

Identification and Characterization of a Bile Acid 7 α -Dehydroxylation Operon in *Clostridium* sp. Strain TO-931, a Highly Active 7 α -Dehydroxylating Strain Isolated from Human Feces

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Clostridium sp. strain TO-931 can rapidly convert the primary bile acid cholic acid to a potentially toxic compound, deoxycholic acid. Mixed oligonucleotide probes were used to isolate a gene fragment encoding a putative bile acid transporter from *Clostridium* sp. strain TO-931. This DNA fragment had 60% nucleotide sequence identity to a known bile acid transporter gene from *Eubacterium* sp. strain VPI 12708, another bile acid-7 α -dehydroxylating intestinal bacterium. The DNA (9.15 kb) surrounding the transporter gene was cloned from *Clostridium* sp. strain TO-931 and sequenced. Within this larger DNA fragment was a 7.9-kb region, containing six successive open reading frames (ORFs), that was encoded by a single 8.1-kb transcript, as determined by Northern blot analysis. The gene arrangement and DNA sequence of the *Clostridium* sp. strain TO-931 operon are similar to those of a *Eubacterium* sp. strain VPI 12708 bile acid-inducible operon containing nine ORFs. Several genes in the *Eubacterium* sp. strain VPI 12708 operon have been shown to encode products required for bile acid 7 α -dehydroxylation. In *Clostridium* sp. strain TO-931, genes potentially encoding bile acid-coenzyme A (CoA) ligase, 3 α -hydroxysteroid dehydrogenase, bile acid 7 α -dehydratase, bile acid-CoA hydrolase, and a bile acid transporter were similar in size and exhibited amino acid homology to similar gene products from *Eubacterium* sp. strain VPI 12708 (encoded by *baiB*, *baiA*, *baiE*, *baiF*, and *baiG*, respectively). However, no genes similar to *Eubacterium* sp. strain VPI 12708 *baiH* or *baiI* were found in the *Clostridium* sp. strain TO-931 *bai* operon, and the two putative *Eubacterium* sp. strain VPI 12708 genes, *baiC* and *baiD*, were arranged in one continuous ORF in *Clostridium* sp. strain TO-931. Intergene regions showed no significant DNA sequence similarity, but primer extension analysis identified a region 115 bp upstream from the first ORF that exhibited 58% identity to a *bai* operator/promoter region identified in *Eubacterium* sp. strain VPI 12708. These results indicate that the gene organization, gene product amino acid sequences, and promoters of the bile acid-inducible operons of *Clostridium* sp. strain TO-931 and *Eubacterium* sp. strain VPI 12708 are highly conserved.

In mammals, the primary bile acids cholic acid and chenodeoxycholic acid are synthesized in the liver and conjugated to either glycine or taurine (31). Conjugated bile acids are required for the proper digestion and absorption of cholesterol, lipids, and other lipid-soluble compounds. Bile acids are actively absorbed in the terminal ileum and returned to the liver (15). However, some bile acids pass into the large intestine and are extensively biotransformed (4). In particular, a minute population of bacteria can 7 α -dehydroxylate the primary bile acids into secondary bile acids, generating potentially toxic products. The bile acid 7 α -dehydroxylation products of cholic acid and chenodeoxycholic acid are deoxycholic acid and lithocholic acid, respectively (4, 15).

In humans, increased levels of deoxycholic acid in the bile acid pool have been associated with an increased risk of cholesterol gallstone disease (6, 9, 17, 19, 24, 27–30) and colon cancer (25, 31, 32). Antibiotic treatment has been shown to inhibit bacterial populations responsible for deoxycholic acid formation and significantly decrease the cholesterol saturation index of bile (7). Despite the potential benefits of such treat-

ment for individuals prone to cholesterol gallstone formation, the selection of antibiotic-resistant bacterial strains during long-term antibiotic administration precludes its effective use. The development of inhibitors specific for the bile acid 7 α -dehydroxylation might be beneficial for preventing cholesterol gallstone formation. However, little is known about the genetics of bile acid 7 α -dehydroxylation in intestinal bacteria.

Specific members of the genera *Eubacterium* and *Clostridium* are the only intestinal bacteria that have been shown to be capable of cholic acid 7 α -dehydroxylation (12). Studies of *Eubacterium* sp. strain VPI 12708, an organism that can rapidly produce deoxycholic acid, identified a multistep pathway responsible for cholic acid 7 α -dehydroxylation (8). Genetic analysis identified a bile acid-inducible operon (*bai*) that encodes enzymes required in this pathway. However, studies have shown that most cholic acid-7 α -dehydroxylating intestinal bacteria belong to the genus *Clostridium* (33). More importantly, recent work found that *Eubacterium* sp. strain VPI 12708 *bai* genes cross-hybridized with DNA from other *Eubacterium* strains, but not with *Clostridium* strains tested (11). Based on this observation, *Clostridium* strains may have genetically distinct *bai* genes.

The present study was designed to identify the bile acid transporter gene from *Clostridium* sp. strain TO-931, a human fecal isolate. In addition, surrounding genes were cloned and sequenced to gain a better understanding of the genetics and enzymology of bile acid 7 α -dehydroxylation in a *Clostridium*

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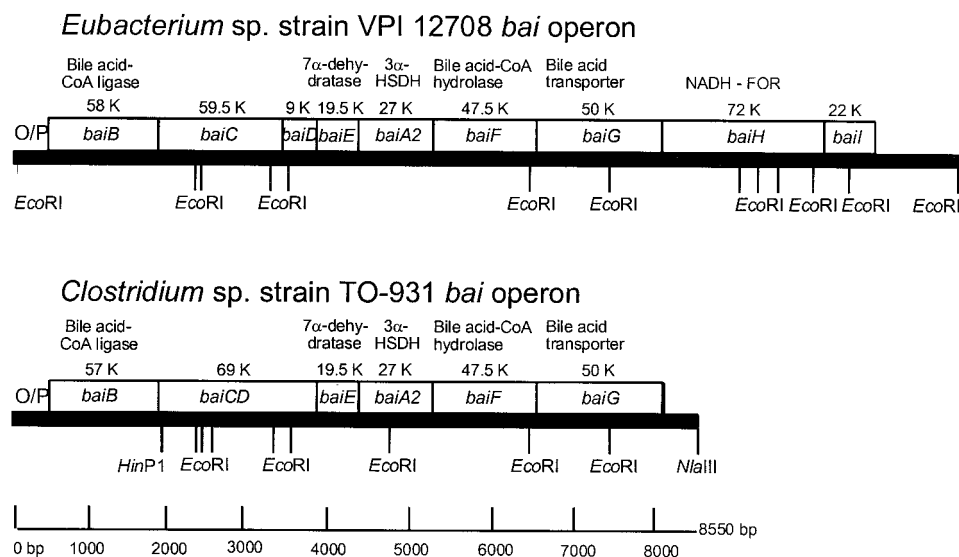


FIG. 1. Overview of gene identity and organization for bile acid-inducible (*bai*) operons of *Eubacterium* sp. strain VPI 12708 and *Clostridium* sp. strain TO-931. No *baiH* or *baiI* gene was found downstream of *Clostridium* *baiG*. O/P, operator/promoter; FOR, flavin oxidoreductase; HSDH, hydroxysteroid dehydrogenase.

strain. Previous work showed that *Clostridium* sp. strain TO-931 had the highest cholic acid-7 α -dehydroxylating activity of any intestinal bacteria tested, including *Eubacterium* sp. strain VPI 12708 (11). Knowledge of the bile acid 7 α -dehydroxylation genetics in *Clostridium* species will allow for a better comparison of genes and gene products, which is necessary for developing specific bile acid 7 α -dehydroxylation inhibitors.

MATERIALS AND METHODS

Isolation of chromosomal DNA. *Clostridium* sp. strain TO-931 was kindly provided by Fusae Takamine (University of Ryukyus, Okinawa, Japan) and had been isolated from a human fecal sample. Cultures (50 ml) were grown in 100-ml volumes of peptone-yeast extract (PY) medium (18) supplemented with sucrose (4 g/liter), using anaerobically sealed serum bottles. Cells were collected by centrifugation (10,000 \times g, 10 min) and suspended in 2-ml volumes of 0.9% saline. Cell suspensions were treated with 2 volumes of buffered phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol; Boehringer-Mannheim) and centrifuged (5,000 \times g, 10 min). Phenol residue was removed by two equal-volume chloroform-isoamyl alcohol (24:1, vol/vol) extractions. Chromosomal DNA was precipitated with 1/20 volume of sodium acetate (3 M, pH 5.5) and 2.5 volumes of ice-cold ethanol and centrifuged. The DNA pellet was washed twice with ice-cold 70% ethanol, dried, dissolved in 250 μ l of H₂O, and stored at 2 to 4°C.

Oligonucleotide probe design. Regions of the *Eubacterium* sp. strain VPI 12708 bile acid transporter sequence with homology to other transporters were scanned, and the nucleotide sequences having the least redundancy (<500) in the DNA sequence were used to design mixed oligonucleotide probes (50KM1 [5'-GARTAYCCNCARGARGAR-3'] and 50KM2 [5'-RCANACCCACATC CATNAC-3']). Subsequent sequence-specific probes needed for cloning, sequencing, and PCR were identified by using Lasergene PrimerSelect software (DNASTAR Inc., Madison, Wis.). All oligonucleotides were commercially synthesized (Genosys Biotechnologies, The Woodlands, Tex.).

Detection of *Clostridium* sp. strain TO-931 *bai* genes. *Clostridium* sp. strain TO-931 DNA (1 to 2 μ g) was digested with *AccI*, *AccI*, *BamHI*, *EcoRI*, *HinPI*, *NlaIII*, *Sau3AI*, or *XbaI* (New England Biolabs, Beverly, Mass.). DNA fragments were separated by gel (1.0% agarose, Tris-acetate-EDTA buffer system) electrophoresis and transferred to a nitrocellulose membrane (Trans-Blot transfer medium; Bio-Rad Laboratories, Hercules, Calif.) for Southern hybridization analysis (13). DNA was cross-linked by using a UV Stratalinker 1800 (Stratagene, La Jolla, Calif.), and the nitrocellulose blots were hybridized for 12 h with probes labeled with [γ -³²P]ATP (NEN, Boston, Mass.) by the use of T₄ polynucleotide kinase. Blots were washed (13) and exposed to BioMax MS film (Kodak, Rochester, N.Y.).

Cloning of *bai* genes. Chromosomal DNA was restriction enzyme digested and separated by agarose gel electrophoresis. DNA fragments were extracted from gel slices by using a GeneClean spin kit (Bio101, Vista, Calif.) and ligated into restriction enzyme-digested pUC19 (New England Biolabs), using T₄ DNA ligase (New England Biolabs). Library Efficiency (*Escherichia coli* DH5 α competent cells (Gibco BRL, Gaithersburg, Md.) were used for DNA transforma-

tions. Clones were identified by colony hybridization analysis (3) by using probes that were labeled with [γ -³²P]ATP by the use of T₄ polynucleotide kinase (New England Biolabs). Clone identities were verified by restriction enzyme digestion and Southern hybridization analysis (13).

Difficult regions were cloned by the PCR technique, using a sequence-specific primer and a random primer (5'-GTTGGTGGCT-3') to anchor downstream. The reaction mixture and conditions used were described previously (3) with the exception of the annealing temperature (35°C). The PCR products were separated by agarose gel electrophoresis, and their identities were verified by Southern hybridization analysis. The PCR products were cloned into the TA cloning vector (Stratagene).

DNA sequencing and sequence analysis. Plasmid DNA was isolated from positive clones and sequenced by using a Dye-Terminator DNA sequencing kit (ABI Prism; Perkin-Elmer [PE] Applied Biosystems, Foster City, Calif.). Se-

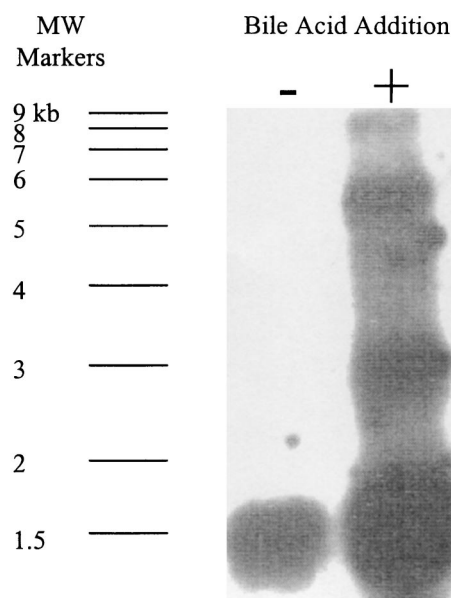


FIG. 2. Northern blot analysis of the *bai* operon of *Clostridium* TO-931. Total RNA was isolated at time zero and at 30 min following addition (+) of cholic acid (50 μ M) to the culture medium. In a control culture, bile acid was not added (-). Approximately 10 μ g of RNA was loaded onto each lane and probed with the *baiCD* gene. MW Markers, molecular size marker positions.

TABLE 1. Comparison of *Eubacterium* sp. strain VPI 12708 and *Clostridium* sp. strain TO-931 *bai* operon DNA sequences

Gene or region	Length (bp), <i>Eubacterium</i> vs. <i>Clostridium</i>	Identity (%)	Protein	Enzymatic function	Identity (%)	Similarity (%)
Operator promoter	218 vs 218	58				
<i>baiB</i>	1,563 vs 1,551	60	BaiB	Bile acid-CoA ligase	60	77
<i>baiB</i> → <i>baiCD</i> (intergene)	45 vs 95	<10				
<i>baiCD</i>	1,920 vs 1,920	71	BaiCD	Unknown	83	91
<i>baiCD</i> → <i>baiE</i> (intergene)	43 vs 62	<10				
<i>baiE</i>	501 vs 504	74	BaiE	Bile acid 7 α -dehydratase	87	93
<i>baiE</i> → <i>baiA</i> (intergene)	33 vs 120	<10				
<i>baiA</i>	747 vs 750	69	BaiA	3 α -Hydroxysteroid dehydrogenase	80	89
<i>baiA</i> → <i>baiF</i> (intergene)	63 vs 85	<10				
<i>baiF</i>	1,281 vs 1,281	75	BaiF	Bile acid-CoA hydrolase	88	94
<i>baiF</i> → <i>baiG</i> (intergene)	5 vs 133	<5				
<i>baiG</i>	1,434 vs 1,422	65	BaiG	Bile acid transporter	71	81

quence reactions were analyzed at the Medical College of Virginia-Virginia Commonwealth University Core Lab, using ABI Prism 373/375 sequence analyzers (PE Applied Biosystems). DNA sequences were submitted via the World-Wide Web to the National Institutes of Health for BLASTX analysis (1, 2). Cloned *Clostridium* sp. strain TO-931 *bai* gene sequences were arranged and managed by using Lasergene software (DNASTAR). Polypeptide analysis of BaiG was performed with the Lasergene software, and a transmembrane model was prepared by using the TMpred transmembrane prediction program operated via the World-Wide Web (<http://ulrec3.unil.ch/software/TMPRED.form.html>).

RNA analysis and manipulations. *Clostridium* sp. strain TO-931 was grown in PY broth with or without cholic acid (100 μ M). RNA was isolated by using an RNeasy Midi kit (Qiagen, Chatsworth, Calif.), separated by 1% agarose gel electrophoresis, and transferred to nitrocellulose membranes (Bio-Rad Laboratories) for Northern hybridization analysis (3). The size of the mRNA transcript was determined by comparison to an RNA ladder (Ambion, Austin, Tex.).

RNA (5 to 10 μ g) was precipitated with 3 M sodium acetate (1/20 volume) and ice-cold ethanol (2.5 volumes) at -20°C . The RNA pellet was dried and resuspended in RNase-free H_2O with ^{32}P -labeled oligonucleotide primer (5'-CATT CATATCGGTATTTTGCTCCCTC-3'). RNA-primer mixtures were heated to 70°C for 10 min and allowed to cool slowly to room temperature to anneal the primers. Primers were extended by using SUPERScript II reverse transcriptase (Gibco BRL) at 42°C for 1 h. To determine the size of the extension product, DNA was manually sequenced using an fmol DNA PCR sequencing kit (Promega, Madison, Wis.) extended from the same ^{32}P -labeled primer. The extension product and corresponding DNA sequencing products were separated by 6% acrylamide-40% urea gel electrophoresis (5). Following electrophoresis, the sequencing gel was dried and exposed to Kodak AR film. Primer extension and sequencing primers were 5'-end labeled with [γ - ^{32}P]ATP (NEN) as discussed above.

Nucleotide sequence accession number. The nucleotide sequence of the *Clostridium* sp. strain TO-931 *bai* operon has been submitted to the GenBank database (accession no. Clobai AF210152).

RESULTS

***Clostridium* sp. strain TO-931 *bai* gene identification.** The *bai* gene of *Eubacterium* sp. strain VPI 12708 encodes a bile acid transporter, and this gene exhibits homology to a large class of ATP-binding cassette transport proteins (20). Two redundant oligonucleotide primers (50KM1 and 50KM2) were based on potential membrane-spanning regions of the *Eubacterium* sp. strain VPI 12708 *baiG* gene product (20), and both sets hybridized to a single 1.0-kb DNA band in *Eco*RI-digested DNA from *Clostridium* sp. strain TO-931. Of 120 colonies, a single *Eco*RI clone was isolated using the 50KM1 probe set. The positive clone was sequenced, and the entire DNA sequence had 64.8% identity to the 5' nucleotide sequence of the *Eubacterium* sp. strain VPI 12708 *baiG* gene.

***Clostridium* sp. strain TO-931 *baiG* gene analysis.** An open reading frame (ORF) similar in size and having 65% DNA sequence identity to the *Eubacterium* sp. strain VPI 12708 *baiG* gene was identified from an overlapping sequence generated from a PCR fragment and restriction enzyme (*Eco*RI and *Nla*III)-generated clones of *Clostridium* sp. strain TO-931

DNA. The full-length polypeptide putatively encoded by *Clostridium* sp. strain TO-931 ORF had 71% identity and 81% similarity to the *Eubacterium* sp. strain VPI 12708 bile acid transporter. The polypeptide sequence of the *Clostridium* sp. strain TO-931 ORF had a hydrophobicity plot similar to that of the bile acid transporter (data not shown), and a two-dimensional model with 14 transmembrane segments was nearly identical to the bile acid transporter model proposed previously (20). Most variation between the peptide sequences was found to be in the C-terminal portion, specifically in the 6th external membrane loop between the 13th and 14th membrane-spanning helices.

***Clostridium* sp. strain TO-931 *bai* operon cloning and sequence analysis.** Nearly 9.2 kb of overlapping DNA sequence surrounding the *baiG* gene was combined from eight clones containing *Clostridium* sp. strain TO-931 DNA (Fig. 1). This large DNA sequence, which contained six ORFs and was expressed as a single mRNA of approximately 8 to 9 kb, was induced within 30 min following addition of 100 μ M cholic acid to the growth medium (Fig. 2). This *Clostridium* sp. strain TO-931 *bai* operon had significant identity (Table 1) to the *bai* operon of *Eubacterium* sp. strain VPI 12708, and their gene orders were very similar (Fig. 1). No *Eubacterium* sp. strain VPI 12708 *baiH*-like or *baiI*-like genes were identified within 500 bp downstream of the *baiG* gene in the *Clostridium* sp. strain TO-931 operon. Interestingly, the *baiC* and *baiD* genes appeared to be encoded by a single continuous ORF rather than by two separate genes as observed for the *Eubacterium* sp. strain VPI 12708 *bai* operon (23). The intergene DNA sequences of *Clostridium* sp. strain TO-931 had little similarity to those observed in the *Eubacterium* sp. strain VPI 12708 *bai* operon and tended to be larger.

Comparison of the putative peptide sequences from each *bai* operon revealed significant identity, with nearly 90% similarity for most of the putative gene products (Table 1). The *Clostridium* sp. strain TO-931 putative gene product BaiCD aligned with both *Eubacterium* sp. strain VPI 12708 BaiC and BaiD putative gene products and had significant homology to *Eubacterium* sp. strain VPI 12708 BaiH (Fig. 3), a protein associated with an NADH:flavin oxidoreductase in *Eubacterium* sp. strain VPI 12708 (12).

Promoter analysis. The initial nucleotide for mRNA transcription was determined by primer extension analysis to lie 106 bases upstream from the *Clostridium* sp. strain TO-931 *baiB* gene (Fig. 4). Only bile acid-induced cultures yielded a primer extension product. The transcription initiation site in the DNA had an 8-bp sequence identical to that observed in

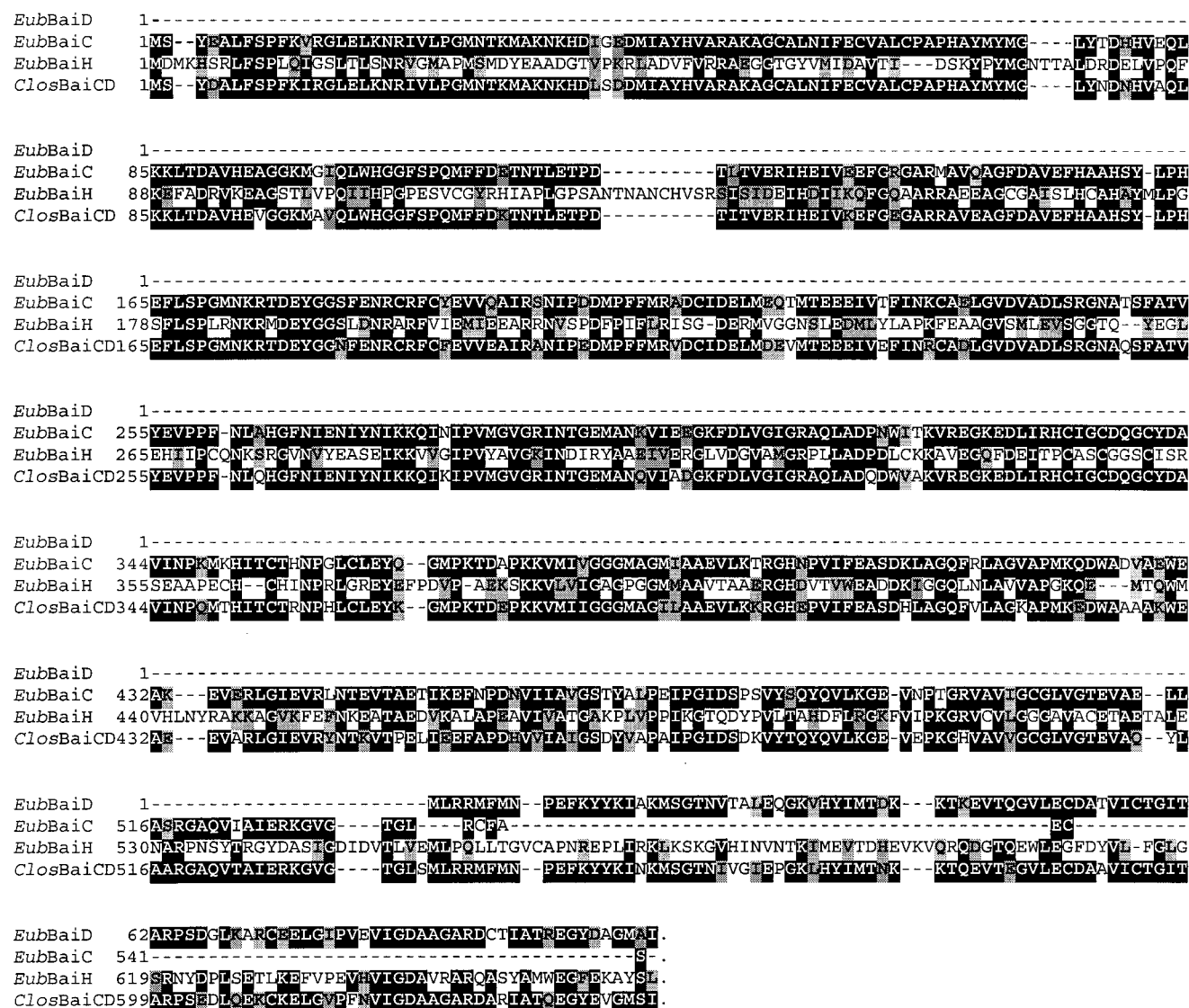


FIG. 3. Clustal alignment of peptide sequences for *Eubacterium* (Eub) sp. strain VPI 12708 bile acid-inducible proteins BaiC, BaiD, and BaiH and *Clostridium* (Clos) sp. strain TO-931 bile acid-inducible protein BaiCD. Darkened residues (white letters on black background) denote identity and shaded residues (black letters on gray background) denote similarity between aligned sequences. The putative BaiCD peptide exhibits 46% identity and 51% similarity to BaiH, an enzyme with NADH:flavin oxidoreductase activity. Neither BaiC nor BaiD has been associated with an enzyme function.

Eubacterium sp. strain VPI 12708, and upstream were two regions identical to those observed in the *Eubacterium* sp. strain VPI 12708 promoter region (Fig. 5). In addition, several regions upstream from the putative promoter region are highly conserved and may be specific to bile acid regulation (5'-TTTGTCTxxxxATxxATTAGxTxTTxxxxxAAAAGGTxATCTxTATxTTGTGTAAGAxGxxATTATx-3'). The transcription initiation site for the *Clostridium* sp. strain TO-931 *bai* operon was surrounded by an inverted-repeat sequence (5'-TATC/AAGATA-3') (Fig. 5) that was not observed in the *Eubacterium* sp. strain VPI 12708 *bai* operon DNA sequence (23).

DISCUSSION

Primary bile acids are rapidly metabolized in the human colon via a 7 α -dehydroxylation pathway (Fig. 6) that appears to be limited to certain strains of the genera *Eubacterium* and

Clostridium (11). Bile acid 7 α -dehydroxylation requires uptake of bile acids (20) and their conjugation to (21) coenzyme A (CoA) followed by two successive oxidation steps yielding a 3-oxo- Δ^4 -bile acid-CoA intermediate (4, 22). The intermediate appears to be deconjugated (34) and rapidly converted to a 3-oxo- $\Delta^{4,6}$ -bile acid intermediate by 7 α -dehydration (10). The 3-oxo- $\Delta^{4,6}$ -bile acid intermediate is sequentially reduced to deoxycholic acid (11), and this end product is released from the cell.

Many of the genes required for bile acid 7 α -dehydroxylation have been identified in *Eubacterium* sp. strain VPI 12708 as part of a large (12-kb) bile acid-inducible operon (Fig. 1) (4, 23). These bile acid 7 α -dehydroxylation genes from *Eubacterium* sp. strain VPI 12708 hybridized to DNA from other *Eubacterium* strains exhibiting bile acid 7 α -dehydroxylation activity but failed to hybridize to a number of *Clostridium* strains (11). In addition, antibodies raised against purified bile acid

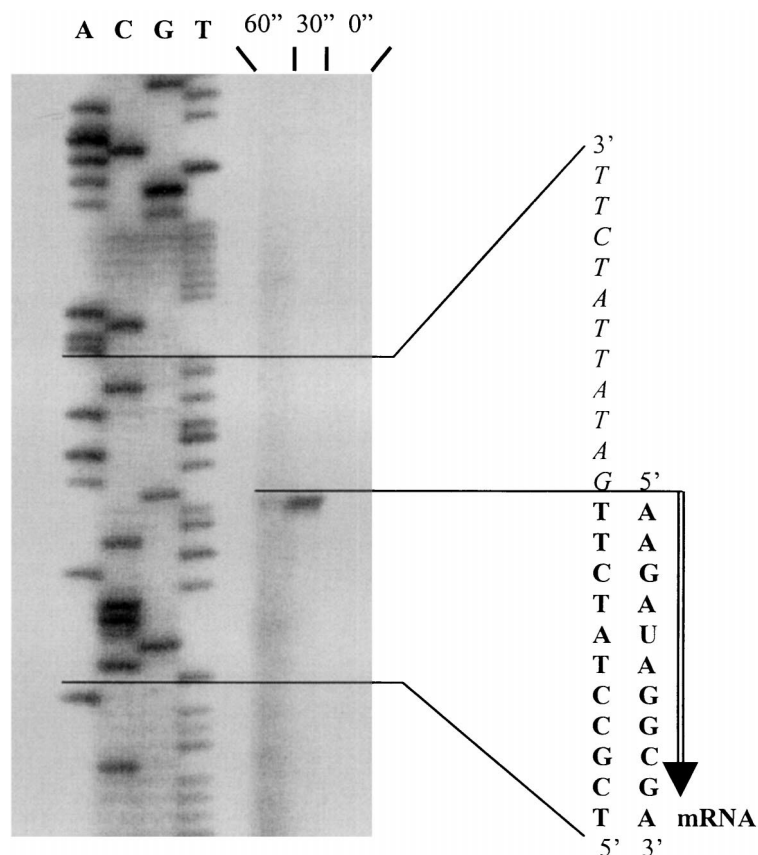


FIG. 4. Autoradiograph of primer extension analysis products. Lanes A, C, G, and T represent dideoxy nucleotide termination of *Clostridium* sp. strain TO-931 DNA sequence reactions. Lanes 60'', 30'', and 0'' denote primer extension of *Clostridium* sp. strain TO-931 mRNA isolated from cultures at 60, 30, and 0 min, respectively, after induction with 100 μ M cholic acid.

7 α -dehydroxylation pathway enzymes from *Eubacterium* sp. strain VPI 12708 did not cross-react with proteins from bile acid-induced *Clostridium* strains (unpublished data). Although all intestinal bile acid 7 α -dehydroxylation appears to proceed via a 3-oxo- Δ^4 -bile acid intermediate (8), these preliminary data suggested that the genes required for bile acid 7 α -dehydroxylation in *Eubacterium* and *Clostridium* strains might be different.

Using a redundant oligonucleotide primer mix based on a membrane-spanning region of the *Eubacterium* sp. strain VPI 12708 bile acid transporter, we identified a *baiG*-like gene fragment in *Clostridium* sp. strain TO-931. Subsequent analysis identified a 1.42-kb ORF similar in size and sequence to the *Eubacterium* sp. strain VPI 12708 *baiG* gene. The *Clostridium* sp. strain TO-931 putative *baiG* gene product was found to

have significant identity and similarity to the *Eubacterium* sp. strain VPI 12708 bile acid transporter (Table 1).

In *Clostridium* sp. strain TO-931, five putative ORFs were identified upstream of the *baiG* gene (Fig. 1), and each of these ORFs was found to exhibit significant identity to a *Eubacterium* sp. strain VPI 12708 bile acid 7 α -dehydroxylation gene upstream of the *baiG* gene (Table 1). *Clostridium* sp. strain TO-931 bile acid 7 α -dehydroxylation genes appears to be more AT biased (36% GC [versus 49% GC for the *Eubacterium* strain]) but were found to be organized in a similar fashion and to be similar in size to those identified in *Eubacterium* sp. strain VPI 12708 (Fig. 1). Nucleotide sequences complementary to *Eubacterium* sp. strain VPI 12708 *baiB*, *baiC*, *baiD*, *baiE*, *baiA2*, *baiF*, and *baiG* were observed, but no *baiH* or *baiI* genes were found. The *baiH* gene has been shown to encode an NADH:

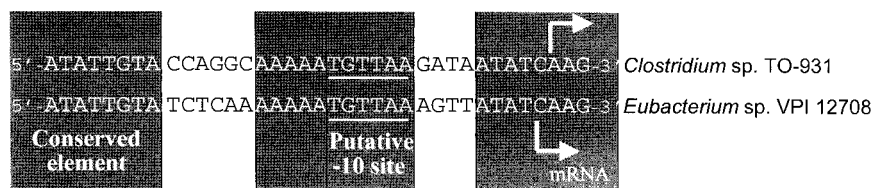


FIG. 5. Alignment of *bai* operon promoter regions from *Clostridium* sp. strain TO-931 and *Eubacterium* sp. strain VPI 12708. Arrows denote transcription start sites as determined by primer extension analysis. The conserved regions are shaded with gray. The underlined sequences are the putative promoter binding (–10) sites. *Clostridium* sp. strain TO-931 DNA also had an inverted repeat (GATA/A/TATC) between the –10 site and the mRNA initiation site.

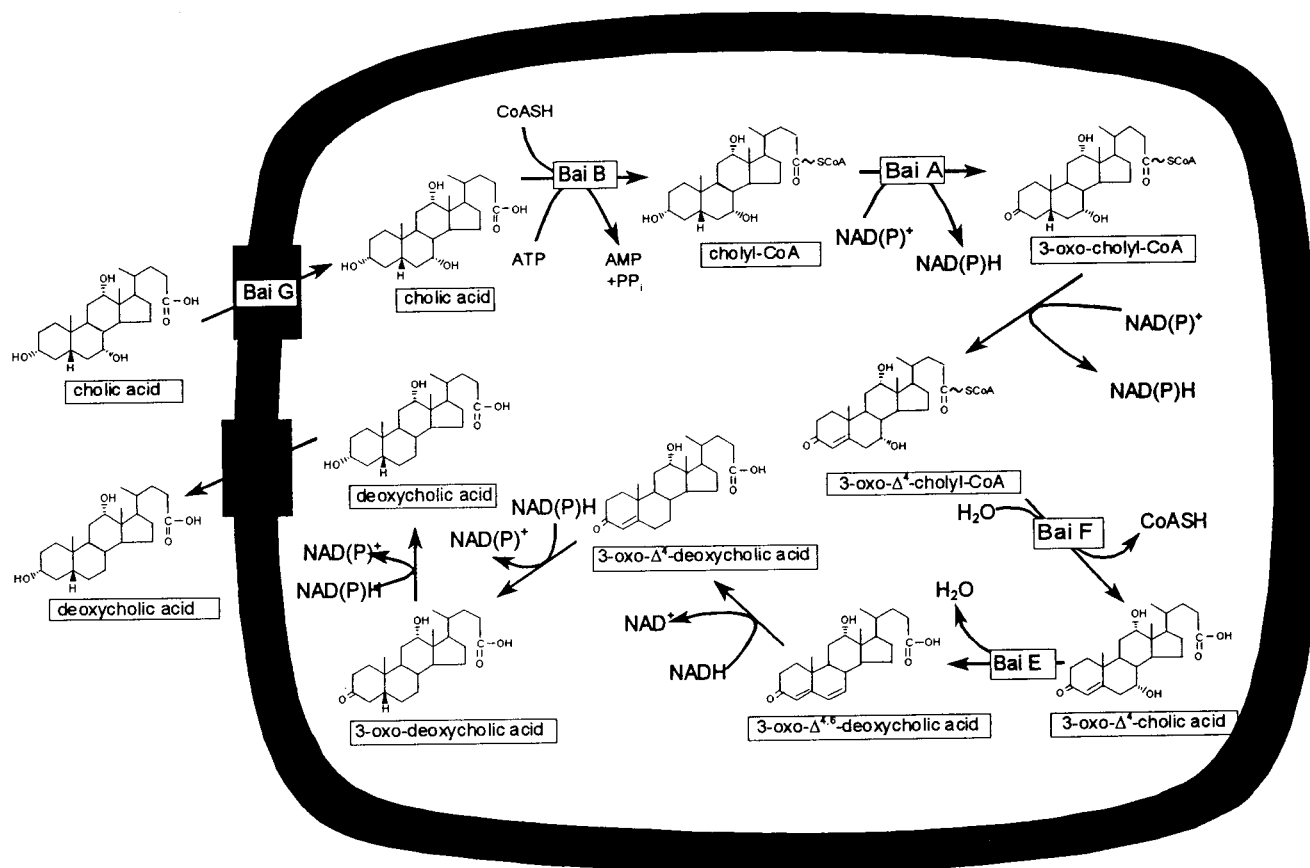


FIG. 6. Cholic acid 7 α -dehydroxylation pathway in intestinal anaerobic bacteria. Bile acid-inducible (*bai*) gene products that participate in this pathway are indicated (also see Fig. 1). CoASH, coenzyme A-SH.

flavin oxidoreductase in *Eubacterium* sp. strain VPI 12708, but its function in bile acid 7 α -dehydroxylation is unclear (12). No function has been assigned to the *bail* gene product.

Although the two bacterial operons were found to have a high degree of individual gene identity, there are some differences. The *baiC* and *baiD* genes from *Eubacterium* sp. strain VPI 12708 were determined to be on overlapping but separate open reading frames (23). In *Clostridium* sp. strain TO-931, the *baiC* and *baiD* genes are fused into one continuous open reading frame that encodes a protein with 84% upstream and 75% downstream identity to the *Eubacterium* sp. strain VPI 12708 *baiC* and *baiD* gene products, respectively. The *Clostridium* sp. strain TO-931 *baiCD* gene aligned in its entirety with the *Eubacterium* sp. strain VPI 12708 *baiH* gene, and the putative gene products were shown to have 46% identity and 51% similarity (Fig. 4). Further analysis of the *baiC*, *baiD*, and *baiE* gene complements from both *Clostridium* sp. strain TO-931 and *Eubacterium* sp. strain VPI 12708 and the *baiH* and *bail* genes from *Eubacterium* sp. strain VPI 12708 revealed a high degree of DNA sequence homology. These results suggest that gene duplication may have occurred in the *Eubacterium* sp. VPI 12708 *bai* operon. Because no *baiH* gene was found in the *Clostridium* sp. strain TO-931 *bai* operon, this enzyme function or a similar function may be associated with the *Clostridium* sp. strain TO-931 *baiCD* gene product. Further studies will be necessary to test this hypothesis.

In spite of the significant DNA sequence identity between

the two *bai* operons, the intergene DNA sequences were determined to have little homology and often were found to be much larger in the *Clostridium* sp. strain TO-931 *bai* operon. The lack of identity, differences in size of the noncoding DNA, and AT bias suggest that there has been some genetic divergence. Despite the intergene differences, the operator/promoter regions upstream of the mRNA initiation site for both *bai* operons exhibit significant identity (Fig. 5). This putative *bai* promoter was shown to have little similarity to a proposed gram-positive promoter motif, but the latter proposed sequence appears to be based on genes expressed during the late-exponential and stationary phases of cell growth (14, 16, 26, 35, 36). Our *bai* promoter region may serve to regulate genes expressed in the presence of bile acids during exponential cell growth and, as a consequence of function, may represent a different class of promoters dependent on alternative sigma factors and/or auxiliary regulatory proteins.

In summary, we have shown that the *bai* operons of *Clostridium* sp. strain TO-931 and *Eubacterium* sp. strain VPI 12708 exhibit nearly 75% DNA sequence identity. More importantly, the putative gene products are homologous, with nearly 90% similarity for many of the proteins. Although the gene sequences may have changed over time, the putative proteins are highly conserved and probably have similar tertiary structures. This latter observation may be important for the development of bile acid 7 α -dehydroxylation-inhibitory drugs.

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