated as described previously (7). cDNA for reverse transcriptase PCR was generated with the Advantage RT-for-PCR kit (Clontech). cDNA for determination of transcription initiation site was generated using the SMART-RACE cDNA synthesis kit (Clontech) according to the manufacturer, with the exception that a gene-specific primer replaced 5' CDS primer. The gene-specific primer for determination of the *baiJ* gene transcription initiation site was SR12708J 5'-TTC TAT GTC CTC ATC CGT TGC CCC TG-3'. The gene-specific primer for determination of the *baiJ* gene transcription initiation site was DSM 15053JRace1 5'-TGC GGT AGA GCC TCC TGT GAT ACC TTG-3'. PCR was performed using the Advantage-GC 2 PCR Kit (Clontech) according to the manufacturer. An amount of 0.5 μl GC-Melt was added to each 50 μl reaction. PCR products were excised from agarose gels,

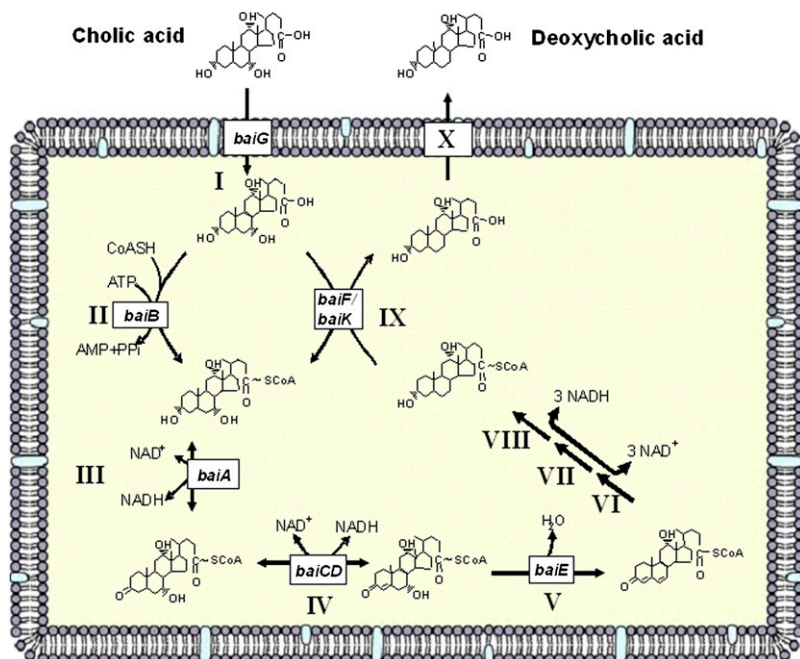


Fig. 1. Current model of the bile acid 7 α -dehydroxylation pathway in *Clostridium scindens* VPI 12708.

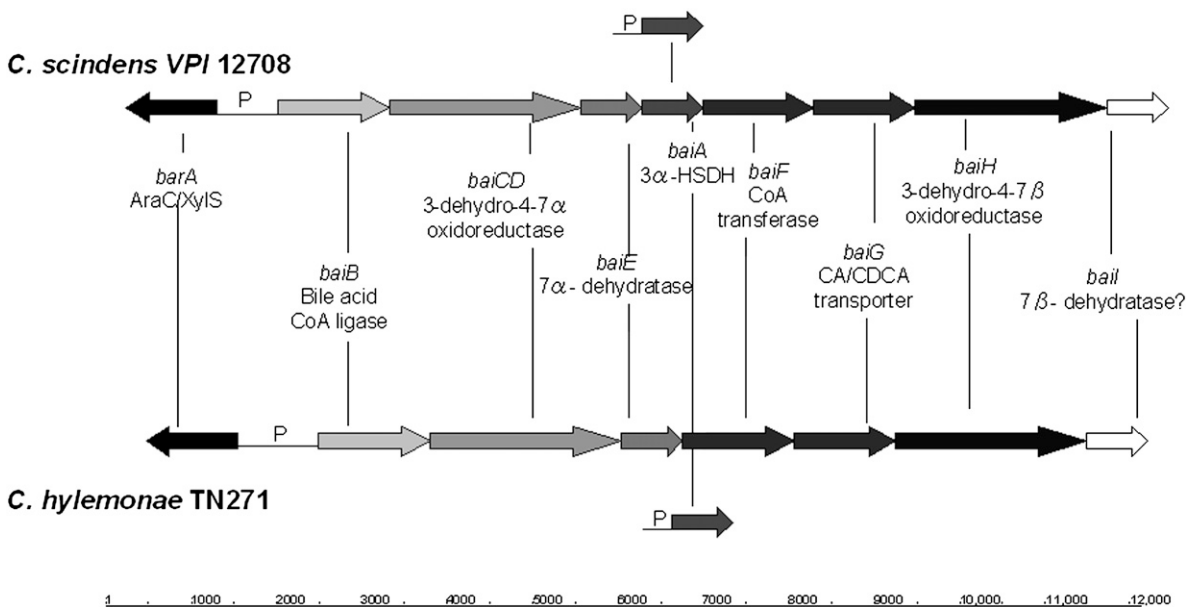


Fig. 2. Schematic representation of bile acid 7 α -dehydroxylation operons from *C. scindens* VPI 12708 and *C. hylemonae* DSM 15053. “P” represents putative promoter regions.

GENECLEAN purified, cloned into a pCR 8 GW TOPO TA vector, and sequenced at the Virginia Commonwealth University Nucleic Acid Research Facility.

Cloning the *baiF* and *baiK* genes

The *baiF* gene insert was amplified with forward primer 5'-CTG-CAGAGAAAAGGAGGTATAAAAAACATGGCTGG-3' (*Pst*I site in italics) and reverse primer 5'GGATCCTATTTTTTCGAAGTGGG-TGGTGGCTCCACTCCTCTTTCTTCTCATATGT-3' (*Bam*HI site in italics). The *baiK* gene insert was amplified with forward primer 5'-CTG CAG GGA AAG GAG GTT ATA GGA ATG AAA GG-3' (*Pst*I site in italics) and reverse 5'-GGA TCC TTA TTT TTC GAA CTG CGG GTG GCT CCA TTC TTC CTC CTT TTT CCA ACG TTC-3' (*Bam*HI site in italics). The streptavidin tag sequence (bold) was incorporated into the C terminus of bile acid CoA transferases for one-step purification (15). PCR products were cloned into a pCR8GW TOPO TA vector (Invitrogen) and transformed by heat shock at 42°C into One Shot Chemically competent *E. coli* that accompanies the vector. Plasmids were isolated using the Qiaprep Miniprep Kit (Qiagen) and double-digested with *Pst*I and *Bam*HI (New England Biolabs). Expression vector pSport-1 was double-digested with the same restriction enzymes, and both inserts and pSport-1 linear plasmid were gel-purified using the GENECLEAN Spin Kit (MP Biomedicals). Ligation was performed using T4 DNA ligase according to the manufacturer (New England Biolabs). Ligated vector was transformed into *E. coli* DH5 (NEB 5 α ; New England Biolabs) and cultivated in LB medium containing 100 μ g/ml ampicillin. pSport vectors containing insert (hereafter referred to as pSport**baiF**CTSBP and pSport**baiK**CTSBP) as determined by double-digestion and agarose gel electrophoresis were sequenced at the Nucleic Acid Core Facility at Virginia Commonwealth University. Expression vectors were transformed into *E. coli* BL21 (DE3)RIL by heat shock.

Expression and purification of bile acid CoA transferases

Colonies were selected and cultivated overnight in 1 ml LB medium containing 100 μ g/ml ampicillin (LB Amp). A 5% inoculum was transferred to LB Amp at 37°C and cultivated until reaching an OD_{600nm} 0.4, at which point gene expression was

induced with 1 mM IPTG. Overexpression occurred for 3 h, at which point cells were centrifuged and washed with Buffer A (two volumes 20 mM sodium phosphate; 0.1 M sodium chloride; 10 mM 2-mercaptoethanol; 15% glycerol, pH 7.0). Cells were frozen at -20°C until further processing. Cell lysates were produced by re-suspending cells in Buffer A containing 700 μ g lysozyme and incubating on ice for 1 h followed by three rounds of sonication (8 s burst, 20 s pause, 2 min total). Benzonase (25 U; Novagen) was added to degrade nucleic acids. Crude lysate was centrifuged 30 min at 16,000 *g*. Lysate was loaded onto Streptactin Superflow Resin (IBA) equilibrated with Buffer A and washed according to the manufacturer instructions. Elution was performed with Buffer A containing 2.5 μ M desthiobiotin. Protein was concentrated on Amicon Ultracel-50K centrifugal filters (Millipore, Cork, Ireland) and stored in 50% glycerol at -20°C. Protein was stable for up to 1 month without detectable loss of activity.

SDS-PAGE and Western immunoblotting

Protein samples were separated by 12% SDS-PAGE and transferred to PVDF Western blotting membranes (Roche). Expression and identity of the recombinant streptavidin-tagged proteins was verified using Strep Tag II antibody HRP conjugate (Novagen).

Synthesis and purification of bile acid CoA conjugates

CoA conjugates of ursodeoxycholic acid (UDCA), chenodeoxycholic acid (CDCA), cholic acid (CA), allocholic acid (ACA), DCA, allodeoxycholic acid (ADCA), lithocholic acid (LCA), and β -muricholic acid (β -MCA) were synthesized according to the method of Killenberg and Dukes (16), with the exception that free bile acids were extracted with ethyl acetate rather than ether. ADCA-CoA synthesized from hydrolyzed rabbit gallstones was separated from contaminating DCA-CoA by reverse-phase HPLC on an HP 1100 series equipped with a Coulter Ultrasphere semi-preparative column (5 μ m silica gel) (10 mm \times 25 cm). Samples (100 μ l) were loaded on the column (40°C) and eluted with 5-40% isopropanol in 20 mM ammonium acetate buffer (pH 7.0) over a 40 min period with a flow rate of 2 ml/min. Free CoA and bile acid CoA conjugates were monitored at 260 nm. Isopropanol from fractions containing ADCA-CoA conjugates was evaporated

under nitrogen and purified by Sep Pak C-18 chromatography. Columns were washed and equilibrated with 10 ml methanol followed by 10 ml deionized water and Buffer B (3 ml 20 mM sodium phosphate buffer; 0.1 M NaCl, pH 6.0). Bile acid CoA conjugates were dissolved in Buffer B and loaded onto the column, followed by washing with 10 ml water and elution with 2 ml absolute methanol. Samples were concentrated under nitrogen and resuspended in 50% methanol. Concentrated bile acid CoA conjugates were resuspended in 0.1 M sodium acetate buffer (pH 5.5) and stored at -20°C . Concentrations were determined by UV spectrophotometry at $A_{260\text{ nm}}$ using a published millimolar extinction coefficient of 15.03 (16).

Bile acid CoA transferase enzyme assays

An HPLC assay was used to measure CoA transferase activity. Assays contained 0.01 μg enzyme of recombinant BaiF-SBP or 0.03 μg recombinant BaiK-SBP and 300-500 μM secondary bile acid CoA conjugate with an equal amount of free bile acid acceptor. Reactions were performed at 37°C and terminated at 1 min by addition of 75 μl 1N HCl. Samples were prepared for HPLC by Sep Pak C-18 chromatography as described above. Concentrated reaction products were resuspended in 125 μl 50% methanol. Samples (100 μl) were autoinjected onto an Agilent 1200 series HPLC equipped with an Agilent Eclipse XDB-C18 column (5 μm , $4.6 \times 250\text{ mm}$). CoA conjugates were separated in a buffer system of 20 mM ammonium acetate buffer (pH 6.0) with a linear gradient 0-55% isopropanol over a 35 min period. CoA conjugates were detected at 260 nm and concentrations determined by comparison of peak areas versus $A_{260\text{ nm}}$ against concentration curves of known quantity.

Assays testing transfer of CoA from DCA-CoA or from ADCA-CoA to CDCA required measurement of the production of $[24\text{-}^{14}\text{C}]\text{CDCA-CoA}$ by liquid scintillation spectrometry instead of reverse-phase HPLC separation because nearly identical hydrophobicities result in comigration of substrate and product. Following termination of reaction by HCl, free $[24\text{-}^{14}\text{C}]\text{CDCA}$ was extracted from the reaction mixture by organic extraction twice with two volumes ethyl acetate. Radioactive counts in the aqueous phase thus measure $[24\text{-}^{14}\text{C}]\text{CDCA-CoA}$, which is not soluble in ethyl acetate. Samples were separated by reverse-phase HPLC, and fractions corresponding to peaks absorbing at 260 nm were collected and counted by liquid scintillation spectrometry. Values were normalized relative to fractions collected from reactions incubated with $[24\text{-}^{14}\text{C}]\text{CA}$. One unit of bile acid CoA transferase activity is defined as the amount of enzyme required to produce 1 μmole of primary bile acid CoA conjugate formed per minute.

Sequence analysis and nucleotide accession numbers

Nucleotide and amino acid sequence alignments were performed using ClustalW (17). Amino acid sequence alignments were represented using Boxshade. Protein M_r calculations were performed using the Compute pI/MW tool on the ExPASy Proteomics Server. Open reading frames (ORF) were detected using the ORF Finder Program at the NCBI website and searched against GenBank by BLAST. GenBank accession numbers for the *baiJKL* operon from *C. hylemonae* DSM 15053 is EU675331, and *baiJKL* operon from *C. scindens* VPI 12708 is EU675330.

RESULTS

Identification of a second multigene operon containing a bile acid CoA transferase

We previously reported multiple copies of the *baiA* gene, which encodes a primary bile acid, CoA-dependent $3\alpha\text{-HSDH}$, in the chromosome of *C. scindens* VPI 12708

(18). One copy of the *baiA* gene is located within in the large *bai* operon, and the other is a single cholic acid-inducible gene at a different chromosomal location (Fig. 2). More recently, we have been able to isolate and identify the *baiA* genes in *C. hylemonae* (7). Subsequent upstream DNA sequencing from the single *baiA* gene in this bacterium revealed a cluster of genes that appeared to be involved in bile acid metabolism by amino acid sequence analysis (Fig. 3). These same genes were also found in *C. scindens* VPI 12708 but not in *C. scindens* ATCC 35704, for which a total genome DNA sequence is available. An ORF (*baiL*) upstream of the *baiA* gene in *C. hylemonae* is predicted to encode a short chain pyridine nucleotide-dependent alcohol/polyol oxidoreductase. Members of this family include the *baiA* gene. However, these two gene products shared only 19% amino acid sequence identity and 35% similarity. A second ORF (*baiK*) was located upstream of the *baiL* gene, and amino acid sequence analysis suggests it may encode a type III CoA transferase. It has very high amino acid sequence identity with the *baiF* gene product from *C. scindens* VPI 12708 (63% identity; 77% similarity), as well as other type III CoA transferases reported in the literature (Fig. 4). Directly upstream from the hypothetical CoA transferase gene was a third ORF (*baiJ*) encoding a putative flavoprotein. BLAST analysis suggested this gene product encodes a flavoprotein similar to fumarate reductases and 3-ketosteroid- Δ^1 -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 (19). These three gene products share 89% amino acid sequence identity between *C. scindens* VPI 12708 and *C. hylemonae* DSM 15053.

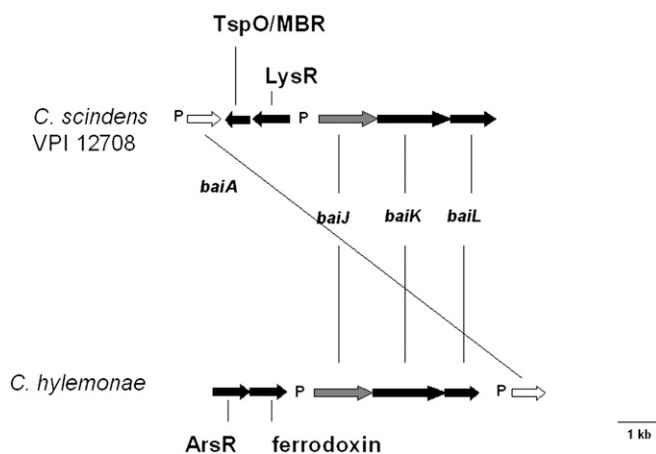


Fig. 3. Schematic representation of *baiJKL* operon from *Clostridium scindens* VPI 12708 and *Clostridium hylemonae* DSM 15053. The *baiA* genes encode 27 kDa 3α -hydroxysteroid dehydrogenases. The *baiJ* genes encode a predicted 62 kDa flavoprotein similar to 3-ketosteroid- Δ^1 -dehydrogenases. The *baiK* genes encode a predicted 49 kDa type III CoA transferase homologous to the *baiF* gene. The *baiL* genes are predicted to encode a 27 kDa protein in the short chain reductase family. “P” represents conserved *bai* “promoter” region. TspO/MBR family acts in signal transduction as well as in transport of steroids/dicarboxylic tetrapyrrole intermediates.

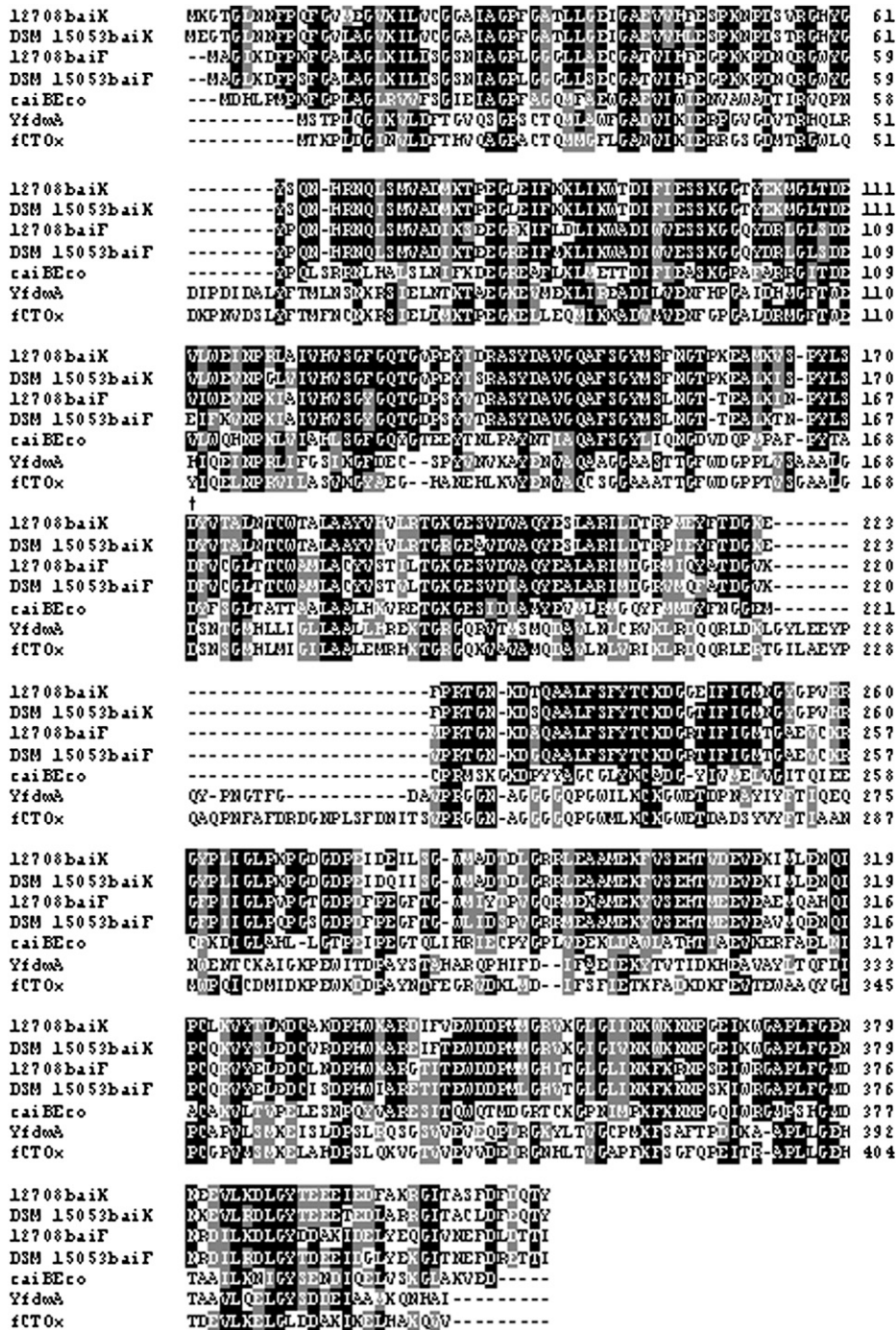


Fig. 4. Boxshade alignment of *baiF* and *baiK* gene products with members of type III CoA transferase family. fCTOx represents formyl CoA transferase from *Oxalobacter formigenes* (AAC45298.1), caiBEco represents carnitine CoA transferase from *E. coli* (BAB96607.1), and YfdwA represents the formyl CoA transferase from *E. coli* (YP_853505). † Active site Asp¹⁶⁹ residue.

In addition, an ORF upstream of the *baiJ* gene on the antisense strand encodes a putative member of the LysR family of transcription factors. A second ORF also located on the antisense strand relative to the *baiJ* gene was found upstream of the putative transcription factor. This ORF was predicted to encode a polypeptide in the tryptophan-rich sensory protein (TspO)/mitochondrial benzodiazepine receptor (MBR) homolog family of signal transduction integral membrane proteins. Farther upstream, and on the

same strand relative to the *baiJ* gene, we located the *baiAI* gene and promoter region that has been previously reported (18). This gene was located directly upstream of the TspO homolog. Note that the single *baiA* genes from both *C. scindens* VPI 12708 and *C. hylemonae* were found in proximity to this novel *bai* operon (Fig. 3).

Some notable features of this novel operon include the similar organization of the *baiJKL* genes, upstream conserved elements with the oxidative *bai* upstream region,

and proximity of the transcription initiation site. 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, which might indicate differences in transcriptional regulation. In addition, the ORF immediately upstream of the *baiJ* gene from *C. hylemonae* DSM 15053 is predicted to encode a 4Fe-4S ferredoxin. This ORF was not located adjacent to the *baiJKL* genes from *C. scindens*. The *baiJKL* genes were searched against total genome sequencing data of *C. scindens* ATCC 35704 and were not detected. Moreover, these genes were not detected by PCR amplification of the *baiK* gene using cloning primers (see *Materials and Methods*). These results suggest that these genes are missing in this strain of bile acid 7 α -dehydroxylating intestinal bacteria.

Transcriptional analysis of multigene operon in *C. scindens* VPI 12708

We located a sequence 24 bp upstream of the *baiJ* ORF in *C. hylemonae* with conserved elements with that of the regions upstream of the *baiBCDAGHI* and *baiA* operons from *C. scindens*, *C. hylemonae*, and *C. hiranonis*. 5' SMART RACE PCR was performed on the *baiJ* gene, and the mRNA start site was determined at the 5' end of the conserved *bai* region, suggesting the promoter is located upstream of this conserved region (Fig. 5). 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. This region was also located directly upstream of the *baiJ* gene in *C. scindens* VPI 12708 and shared a high degree of sequence identity with the putative *bai* promoter region found upstream of the *baiJ* gene of *C. hylemonae*. Finally, cDNA was prepared from CA versus uninduced control cells and primers were designed at the 3' end of the *baiJ* gene and the 5' end of the *baiK* gene and between *baiK* and *baiL* for determination of coexpression. Indeed, the *baiJKL* genes appear to be expressed as a polycistronic (single mRNA) operon (Fig. 5D).

Cloning, expression, and purification of bile acid CoA transferases in *E. coli*

The *baiF* gene was PCR amplified from purified genomic DNA from *Clostridium scindens* ATCC 35704 and cloned into a pSport-1 expression vector. The *baiF* gene was overexpressed in *E. coli* BL21(DE3)RIL, producing a C-terminal streptavidin-tagged recombinant protein. One-step purification from 300 mg protein yielded 600 μ g BaiF-Strep tag fusion protein, which matches 2% total protein expression estimated by SDS-PAGE gel (Fig. 6A). Western blot analysis using Strep Tag II antibody confirmed identity of the recombinant BaiF-Strep tag fusion protein.

The *baiK* gene was also cloned into a pSport-1 expression vector and expressed as a C-terminal streptavidin-tagged recombinant protein. Expression and one-step purification from 300 mg crude extract resulted in recovery of 500 μ g purified enzyme. The deduced molecular weight of the BaiK is 49.5 kDa, which was confirmed by SDS-PAGE and Western immunoblotting using Strep-Tag II antibody (Fig. 6B).

The *baiF* gene encodes a bile acid CoA transferase

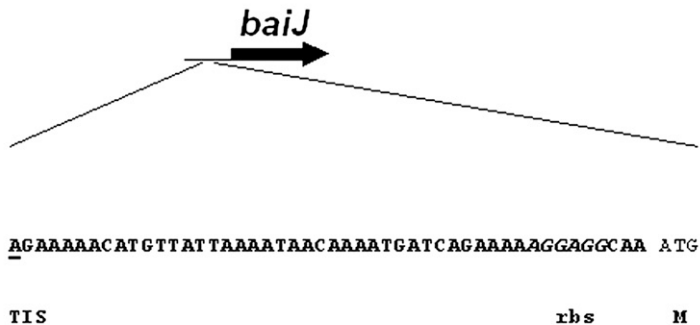
The adenosine moiety in CoA absorbs at 260 nm, whereas bile acids do not. In addition, secondary bile acid-CoA conjugates are with one exception readily separable from primary bile acid-CoA conjugates by reverse-phase chromatography. We hypothesized that bile acid CoA transfer would proceed from secondary bile acid CoA conjugates to free primary bile acids (Fig. 1). We could thus detect the formation of primary bile acid CoA conjugates by reverse-phase HPLC by monitoring at 260 nm. Substrate saturation curves were prepared for the recombinant *baiF* and *baiK* gene products with DCA-CoA, LCA-CoA, and ADCA-CoA with CA as CoA acceptor (data not shown). Saturating substrate concentrations appeared to be inversely related to hydrophobicity. As a result, reactions were performed at concentrations of 500 μ M DCA-CoA, 400 μ M ADCA-CoA, and 300 μ M LCA-CoA with equimolar concentrations of primary bile acid acceptors in each case. LCA-CoA did not appear to be a substrate for the recombinant *baiK* gene product (Table 1). The reaction was linear over a 2 min period and over protein concentrations between 0.005 μ g and 0.05 μ g at 37°C.

We determined that synthesized DCA-CoA eluted at 27.48 min on reverse-phase HPLC. Addition of the recombinant BaiF to a reaction mixture containing DCA-CoA and CA resulted in a product eluting at 24 min (Fig. 7). We repeated the assay with radiolabeled CA, and after termination of the reaction, free bile acids were extracted from the aqueous reaction mixture by ethyl acetate. Remaining water soluble radioactive counts represent [24-¹⁴C]CA-CoA conjugates. We collected fractions from 15 to 35 min, measured radioactivity by liquid scintillation spectrometry, and compared BaiF catalyzed reaction to the no-enzyme control. Fractions corresponding to the HPLC peak detected at 24 min also contained [24-¹⁴C] CA, whereas no such peak or radioactivity above background was detected in the no-enzyme control (Fig. 7). This data suggests that the *baiF* gene product transfers CoA from DCA-CoA to CA.

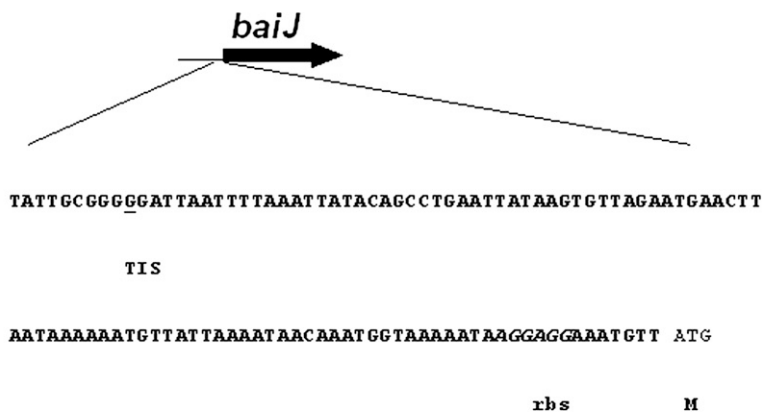
Bile acid substrate specificity of recombinant bile acid CoA transferases

We then wanted to determine the substrate specificity for the recombinant *baiF* and *baiK* gene products using secondary bile acid CoA substrates and primary bile acid acceptors. The 3,7,12-trihydroxy bile acids CA and ACA were excellent acceptors, regardless of secondary bile acid CoA donor (Table 1). CDCA and CA had similar reaction rates when LCA-CoA was donor; however, transfer to CDCA was significantly diminished when DCA-CoA was donor (91% relative activity versus 4%, respectively). This was observed with both the *baiF* and *baiK* gene products. Although both the *baiF* and the *baiK* gene products were able to transfer CoA to UDCA, the *baiK* had the greatest reaction rate with this tertiary bile acid. Interestingly, the BaiK gene product demonstrated CoA transferase activity with DCA-CoA and ADCA-CoA but not with LCA-CoA. To determine whether lower concentrations of LCA-CoA were required, experiments were repeated with concentrations of LCA-CoA lowered from 300 μ M to between 100 μ M and 200 μ M, with

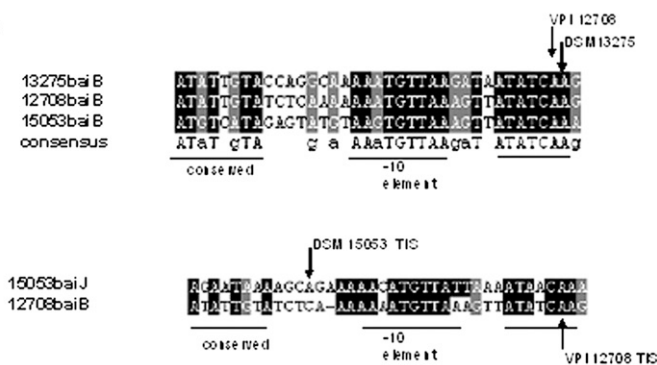
A *C. hylemonae* DSM 15053



B *C. scindens* VPI 12708



C



D

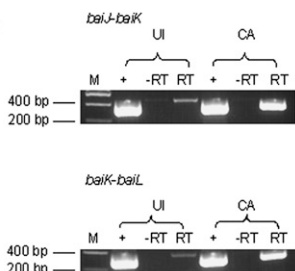


Fig. 5. Transcriptional analysis of *baiJ**KL* operons from *C. hylemonae* and *C. scindens* VPI 12708. A: Transcriptional initiation site for *baiJ* gene from *C. hylemonae* (underlined). B: Transcriptional initiation site for *baiJ* gene from *C. scindens* VPI 12708. C: Boxshade alignment of conserved putative regulatory elements upstream of the *baiB* between *C. scindens* VPI 12708, *C. hylemonae* DSM 15053, and *C. hirononis* 13275 (above). The *baiJ*-conserved regulatory region from *C. hylemonae* DSM 15053 compared with that of the *baiB* upstream element (below) D: RT-PCR using primers spanning intergenic region of *baiJ*-*baiK* and *baiK*-*baiL* genes of *C. scindens* VPI 12708. “+” represents 10 ng *C. scindens* VPI 12708 genomic DNA, “-RT” represents negative control in which reverse transcriptase is not added, and “RT” represents cDNA prepared from uninduced (UI) and 50 μM cholic acid-induced (CA) cultures of *C. scindens* VPI 12708. M, methionine; rbs, ribosome binding site; TIS, transcription initiation site.

no product detected (data not shown). We tested the 3,6,7-trihydroxy bile acid β-MCA, which had a specificity similar to UDCA for the *baiF* gene product, and it was recognized to a lesser extent by the *baiK* gene product. In addition, unlike the *baiF* gene product, the *baiK* gene product appeared to prefer UDCA when DCA-CoA was donor,

whereas ACA was the best acceptor when ADCA-CoA was donor (Table 1). We expected to see a broad substrate specificity as CA, CDCA, UDCA are common in human bile and the secondary bile acids LCA, DCA, and the 5α-epimer of DCA, ADCA, are produced by human bile acid 7α-dehydroxylating bacteria.

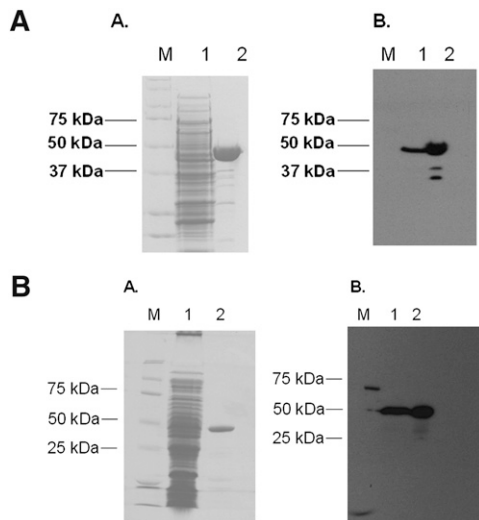


Fig. 6. Purification of the streptavidin-tagged recombinant bile acid-CoA transferases from *E. coli*. A. Purification of the recombinant C-terminal streptavidin tagged BaiF. B. Purification of the recombinant C-terminal streptavidin tagged BaiK. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 30 μ g crude (1) and 5g Streptactin-purified (2) fractions. Molecular weight standards are indicated to the left (M). (B) Detection of streptavidin-tagged recombinant transferases in crude (1) and purified (2) fractions using Strep-tag II antibody.

DISCUSSION

In the current study, we cloned and expressed two genes encoding bile acid CoA transferases from *C. scindens* VPI 12708 in *E. coli*. We tested several combinations of secondary bile acid CoA donors and primary bile acid CoA acceptors and found that the *baiF* and *baiK* gene products have broad bile acid substrate specificity. However, the *baiK* gene product had no activity when LCA-CoA was used as the CoA donor (Table 1). Generally, the highest bile acid CoA transferase activity occurred when a homologous secondary bile acid was used as the bile acid CoA donor (i.e., cholic acid was the best acceptor when deoxycholy-CoA was used as the CoA donor).

Bile acid 7 α -dehydroxylating bacteria express a number of genes in the presence of primary bile acids (1). Upon entering the cell, bile acids are rapidly conjugated to CoA.

Previously, we showed that the *baiB* gene product ligated CoA to primary bile acids by an ATP-dependent manner (9). We also demonstrated that the *baiF* gene product catalyzes the hydrolysis of cholyl-CoA (13). However, in our original experiments, we did not test for CoA transferase activity. In 2001, Heider proposed a new family of CoA transferases that, based on amino acid sequence analysis and knowledge of reaction mechanisms at the time, could not be placed into family I or II (14). This third family, involved in anaerobic metabolic pathways, includes the *baiF* gene and has been referred to as the “CaiB/BaiF” family. During anaerobic carnitine metabolism, (R)-carnitine is activated for degradation to butyrobetaine by ATP-dependent ligation by the *caiC* gene. ATP-dependent CoA ligation is thought to be required to generate a pool of CoA-thioesters that can later be usurped by an ATP-independent CoA-transferase encoded by the *caiB* gene product (14). In bile acid 7 α -dehydroxylation, the *baiB* gene product would serve to generate a pool of bile acid-CoA conjugates, which could then be acted on by the bile acid CoA transferases encoded by the *baiF* and *baiK* genes (Fig. 1).

Type III CoA transferases, such as the crotonoyl-CoA (*caiB*) and *yfaW* gene products from *E. coli* (19–21) as well as the formyl-CoA transferase (FRC) from *Oxalobacter formigenes* (22), share a conserved Asp¹⁶⁹ residue (D169 formyl CoA) involved in catalytic formation of oxo-acid mixed anhydrides as well as covalent CoA thioesters. The *baiF* and *baiK* gene products share this conserved residue (Fig. 4). It has been proposed that CoA transfer proceeds through Asp¹⁶⁹ acylation (22–25). Mutagenesis of Asp¹⁶⁹ (D169A, D169S, D169E) in the FRC from *O. formigenes* resulted in abolition of or appreciable decrease in specific activity despite no significant structural alterations (22). Recently, a catalytic mechanism has been proposed based on crystallographic data for FRC from *O. formigenes* (26). The proposed catalytic mechanism for FRC suggests that formyl-CoA forms an acid anhydride with Asp¹⁶⁹, resulting in hydrolysis of CoAS⁻. Gln-17 and a glycine loop alternate between “open” and “closed” conformations, shielding aspartyl mixed anhydrides and aspartyl-CoA thioesters from hydrolysis between steps. -SCoA nucleophilically attacks the β -aspartyl-formyl anhydride, releasing formate. The second substrate, oxalate, then nucleophilically attacks

TABLE 1. Substrate specificity of bile acid CoA transferase

CoA Acceptor	BaiF ^a						BaiK ^a				
	Relative Activity			Position and Substitutions			Relative Activity				
	CoA Donor			3	5	6	7	12	CoA Donor		
	DCA-CoA	LCA-CoA	ADCA-CoA						DCA-CoA	LCA-CoA	ADCA-CoA
CA	100 \pm 15.4	91.6 \pm 3.1	100 \pm 9	α OH, β H	β H	α H, β H	α OH, β H	α OH, β H	69.6 \pm 11.2	ND	44.5 \pm 2.98
ACA	87 \pm 4.3	100 \pm 1.5	88.9 \pm 0.9	α OH, β H	α H	α H, β H	α OH, β H	α OH, β H	28.1 \pm 3	ND	100 \pm 3
UDCA	51.35 \pm 4.35	35 \pm 1.2	44.38 \pm 8.0	α OH, β H	β H	α H, β H	α H, β OH	α H, β H	100 \pm 12	ND	73.61 \pm 11.9
CDCA	4.1 \pm 2	90.14 \pm 6.5	NA	α OH, β H	β H	α H, β H	α OH, β H	α H, β H	2.15 \pm 1.2	ND	NA
β -MCA	53.6 \pm 2.6	32.87 \pm 3.7	35 \pm 2.8	α OH, β H	β H	α H, β OH	α H, β OH	α H, β H	11.4 \pm 2.4	ND	NA

Reactions performed in triplicate, represented as average with standard deviation. NA, reaction not performed; ND, not detected.

^aAmounts of 0.02 U BaiF and 0.012 U BaiK were used per reaction. One unit of activity is defined as the amount of enzyme required to transfer CoA from 1 μ mol DCA-CoA to CA min⁻¹.

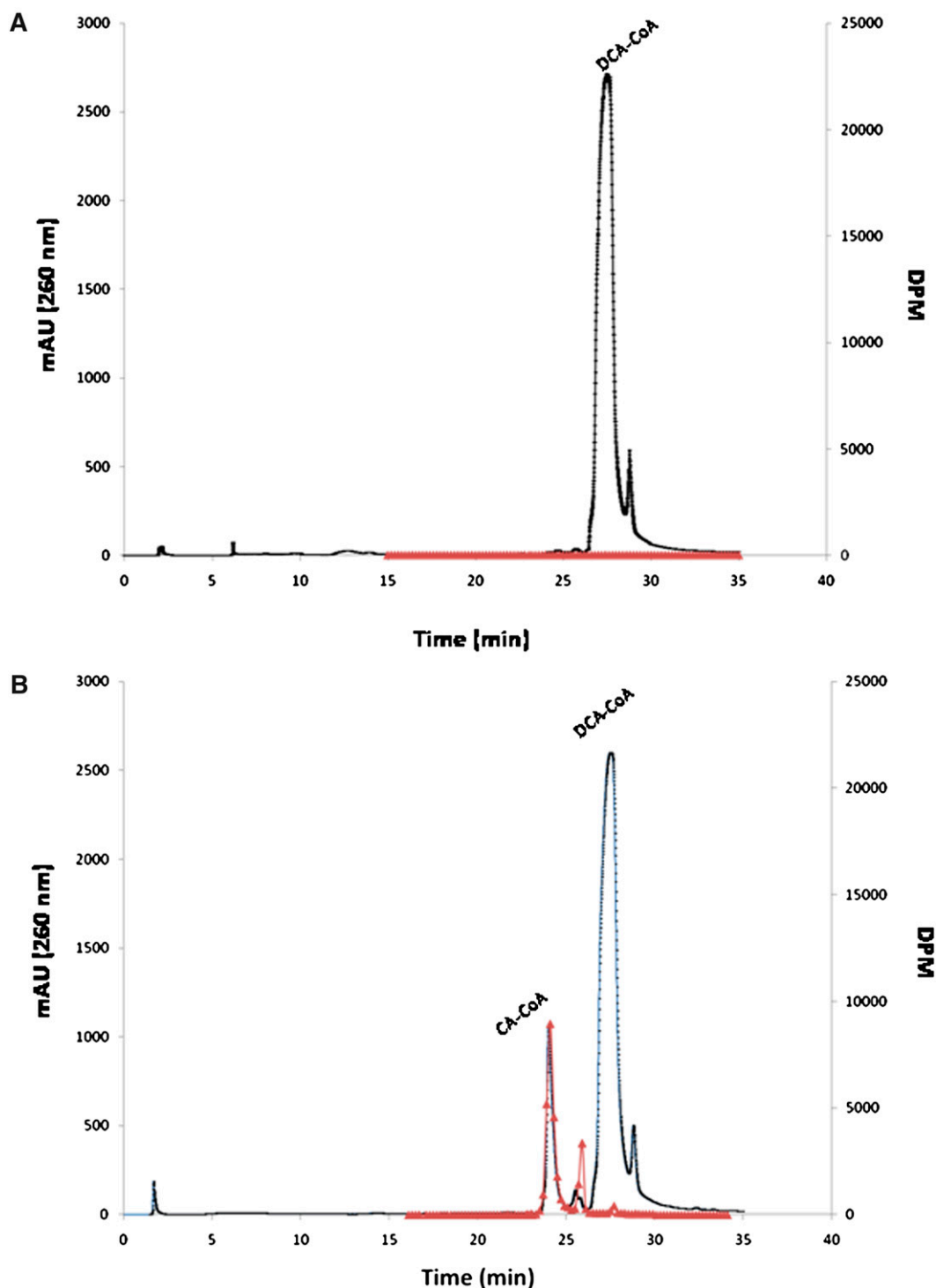


Fig. 7. Reverse phase C-18 HPLC elution profile of CoA transferase reaction products catalyzed by BaiF. Reactions contained 500 μM cholic acid (17,600 DPM/nmole [24- ^{14}C] cholic acid) and 500 μM deoxycholy-CoA. A: No enzyme control elution profile. B: 0.01 μg BaiF catalyzed reaction. Reactions were acidified and extracted with ethyl acetate to remove free bile acids prior to HPLC. Radioactivity represented by triangles; absorption at 260 nm represented by dots.


the aspartyl-CoA thioester, forming the second aspartyl-oxalyl anhydride, which is hydrolyzed in a final attack by CoAS^- , releasing oxalyl-CoA and regenerating Asp^{169} . This mechanism suggests that a ternary complex is not formed as observed in family II transferases. Instead, the reaction is completed before reaction products are released (26).

Crystallization of CaiB yielded crystals with only CoA bound, suggesting hydrolysis occurs in the absence of the CoA-accepting substrates (21). This would provide a plausible explanation for observed CoA hydrolase activity shown for the *baiF* gene product in the absence of CoA substrate (13). We speculate that a similar mechanism is

involved in bile acid CoA transfer by the *baiF* and possibly the *baiK* genes to that proposed for the FRC. However, the *baiF* and *baiK* gene products appear to lack the ²⁵⁸GGGGQ²⁶¹ glycine loop; therefore, they use a different means to shield intermediates from premature hydrolysis.

We have demonstrated that the *baiF* and *baiK* gene products have bile acid CoA transferase activity with broad bile acid substrate specificity. However, in the current study, we focus only on end-product secondary bile acid donors. It is possible that intermediates following 7 α / β -dehydration, including 3-dehydro-4, 3-dehydro-4,6 and 3-dehydro-intermediates of DCA, LCA, and ADCA, are the preferred substrates. Chemical synthesis of these intermediates and future kinetic studies of the *baiF* and *baiK* genes will be required to answer this question. Additionally, identification of genes in the “reductive” arm of the pathway and kinetic analysis of free bile acid versus bile acid-CoA thioesters will allow determination of the step in the pathway where CoA is transferred. Previously, we isolated a 3-dehydro-4-DCA~CoA intermediate, produced after reduction of the Δ^6 -bond following 7 α -dehydration, from cell extracts of CA-induced cultures of *C. scindens* VPI 12708 (27). This suggests that CoA is transferred late in the pathway; however, additional work will be required to determine which intermediate is the preferred donor.

We have discovered a polycistronic operon that appears to be involved in bile acid metabolism in *C. scindens* VPI 12708 and *C. hylemonae* DSM 15053. The *baiJ* gene is similar to fumarate reductases and 3-keto steroid- Δ^1 -dehydrogenases, and the *baiL* is suggested to encode a 3 α -hydroxysteroid dehydrogenase. The *baiK* gene located on this operon encodes a bile acid CoA transferase that may be required to enhance transferase activity when bile acids are prevalent. Coexpression of the *baiK* gene with genes in the “reductive” arm of the 7 α / β -dehydroxylation pathway may help maintain a steady ATP-independent recycling of CoA to maximize conservation of energy in this reductive anaerobic process. Interestingly, genes within this operon have been identified in the 7 α -dehydroxylating strains *C. hylemonae* DSM 15053, *C. scindens* VPI 12708, and *C. hiranonas* DSM 13275, but these genes were not detected in the 7 α -dehydroxylating strain *C. scindens* ATCC 35704. Further work will be necessary to determine the function of this novel operon.

In summary, we report the first bile acid CoA transferases (*baiF*, *baiK*) found in bacteria and suggest they function to conserve an ATP in the bile acid 7 α -dehydroxylation pathway (Fig. 1). 

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