Expression and characterization of a C_{24} bile acid 7α-dehydratase from *Eubacterium* sp. strain VPI 12708 in *Escherichia coli*

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Abstract The intestinal bacterium *Eubacterium* sp. strain VPI 12708 has been shown to have a bile acid 7α/7β-dehydroxylation pathway. A large bile acid inducible (bai) operon encoding at least 9 open reading frames has been cloned and sequenced from this bacterium. The *baiE* gene from this operon has been subcloned and expressed in *E. coli* and found to encode a bile acid 7α-dehydratase (BA7αD). The purified BA7αD was shown to have a calculated subunit mass of 19 kD and a relative native molecular weight of 36,000. The K_m and V_{max} for 7α,12α-dihydroxy-3-oxo-4-cholenoic acid was 0.16 mM and 0.48 mmol/min per mg protein, respectively. Of the substrates tested, the BA7αD used only 7α,12α-dihydroxy-3-oxo-4-cholenoic acid and 7α-hydroxy-3-oxo-4-cholenoic acid as substrates. A molecular modeling program (SYBYL) was used to calculate the energy differences between the various intermediates in the 7α-dehydroxylation pathway. A marked energy difference (-9.4 kcals/mol) was observed between 7α,12α-dihydroxy-3-oxo-4-cholenoic acid and 12α-hydroxy-3-oxo-4,6-choldienoic acid, possibly accounting for the apparent irreversibility of the bile acid 7α-dehydratase reaction under our experimental conditions. No significant amino acid sequence homologies were found between BA7αD and other proteins in the data base; however, BA7αD does contain a lipocalin signature sequence, possibly indicating a bile acid binding domain. The bile acid 7α-dehydratase appears to be a unique enzyme in the bacterial bile acid 7α-dehydroxylation pathway.—**Dawson, J. A., D. H. Mallonee, I. Björkhem, and P. B. Hylemon.** Expression and characterization of a C_{24} bile acid 7α-dehydratase from *Eubacterium* sp. strain VPI 12708 in *Escherichia coli*. *J. Lipid Res.* 1996. **37:** 1258–1267.

Supplementary key words 7α-dehydroxylation • secondary bile acids

Cholic and chenodeoxycholic acids are primary bile acids synthesized in the liver from cholesterol in humans. During their enterohepatic circulation they are 7α-dehydroxylated by the intestinal microflora yielding deoxycholic and lithocholic acids, respectively. Although both cholic acid and chenodeoxycholic acid are 7α-dehydroxylated, deoxycholic acid is absorbed to a greater extent than lithocholic acid. Deoxycholic acid normally makes up approximately 20–25% of the biliary bile acid pool of man. However, the % deoxycholic acid in bile can vary from 10 to 60% (1).

Eubacterium sp. strain VPI 12708 is an intestinal anaerobic bacterium isolated from human fecal flora that has been shown to contain both 7α- and 7β-dehydroxylation activities (2). This bacterium has been reported to 7-dehydroxylate only unconjugated bile acids (3). Although both 7α- and 7β-dehydroxylation activities are present, 7β-dehydroxylation proceeds at a rate 3- to 5-fold slower than that of 7α-dehydroxylation (2). In 1960, Samuelsson (4) demonstrated a mechanism for the 7α-dehydroxylation of cholic acid to deoxycholic acid that included a diaxial *trans* elimination of water and a proposed Δ^6-intermediate. Coleman et al. (5) initially proposed a new pathway by which 7α-dehydroxylation occurs in *Eubacterium* VPI 12708. The first step was hypothesized to involve the formation of a bile acid nucleotide that was identified using reverse phase-HPLC (5). Further investigation has shown that the initial step in this pathway is the ligation of cholic acid to Coenzyme A with the formation of a bile acid-AMP nucleotide intermediate in this reaction (6) (**Fig. 1**). The ligation reaction is followed by a two-step oxidation to produce a 3-oxo-Δ^4-7α-hydroxy-bile acid intermediate (7). The fourth step in the 7α-dehydroxylation pathway

Abbreviations: BA7αD, bile acid 7α-dehydratase; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; DTNB, 5,5′-dithio-bis-2-nitrobenzoic acid.

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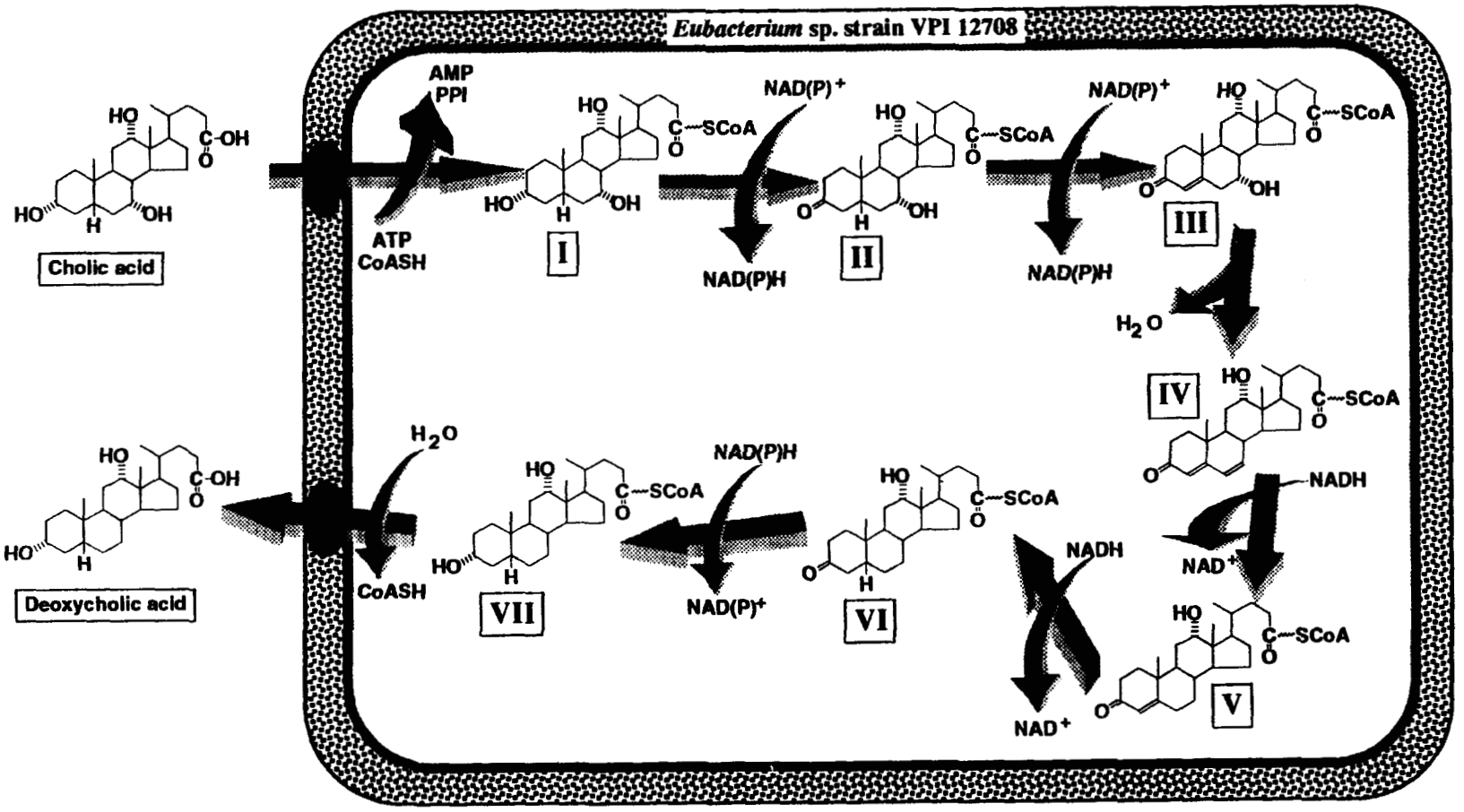

Fig. 1. Proposed pathway for the 7α-dehydroxylation of cholic acid in *Eubacterium* sp. strain VPI 12708. I: Cholic acid; II: 7α,12α-dihydroxy-3-oxo-5β-cholanoic acid; III: 7α,12α-dihydroxy-3-oxo-4-cholenoic acid; IV: 12α-hydroxy-3-oxo-4,6-choldienoic acid; V: 12α-hydroxy-3-oxo-4-cholenoic acid; VI: 12α-hydroxy-3-oxo-5β-cholanoic acid; VII: 3α12α-dihydroxy -5β-cholanoic acid.

is the 7-dehydration of the 3-oxo-Δ^4-7α-hydroxy bile acid to yield the 3-oxo-$\Delta^{4,6}$ intermediate which is then reduced in three steps to deoxycholic acid. It has not been determined how long the CoA remains conjugated to the bile acid; however it is known that the second enzyme in the pathway, 3α-hydroxysteroid dehydrogenase, prefers the CoA-conjugated bile acid substrate to the free bile acid (8). A large bile acid inducible (*bai*) operon has been cloned and sequenced and has been shown to encode several genes involved in bile acid 7α-hydroxylation (6, 8–11) (**Fig. 2**). In this communication, we report the indentification and characterization of a unique enzyme that catalyzes the bile acid 7α-dehydration reaction.

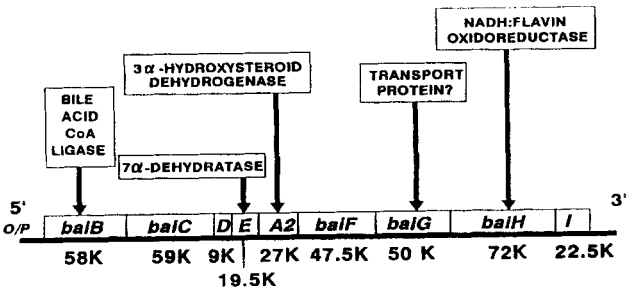

Fig. 2. *bai* Operon from *Eubacterium* sp. strain VPI 12708. BA7αD is encoded by *baiE*. The known functions of several gene products are labeled.

MATERIAL AND METHODS

Labeled and unlabeled bile acid substrates

[24-^{14}C]7α,12α-dihydroxy-3-oxo-4-cholenoic acid with a specific radioactivity of 100,000 cpm/μg was synthesized from 24-^{14}C-labeled cholic acid (obtained from Radiochemical Center, Amersham, England) by selective oxidation at C-3 according to Oppenhauer followed by dehydrogenation with selenium dioxide as described previously (7, 12). The material was purified by preparative thin-layer chromatography using trimethylpentane–isopropyl alcohol–acetic acid 60:20:0.05 (v/v/v) (13).

[24-^{14}C]7α-hydroxy-3-oxo-4-cholenoic acid with a specific radioactivity of 250,000 cpm/μg was prepared by the same method as above using 24-^{14}C-labeled chenodeoxycholic acid (obtained from Radiochemical Center) as starting material. The material was purified by thin-layer chromatography, using solvent system trimethylpentane–isopropyl alcohol–acetic acid 30:10:1 (v/v/v) (13). The mass spectrum (as methyl ester trimethylsilyl ether) was identical to that of authentic 7α-hydroxy-3-oxo-4-cholenoic acid (a generous gift from Dr. Anders Kallner) and the compound had the expected UV-absorbance at 244 nm.

Unlabeled 7β-hydroxy-3-oxo-4-cholenoic acid was prepared from ursodeoxycholic acid by selective oxidation at C-3 according to Oppenhauer followed by dehydrogenation with selenium dioxide as above. The yield of the Δ^4-unsaturated product was, however, very low in the latter reaction. After treatment of 10 mg of 7β-hydroxy-3-oxo-5β-cholanoic acid with 5 mg selenium dioxide in 2 ml of ethanol for 72 h at room temperature under stirring, the yield of the desired product was below 5%. Attempts to increase the yield by increasing the temperature, the amount of selenium dioxide or duration of the treatment did not increase the yield of the desired product and led to a number of more extensively oxidized products. Milder treatment gave little or no product. The small amount of 7β-hydroxy-3-oxo-4-ch olenoic acid obtained (about 0.5 mg) was purified by thin-layer chromatography using solvent system trimethylpentane–isopropyl alcohol–acetic acid 30:10:1 (v/v/v) (13). The preparation obtained after the purification was, however, contaminated to about 30% with the precursor, 7β-hydroxy-3-oxo-5β-cholanoic acid. The identity of the product was confirmed by its UV-absorbance at 244 nm and by its mass spectrum (as methyl ester trimethylsilyl ether). The latter spectrum was similar to that of the corresponding derivative of the isomeric compound 7α-hydroxy-3-oxo-4-cholenoic acid with a dominating molecular ion at *m/z* 384. Further evidence for presence of a Δ^4-double bond in the compound was obtained by treatment with 2 M KOH in methanol, which caused the expected shift in UV absorbance from 244 nm to 285 nm (corresponding to a 3-oxo-$\Delta^{4,6}$-structure).

7α,12α-Dihydroxy-3-oxo-5β-cholanoic acid was synthesized enzymatically from cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid) (Calbiochem, La Jolla, CA) using a commercially available *Pseudomonas testosteroni* 3α-hydroxysteroid dehydrogenase (Sigma Chemical Company, St. Louis, MO). 3α,12α-Dihydroxy-7-oxo-5β-cholanoic acid was obtained from Steraloids (Wilton, NH). [24-^{14}C]cholic acid was obtained from New England Nuclear (Boston, MA).

Bacterial strains

E. coli BL21 (DE3) (Novagen, Madison, WI) was used as the host strain for the recombinant plasmid. The *E. coli* strain was grown on LB medium supplemented with ampicillin (100 μg/ml) as needed. *Eubacterium* sp. strain VPI 12708 was grown anaerobically as previously described (10).

Expression of the *baiE* gene in *E. coli*

The strategy used previously to overexpress the *baiB* gene was used with some modifications (6). The PCR

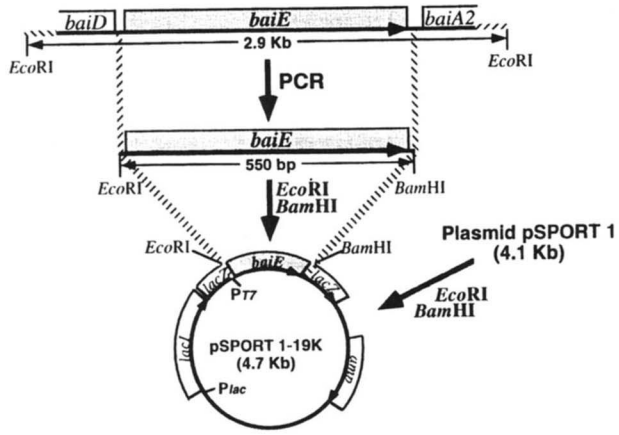

Fig. 3. Construction of an expression vector for the *baiE* gene from *Eubacterium* sp. strain VPI 12708. A 2.9-kb *Eco*RI fragment containing the *baiE* gene was used as the starting material for PCR amplification of the *baiE* gene. The amplified material was purified, digested with *Eco*RI and *Bam*HI, and ligated into a pSPORT 1 plasmid vector.

primers were 5′-AAGAATTCAAGGAGGATACATATGACATTAGAAGAGAG and 5′-AAGGATCCTGCCTTTCTTTTACAATATTC. These primers changed the ribosome binding site from AAGGATG to AAGGAGG and added *Eco*RI and *Bam*HI sites (**Fig. 3**). The PCR template was a 2.9 Kb *Eco*RI fragment containing the *baiE* gene (9, 11). This system allows for IPTG-inducible expression of genes which are placed downstream of the lac and T7 promoters found in pSPORT 1 (Gibco BRL, Gaithersburg, MD). The plasmid containing the *baiE* gene was designated pSPORT 1-19K.

Purification of the bile acid 7α-dehydratase

Overnight cultures of *E. coli* BL21 (DE3) containing the pSPORT 1-19K plasmid were used to inoculate 6 L of LB medium. Bacteria were grown and induced as described previously (6). Cells were harvested by centrifugation at 10,000 *g* for 15 min at 10°C. Cell pellets were suspended in buffer A (25 mM sodium phosphate, pH 6.8; 5% (v/v) glycerol) and disrupted by sonication in a Branson sonifier (samples were kept on ice during three 30-sec cycles at 40% output). Soluble cell extracts were collected after centrifugation for 2 h at 105,000 *g* at 4°C. Cell extracts were loaded onto a DE52 DEAE-cellulose column (2 × 6 cm) (Whatman LabSales, Hillsboro, OR) previously equilibrated with buffer A. The column was washed with 60 ml of buffer A and eluted with increasing concentrations (0 to 500 mM) of NaCl in buffer A. The 7α-dehydratase activity eluted at approximately 100 mM NaCl. Fractions containing enzyme activity were pooled, brought to 45% $(NH_4)_2SO_4$, and loaded onto a phenyl Sepharose CL-4B column (2 × 5 cm) (Sigma Chemical Co., St. Louis, MO) previously equilibrated with buffer A containing 45% $(NH_4)_2SO_4$. The column was washed with the 45% buffer and eluted step-wise with buffers containing decreasing amounts of $(NH_4)_2SO_4$. Enzyme activity eluted in the 0% $(NH_4)_2SO_4$ buffer. Fractions containing the enzyme activity were pooled, concentrated, and de-salted using Centriprep 10 concentrators (Amicon Corp., Danvers, MA). The pH was decreased to 5.0 with 0.1 N HCl prior to loading onto an HPLC-DEAE 5PW column (7.5 mm × 7.5 cm) (Beckman, Palo Alto, CA) previously equilibrated with buffer B (25 mM sodium phosphate, pH 5.0; 5% glycerol). The column was eluted with a linear NaCl gradient in buffer B from 0 to 500 mM. The enzymatic activity eluted with 190 mM NaCl. Further purification was obtained using a Superose 12 HR 10/30 HPLC size exclusion chromotography column (10 mm × 30 cm) (Pharmacia Biotech, Piscataway, NJ) previously equilibrated with buffer C (50 mM sodium phosphate, pH 7.0; 150 mM NaCl; 5% glycerol). Purification was monitored using SDS-PAGE (12% resolving gels), and the enzyme was judged to be more than 90% pure. Protein concentrations were estimated using the method of Bradford (14) (Bio-Rad Laboratories, Hercules, CA).

Enzyme assay

Bile acid 7α-dehydratase activity was measured aerobically by monitoring the conversion of the ^{14}C-labeled steroid substrate, 7α,12α-dihydroxy-3-oxo-4-cholenoic acid, into its dehydrated product, 12α-hydroxy-3-oxo-4,6-choldienoic acid using thin-layer chromatography (TLC). The standard reaction mixture contained 25 mM

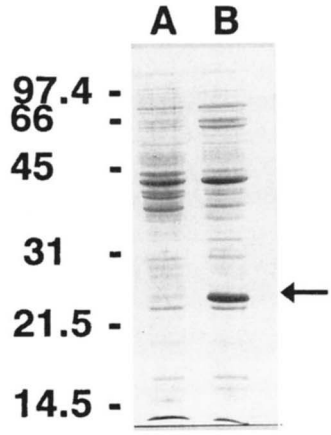

Fig. 4. SDS-polyacrylamide gel electrophoresis showing expression of the *baiE* gene in *E. coli* BL21 (DE3). Aliquots of cell extract were subjected to electrophoresis using a 12% slab gel. Gels were stained with Coomassie blue. Lanes: A, *E. coli* BL21 (DE3) pSPORT 1 (35 μg); B, *E. coli* BL21 (DE3) pSPORT 1-19K (35 μg). Molecular mass markers are listed in kilodaltons on the left. The BaiE protein is indicated by the arrow.

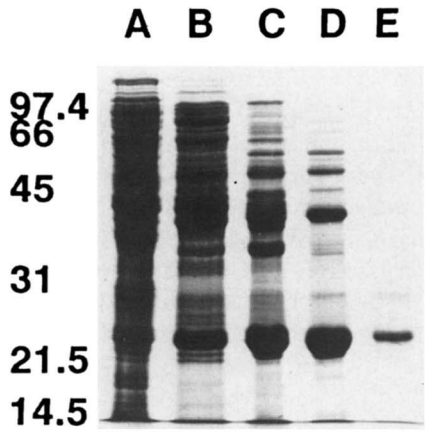

Fig. 5. Purification of bile acid 7α-dehydratase. Aliquots from each step of the purification were subjected to electrophoresis using a 12% polyacrylamide slab gel. Gels were stained with Coomassie blue. Lanes: A, cell extract from *E. coli* BL21 (DE3) pSPORT 1-19K (40 μg); B, pooled DE52 DEAE fractions (35 μg); C, pooled phenyl Sepharose fractions (15 μg); D, pooled HPLC-DEAE fractions (10 μg); E, pooled HPLC-gel filtration fractions (1 μg). Molecular mass markers are shown in kilodaltons at the left.

sodium acetate/MOPS buffer, pH 7.5, ^{14}C-labeled and unlabeled substrate in varying amounts, enzyme sample, and distilled water to a final volume of 0.05 ml. The reaction was started by the addition of the enzyme. The reaction was terminated by the addition of 5.5 μl of 0.5 N HCl, and the substrate and product were extracted with ethyl acetate. The organic phase was recovered and dried under a nitrogen gas atmosphere. The residue was dissolved in ethyl acetate and spotted onto a silica TLC plate. Bile acids were separated by TLC using a solvent system of benzene–dioxane–acetic acid 75:20:2 (v/v/v) (13). The TLC plate was allowed to air dry and then exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) overnight. Quantitation of radioactivity was done using the Betascope 603 Blot Analyzer (Betagen, a division of IntelliGenetics, Mountain View, CA). R_f values were calculated for bile acid substrates and products. The dehydratase product was further identified as 12α-hydroxy-3-oxo-4,6-choldienoic acid by mass spectrometry as described previously (15).

Determination of apparent kinetic constants

The bile acid 7α-dehydratase activity was determined to be linear with respect to time up to 1 min and linear with respect to protein concentration up to 50 μg. These data were used in determining the apparent V_{max} and K_m values according to the method of Hanes (16). Enzyme assays were performed using 0.5 μg of pure enzyme preparation. Concentrations of substrate were only about twice the K_m value because of the limited availability of the bile acid substrate.

Characterization of substrates and inhibitors

Additional bile acids were tested as possible substrates for the bile acid 7α-dehydratase. The standard enzyme assay was used and 7α-hydroxy-3-oxo-4-cholenoic acid, 7α,12α-dihydroxy-3-oxo-5β-cholanic acid, 3α,7α,12α-trihydroxy-5β-cholanic acid, 7β-hydroxy-3-oxo-4-cholenoic acid, and 12α-hydroxy-3-oxo-4,6-choldienoic acid were tested as substrates. Bile acids and other reagents were also tested for their ability to inhibit the 7α-dehydration of 7α,12α-dihydroxy-3-oxo-4-cholenoic acid. Bile acids (3α,7α,12α-trihydroxy-5β-cholanic acid, 7α,12α-dihydroxy-3-oxo-5β-cholanic acid, 3α,12α-dihydroxy-7-oxo-5β-cholanic acid) were added to the enzyme assays in a concentration equal to that of the substrate. Other putative enzyme inhibitors (DTNB, iodoacetate, N-bromo-succinimide, N-ethylmaleimide, p-hydroxymercuribenzoic acid) were incubated with the enzyme for 10 min at 37°C at a concentration of 10 mM.

Fractionation of the 7α- and 7β-dehydratase activities using gel filtration chromatography

An HPLC-gel filtration column (Superose HR 10/30, Pharmacia-Hoefer Biotech, Piscataway, NJ) was used to fractionate 7α- and 7β-dehydratase activities in induced *Eubacterium* sp. 12708 cell extracts.

Molecular modeling and calculation of the approximate energies of the bile acids

Bile acid substrates and products were analyzed using the SYBYL molecular modeling software, version 6.2, developed by Tripos, Inc. (St. Louis, MO) on a Silicon Graphics workstation. The SKETCH function of this program was used to draw a stick model for the individual bile acids. The bond-stretching, angle-bending torsional, 6–12 Lennard-Jones potential parameters and out-of-plane bending terms for the Tripos Force field were described by Clark et al. (17). The atomic charges were computed using the Gasteiger-Hückel method (17). The standard energy minimizer, MAXIMIN2, was

TABLE 1. Purification of the bile acid 7α-dehydratase

Purification Step	Protein	Specific Activity	Purification	Yield
	mg	*units*[a]*/mg*	*fold*	%
Cell-free extract	542	1.4	1.0	100
DE-52	77	14.2	10	14.1
Phenyl Sepharose	28	31.6	23	11.4
HPLC-DEAE	1.76	76.4	55	1.73
HPLC-GF	0.73	131	94	1.23

[a]One unit of activity is defined as 1 μmol of 12α-hydroxy-3-oxo-4,6-choldienoic acid produced from 7α,12α-dihydroxy-3-oxo-4-cholenoic acid per minute.

used to calculate the lowest possible energy for each molecule. The energy is a function of the atomic coordinates, and the program attempts to generate the coordinates that correspond to a minimum of energy (17). The parameters used were all default settings except that the number of iterations was 10,000 and the conjugate-gradient termination value was 0.002 kcal/mol. It was hypothesized that these energy calculations would provide some information regarding the equilibrium of the 7-dehydration reaction lying far to the right. Energies were also calculated for the various intermediates in the 7α-dehydroxylation pathway as well as for other steroid dehydratase substrates and products (18, 19). It should be noted that the numbers obtained from the computer are approximate energy values and cannot be equated with standard free energy because the computer calculation includes no consideration of entropy.

Sequence analysis

Analysis of protein sequence data was performed using the GCG program (University of Wisconsin Biotechnology Center, Madison, WI) and the BLAST program (National Cancer Institute, Bethesda, MD).

RESULTS

Expression of the *baiE* gene in *E. coli*

Expression of the *baiE* gene in *E. coli* allowed us to identify and produce large quantities of the bile acid 7α-dehydratase. Seven liters of *E. coli* BL21 (DE3) pSPORT 1-19K usually yielded approximately 550 mg of total soluble protein, with the bile acid 7α-dehydratase representing 11% of the total cellular protein (**Fig. 4**).

Purification of the bile acid 7α-dehydratase

The bile acid 7α-dehydratase was purified using four chromatographic steps. A typical bacterial preparation yielded more than 500 units of enzyme activity with a specific activity of 1.4 units/mg (**Table 1**). Samples of each of the four purification steps were analyzed by SDS-PAGE and the results are shown in **Fig. 5.** Although large quantities of the enzyme were produced by the organism, the overall purification yield remained relatively low. Because of the difficulty in obtaining substrate for the enzyme, the protein was purified based on SDS-PAGE analysis of the fractions collected from the various chromatographic steps. Therefore, some en-

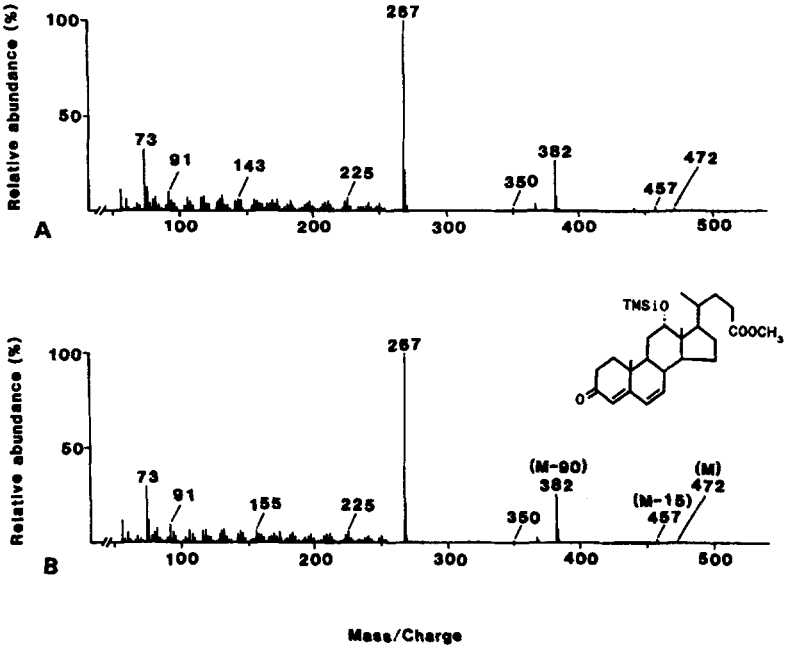

Fig. 6. Mass spectrum of 12α-hydroxy-3-oxo-4,6-choldienoic acid standard (B) and unknown 7α-dehydration reaction product (A). [^{14}C]7α,12α-dihydroxy-3-oxo-4-cholenoic acid was incubated with purified BA7αD for 1 min. Bile acids were extracted with ethyl acetate and separated by TLC as described in Materials and Methods. The unknown dehydration reaction product was extracted from TLC plates and subjected to gas–liquid chromatography–mass spectrometry.

TABLE 2. Substrate specificity of the bile acid 7α-dehydratase

Substrate	Relative Activity
7α,12α-Dihydroxy-3-oxo-4-cholenoic acid	100%
7α-Hydroxy-3-oxo-4-cholenoic acid	84%
7α,12α-Dihydroxy-3-oxo-5β-cholanoic acid	<1%
3α,7α,12α-Trihydroxy-5β-cholanoic acid	<1%
7β-Hydroxy-3-oxo-4-cholenoic acid	<1%

Assays contained sodium acetate/MOPS buffer, pH 7.5, 0.5 μg of pure bile acid 7α-dehydratase, ^{14}C-labeled bile acid substrate, and distilled water in a final volume of 0.05 ml. The amount used was determined by specific radioactivity so that comparable amounts of each bile acid were used. Assays were incubated at 37°C for 1 min, then stopped with 5.5 μl of 0.5 N HCl. Radiolabeled 7β-hydroxy-3-oxo-4-cholenoic acid was not available so the minimum amount detectable by UV irradiation of the TLC plate was used (14 nmol/280 μM).

zyme units were probably lost due to the inability to monitor activity in each of the column fractions.

Although the enzyme appears to be a 23-kilodalton protein on SDS-PAGE, the size of the protein calculated from translated DNA sequence is 19.5 kilodaltons. The protein, when purified from *Eubacterium* sp. strain VPI 12708, has consistently migrated as a larger protein on SDS-PAGE (9). In fact, the protein was originally identified as a 23.5 kilodalton protein by White et al. (20). The native molecular weight of the enzyme was estimated to be 36,000 using gel filtration chromatography suggesting that the enzyme is a dimer (data not shown).

Identification of the 7-dehydratase product

R_f values for each bile acid substrate and product were determined: 7α,12α-dihydroxy-3-oxo-4-cholenoic acid, 0.18; 12α-hydroxy-3-oxo-4,6-choldienoic acid, 0.52; 7α-hydroxy-3-oxo-4-cholenoic acid, 0.49; 3-oxo-4,6-choldienoic acid, 0.76; 7β-hydroxy-3-oxo-4-cholenoic acid, 0.53. The identity of 12α-hydroxy-3-oxo-4,6-choldienoic acid was confirmed by mass spectrometry (**Fig. 6**).

Determination of apparent kinetic constants

K_m and V_{max} values were obtained under initial velocity conditions. Using 7α,12α-dihydroxy-3-oxo-4-cholenoic acid as substrate, the apparent K_m was 0.16 mM and the apparent maximal velocity was 0.48 mmol/min per mg protein.

Characterization of substrates and inhibitors

Several additional bile acids were tested as potential substrates for the bile acid 7α-dehydratase. Of those tested, only 7α-hydroxy-3-oxo-4-cholenoic acid was a detectable substrate for BA7αD. The bile acids tested and their activities relative to the activity observed with 7α,12α-dihydroxy-3-oxo-4-cholenoic acid are shown in **Table 2.** Using 12α-hydroxy-3-oxo-4,6-choldienoic acid as substrate, the reaction catalyzed by BA7αD was not detectably reversible under the conditions used here. None of the bile acids (280 μM) or putative enzyme inhibitors (10 mM) tested (see Materials and Methods) inhibited the activity of BA7αD when added at the concentrations indicated (data not shown).

Fractionation of the 7α- and 7β-dehydratase activities by gel filtration chromatography

Both bile acid 7α- and 7β-dehydratase activity were found to be induced by cholic acid. This is consistent with the results of a control experiment using ursodeoxycholic acid in which only the induced cell extract 7β-dehydroxylated the ursodeoxycholic acid.

The result of the gel filtration chromatography of *Eubacterium* sp. 12708 cell extract is shown in **Fig. 7.** The fraction containing the greatest 7α-dehydratase activity eluted at 34 min. In contrast, the fraction containing the greatest 7β-dehydratase activity, determined using 7β-hydroxy-3-oxo-4-cholenoic acid as substrate, eluted at 37 min. An estimated molecular weight of 17,000 was calculated for the 7β-dehydratase (data not shown).

Molecular modeling and calculation of the approximate energies of the bile acids

The computer program SYBYL was used to calculate the lowest possible energy for each steroid molecule. While the approximate energy values obtained are not equivalent to free energy values, they do provide a basis

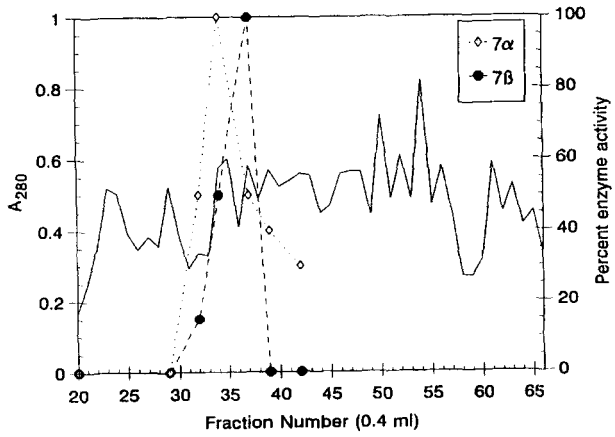

Fig. 7. The 7α- and 7β-dehydratase activities in *Eubacterium* sp. strain VPI 12708 were fractionated using gel filtration chromatography. An HPLC-gel filtration column was previously equilibrated in buffer C (50 mM sodium phosphate, pH 7.0; 150 mM NaCl; 5% glycerol). A total of 8 mg of protein was loaded at a flow rate of 0.4 ml/min, and 1-min fractions were collected. Total protein was monitored at 280 nm and is represented by the solid line. Bile acid 7α-dehydratase activity is represented by the dotted line. 7β-Dehydratase activity is represented by the dashed line. Enzymatic activities are relative, with the fraction containing the most activity equal to 100%.

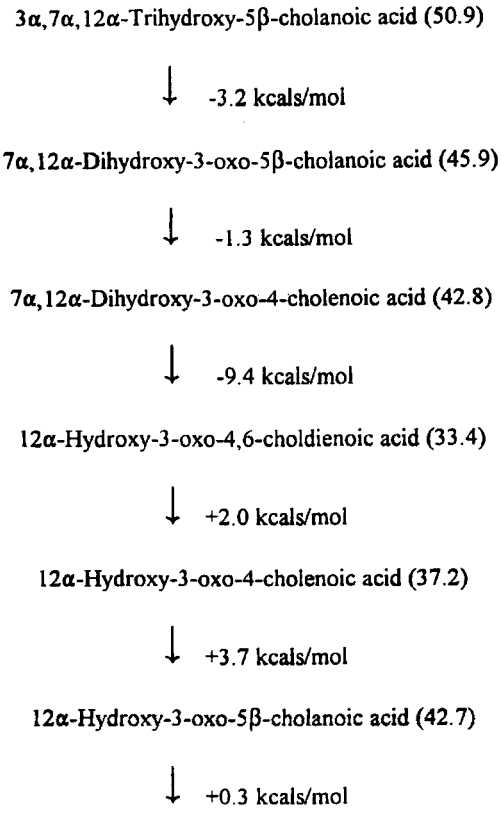

Fig. 8. Computer calculated energies of bile acid 7α-dehydroxylation pathway intermediates. The approximate energy values determined for the bile acid intermediates, in kcals/mol, are shown in parentheses after each bile acid. The energy difference calculated for each reaction is shown beside the arrows and includes the energy difference of any cofactors involved. The energy difference between the oxidized and reduced forms of the cofactors is 1.8 kcals/mol. Overall, the energy difference for the entire pathway is -6.0 kcals/mol.

of comparison for similar reactions. The energies of steroid substrates and products were compared for information on the energetics of the 7-dehydration reaction. The energies were calculated for each step in the bile acid 7α-dehydroxylation pathway, and these results are shown in **Fig. 8.** Overall, the calculated energy change for the pathway is -6.0 kcals/mol. The biggest energy difference occurs during the dehydration reaction. The bile acid 7β-dehydratase reaction was also analyzed. The energy calculated for the 7β-hydroxy-3-oxo-4-cholenoic acid substrate was 38.34 kcals/mol. The energy for the product, 3-oxo-4,6-choldienoic acid, was 31.09 kcals/mol, giving a somewhat smaller energy difference (-7.25 kcals/mol) than that calculated for the BA7αD reaction.

Energies were also calculated for hepatic C-27 steroid 7α-dehydratase (18) and 5α-hydroxysterol dehydrase (19). The calculated energies of the substrate and product of the hepatic C-27 steroid 7α-dehydratase were 47.93 kcals/mol (7α-hydroxy-4-cholesten-3-one, substrate) and 39.23 kcals/mol (cholesta-4,6-dien-3-one, product). The energy difference for hepatic C-27 steroid 7α-dehydratase (-8.7 kcals/mol) was comparable to that obtained for BA7αD (-9.4 kcals/mol). The energy calculated for the 5α-hydroxysterol dehydratase reaction was 27.85 kcals/mol for the substrate, ergosta-7,22-diene-3β,5α-diol, and 27.66 for the product, ergosterol. The energy difference between the substrate and product was negligible.

Amino acid sequence analysis

The amino acid sequence of the bile acid 7α-dehydratase revealed no significant homologies with other dehydratases or other amino acid sequences in the database (8) (**Fig. 9**). However, analysis using the GCG motifs program revealed that amino acids 127–140 of the bile acid dehydratase contain the lipocalin signature motif with one mismatch (21) (Fig. 9). Lipocalins are proteins that bind and transport small hydrophobic molecules, and the presence of the lipocalin motif might suggest a bile acid binding site for the bile acid dehydratase. However, attempts to investigate this hypothesis using specific amino acid modifiers (see Materials and Methods) provided no evidence for a binding site.

DISCUSSION

The bacterial 7α-dehydroxylation pathway results in the production of the secondary bile acids, deoxycholic and lithocholic acids, which are more toxic than their primary bile acid precursors (22). Deoxycholic acid has also been shown to be a co-carcinogen in animal model studies (23) and has been implicated in cholesterol gallstone disease (24).

Purified bile acid 7α-dehydratase showed no activity with 7β-hydroxy-3-oxo-4-cholenoic acid. Further evidence for a second dehydratase specific for 7β-hydroxy bile acids is suggested by size exclusion chromatography of the two activities in *Eubacterium* sp. strain VPI 12708 cell extract. The mechanism of 7β-dehydroxylation has not been elucidated. However, as both activities are inducible by cholic acid, they may have similar pathways. If a comparable pathway for 7β-dehydroxylation does exist, then the 7β-dehydratase might also be a unique enzyme.

The gene encoding the bacterial bile acid 7α-dehydratase appears to belong to a unique gene family. No other proteins with significant homology were found in amino acid sequence database searches. Additionally, no other bile acid or steroid dehydratases have been cloned or sequenced. Therefore, amino acid sequence comparisons are not yet possible. The only bacterial

steroid dehydratase reported in the literature is the 16α-dehydratase (25, 26). This enzyme has been purified from an intestinal *Eubacterium* species and has a reported subunit molecular weight of 42,000 (27). This enzyme uses both 16α-hydroxyprogesterone and 16α-hydroxypregnenolone as substrates.

Of the four other steroid dehydratases reported in the literature (18, 19, 25, 26, 28), the rat hepatic C-27 sterol 7α-dehydratase described by S. Skrede, Buchmann, and Björkhem (18) appears to be most similar to the bacterial 7α-dehydratase in terms of mechanism. Skrede and Björkhem (29) initially proposed that the pathway involved in cholestanol biosynthesis occurred in the intestine, analogous to the 7-dehydroxylation of bile acids by intestinal bacteria. However, the rat hepatic sterol 7α-dehydratase has been reported to be a membrane bound protein (18) while BA7αD has been shown to be a cytosolic protein.

The mechanism proposed for the 7α-dehydroxylation of bile acids indicates that the dehydration of the 3-oxo-Δ^4-7α-hydroxy-bile acid proceeds via a diaxial *trans* elimination of water to yield a $\Delta^{4,6}$-intermediate (4). Both the 3-oxo-Δ^4-7α-hydroxy-bile acid and the $\Delta^{4,6}$-bile acid intermediates have been specifically identified (7, 15). The exact mechanism of BA7αD has not been investigated using the appropriately radiolabeled bile acids (i.e., [6β-^{3}H]7α,12α-dihydroxy-3-oxo-4-cholenoic acid). However, some evidence suggests that the mechanism of this enzyme is indeed a diaxial *trans* elimination of water as originally proposed by Samuelsson (4). Skrede et al. (18) have studied the 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one in mammalian liver, an important step in the formation of cholestanol. The 7α-dehydroxylation activity in human liver is important to patients with cerebrotendinous xanthomatosis (CTX), an inborn error of bile acid side chain oxidation. 7α-Hydroxy-4-cholesten-3-one can accumulate in the liver of CTX patients because of the interruption of normal bile acid synthesis. Experiments using [6β-^{3}H]7α-hydroxy-4-cholesten-3-one demonstrate that the mechanism of dehydroxylation involves a diaxial *trans* elimination of the 7α-hydroxy group and the 6β-hydrogen producing the cholesta-4,6-dien-3-one product (18).

A molecular modeling computer program (SYBYL) (17) was used to calculate the approximate energies of the bile acid 7α-dehydratase substrates and products. We hypothesized that energy differences between substrate and product might suggest why the BA7αD reaction was not detectably reversible under our assay conditions. The calculated values (Fig. 8) for 7α,12α-dihydroxy-3-oxo-4-cholenoic acid and 12α-hydroxy-3-oxo-4,6-choldienoic acid were 42.8 kcal/mol and 33.4 kcal/mol, respectively. The difference of -9.4 kcals/mol suggests that the equilibrium of BA7αD strongly favors dehydration. If 1 kcal/mol of energy is approximately equal to an order of magnitude difference in concentrations between the substrate and product at equilibrium, then, at the concentrations of substrate we are using, we would be unable to detect the reverse reaction because the amount of bile acid formed by the reverse reaction would be less than our lower limit of detection. In examining the energy values for each step in the 7α-dehydroxylation pathway, we found that the dehydration reaction is accompanied by the largest energy change (Fig. 8). Such a large energy difference at this step in the pathway could suggest that the dehydration reaction catalyzed by the BA7αD drives the pathway in the reductive direction.

In summary, we report the identification, expression, and characterization of a bile acid 7α-dehydratase in *Eubacterium* sp. strain VPI 12708. The enzyme catalyzes the fourth step in bile acid 7α-dehydroxylation by this intestinal organism, and the reaction is not detectably reversible under the conditions used. The gene encoding the BA7αD is unique in that it shares no homology with other known protein sequences. Furthermore, we have shown that *Eubacterium* VPI 12708 has two separate 7-dehydratase enzyme activities. One is specific for 7α-hydroxy bile acids and the other appears to be specific for 7β-hydroxy bile acids. However, the specificity of the 7β-dehydratase cannot be determined completely until the enzyme is purified and characterized.

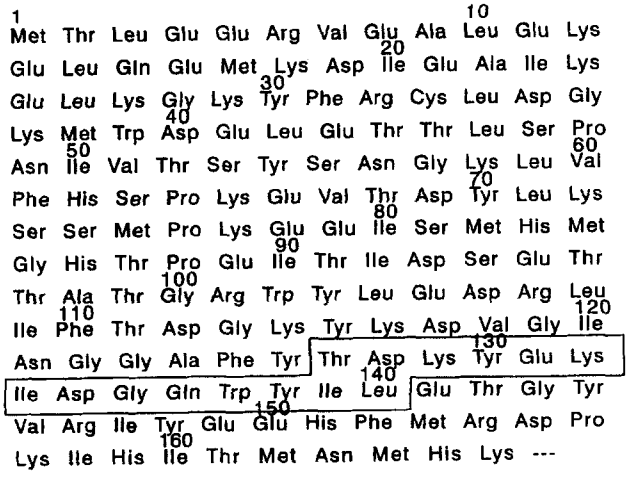

Fig. 9. Predicted amino acid sequence of the bile acid 7α-dehydratase, deduced from the nucleotide sequence (8) (GenBank/EMBL Accession No. M36292). Computer database searches for significant amino acid sequence homologies revealed none. The lipocalin motif is shown by the boxed amino acids.

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