JOURNAL OF BACTERIOLOGY, Apr. 1992, p. 2065–2071
0021-9193/92/072065-07$02.00/0

Vol. 174, No. 7

The Bile Acid-Inducible *baiB* Gene from *Eubacterium* sp. Strain VPI 12708 Encodes a Bile Acid-Coenzyme A Ligase

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Received 10 October 1991/Accepted 24 January 1992

The *baiB* gene from *Eubacterium* sp. strain VPI 12708 was previously cloned, sequenced, and shown to be part of a large bile acid-inducible operon encoding polypeptides believed to be involved in bile acid 7α-dehydroxylation. In the present study, the *baiB* gene was subcloned and expressed in *Escherichia coli* and shown to encode a bile acid-coenzyme A (CoA) ligase. This ligase required a C-24 bile acid with a free carboxyl group, ATP, Mg^{2+}, and CoA for synthesis of the final bile acid-CoA conjugate. Product analysis by reverse-phase high-performance liquid chromatography revealed final reaction products that comigrated with cholyl-CoA and AMP. A putative bile acid-AMP intermediate was detected when CoA was omitted from the reaction mixture. The bile acid-CoA ligase has amino acid sequence similarity to several other polypeptides involved in the ATP-dependent linking of AMP or CoA to cyclic carboxylated compounds. The bile acid-CoA ligation is believed to be the initial step in the bile acid 7α-dehydroxylation pathway in *Eubacterium* sp. strain VPI 12708.

The intestinal bacterium *Eubacterium* sp. strain VPI 12708 possesses a bile acid 7-dehydroxylation activity that is induced by culturing in the presence of unconjugated C-24 bile acids that possess a 7α-hydroxyl group (37). This bacterium synthesizes several new polypeptides after cholic acid induction (24, 36). Three of these inducible polypeptides have been purified, and the genes encoding them have been cloned and sequenced (5, 6, 20, 38). One of the purified polypeptides, a 27,000-M_r species, is encoded by three separate genes (*baiA1*, *baiA2*, and *baiA3*) (5, 6, 13, 39). One of these three gene copies (*baiA2*) resides on a large bile acid-inducible operon (20). The genes (*baiE* and *baiF*) encoding two other verified bile acid-inducible polypeptides are also included among the six or more open reading frames present in this operon. Most of the enzymes involved in bile acid 7α-dehydroxylation are thought to be encoded from this operon (20). It has been suggested that the 7-dehydroxylation reaction may provide an ancillary electron acceptor for bacteria capable of expressing this activity (15). Bile acids modified by 7-dehydroxylation constitute 20 to 25% of the biliary pool in humans (29).

The 7α-dehydroxylation activity has been proposed to proceed by a multistep pathway where the bile acid is first linked to an adenosine nucleotide upon entering the cell (4, 6). The bile acid then undergoes a pair of sequential oxidation reactions followed by loss of the 7α-hydroxyl group in a dehydration step, and finally the 3-oxo-$\Delta^{4,6}$-steroid intermediate undergoes a three-step reduction, yielding the 7-dehydroxylated product. A previous report (4) identified a proposed bile acid-adenosine nucleotide conjugate that was produced from crude extracts of cholic acid-induced *Eubacterium* sp. strain VPI 12708.

The *baiB* gene is the first open reading frame in the bile acid-inducible operon from *Eubacterium* sp. strain VPI 12708 and is thought to encode a 58,000-M_r polypeptide that has significant sequence similarity to several polypeptides from divergent sources. The activities of these homologous polypeptides all involve the ATP-dependent linking of AMP or coenzyme A (CoA) to cyclic carboxylated compounds. It has therefore been suggested (20) that the *baiB* gene encodes an enzyme that activates bile acids as the initial step in the 7α-dehydroxylation pathway. In this study, we expressed the cloned *baiB* gene in *Escherichia coli* and analyzed its expressed activities.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* Y1090 (40) was used as the host strain for λgt11 recombinant bacteriophage, and *E. coli* DH5α (GIBCO BRL, Gaithersburg, Md.) and HMS174(DE3) (31) were used as the host strains for recombinant plasmids. The *E. coli* strains were grown on LB medium (1% tryptone [Difco Laboratories, Detroit, Mich.], 1% NaCl, 0.5% yeast extract, pH 7.5), supplemented with 100 μg of ampicillin per ml as required.

Subcloning and expression of the *baiB* gene in *E. coli*. The *baiB* gene from *Eubacterium* sp. strain VPI 12708 is contained in a 2.4-kb chromosomal *Eco*RI fragment that was initially cloned in *E. coli* Y1090 by using a λgt11 vector (20). This 2.4-kb fragment was subsequently subcloned in *E. coli* DH5α by using a pUC19 vector (8). The 2.4-kb insert was used for Sanger dideoxy sequencing (26) and as the starting material for polymerase chain reaction (PCR) amplification of the *baiB* gene with synthetic oligonucleotide primers that added a *Pst*I restriction site to the 5′ upstream end of the gene and an *Eco*RI site to the 3′ downstream end of the gene (Fig. 1). An *E. coli* ribosome binding site was also added to the 5′ sequence. Approximately 10 to 20 ng of the purified 2.4-kb *Eco*RI fragment was subjected to PCR amplification with 1 μg of each oligonucleotide primer. A Perkin Elmer (Norwalk, Conn.) PCR kit was used as per the accompanying instructions with cycling temperatures of 94°C (1 min), 55°C (2 min), and 72°C (1 min) on a Perkin Elmer thermal cycler for 25 cycles. The DNA generated from this reaction was run on an agarose gel, and the fragment corresponding to the amplified *baiB* gene was cut out and purified with GeneClean (Bio 101 Inc., La Jolla, Calif.). The purified

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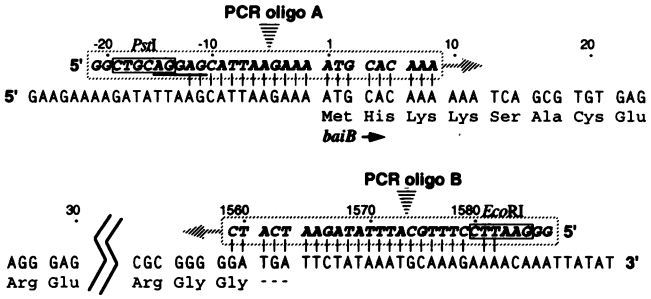

FIG. 1. Sequences of oligonucleotide (oligo) primers used for PCR amplification of the *baiB* gene from *Eubacterium* sp. strain VPI 12708. The added ribosome binding site is underlined, and the added *Pst*I and *Eco*RI restriction sites are boxed.

material was digested with *Eco*RI and *Pst*I and ligated to plasmid pSPORT 1 (GIBCO BRL), which had been digested with *Eco*RI and *Pst*I and treated with bacterial alkaline phosphatase. The ligated material was used to transform *E. coli* DH5α and HMS174(DE3) with selection for ampicillin resistance and screening for white colonies in the presence of isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Plasmid isolation was performed by the method of Birnboim and Doly (2). The plasmid containing the *baiB* gene was designated pSPORT 1-58. A schematic of the construction of plasmid pSPORT 1-58 is presented in Fig. 2.

Partial purification of the bile acid-CoA ligase. For purification of the bile acid-CoA ligase, *E. coli* HMS174(DE3) containing the pSPORT 1-58 plasmid was used to inoculate 3 liters of LB medium containing ampicillin. The strain was grown with shaking at 37°C to a reading of 80 Klett units before induction with 0.5 mM IPTG. After 2 h of additional growth, the cells were harvested by centrifugation, resuspended in 1/100 volume of distilled water containing 100 μg of DNase I and 20 mM 2-mercaptoethanol (2ME), and disrupted by sonication. The cell lysate was centrifuged at 105,000 × *g* for 2 h, and the supernatant was collected and filtered extensively with Centriprep-10 concentrators (Amicon Corp., Danvers, Mass.) to remove low-molecular-weight components. The suspension was then applied to a Waters AP-2 DEAE high-performance liquid chromatography (HPLC) column (Millipore Corp., Burlington, Mass.). Fractions eluted from a 0 to 500 mM salt gradient in 20 mM sodium phosphate buffer (pH 6.5)–20 mM 2ME were collected (1-min fractions at a flow rate of 3.5 ml/min). Fractions containing bile acid ligase activity were pooled, concentrated with Centriprep-10 concentrators, and stored overnight with 10% glycerol at −70°C. The samples were then passed through an Altex TSK 3000 SW gel filtration column equilibrated with 50 mM sodium phosphate buffer (pH 6.8)–20 mM 2ME–100 mM NaCl (1-min fractions at a flow rate of 0.85 ml/min). Samples containing ligase activity were concentrated with Centriprep-10 units before being applied to a Mono-Q ion-exchange column (Pharmacia LKB Biotechnology, Piscataway, N.J.) equilibrated with 20 mM Tris-HCl buffer (pH 7.5)–20 mM 2ME. Fractions containing the ligase activity from a 0 to 1 M NaCl gradient (1-min fractions at a flow rate of 1 ml/min) were concentrated with Centricon-10 microconcentrators (Amicon), brought to 50% glycerol, and stored at −20°C. Fractions were analyzed by electrophoresis on 7 to 20% exponential-gradient denaturing polyacrylamide gels. Protein concentrations were determined with a Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, Calif.) (3).

Bile acid-CoA ligation assay. The standard reaction mixture for analysis of bile acid-CoA ligase activity contained 10 mM MOPS (morpholinepropanesulfonic acid; pH 6.8), 10 mM 2ME, 5 mM $MgCl_2$, 2.5 mM ATP, 2.5 mM CoA (some reactions were performed with 250 μM CoA), 5 μM [24-^{14}C]-cholic acid (0.05 μCi), and bile acid-CoA ligase. The reaction was stopped by addition of 10 μl of 1 M HCl per 250-μl reaction volume and extracted three times with equal volumes of ethyl acetate to remove unconjugated bile acids. The radioactivity in the aqueous phase was assayed by liquid scintillation counting.

Reverse-phase HPLC of reaction products. A reverse-phase C18 column (4.6 mm by 25 cm) (10-μm silica gel) was used for analysis of bile acid ligation products. Standard reaction mixtures with and without CoA were used. Additional unlabeled cholic acid was added at a 1 mM concentration in some experiments.

Spectrophotometric analysis. Spectrophotometric analysis of the purified cholyl-CoA conjugate was performed with a Shimadzu UV160U spectrophotometer.

TLC of reaction products. Analysis of the bile acid component of the bile acid conjugates was carried out on silica gel 1B thin-layer chromatography (TLC) plates (J. T. Baker, Inc., Phillipsburg, N.J.). Ligation reactions were performed with standard reaction mixtures for 30 min at 37°C before being stopped with 10 μl of 1 M HCl. Three extractions to remove unconjugated bile acids were performed with 250-μl volumes of ethyl acetate before an equal volume (260 μl) of 2 M NaOH was added to the aqueous phase and the mixture was boiled for 30 min (28). The suspension was acidified by adding 160 μl of 4 M HCl and extracted twice with 500 μl of ethyl acetate. The pooled ethyl acetate fractions were spotted on the TLC plates and chromatographed with solvent system S4 (11). The plates were then dried and exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) for 24 h at room temperature before the film was developed.

Analysis of sequence data. Analysis of nucleic acid and protein sequence data was performed with the IBI/Pustell DNA analysis program (International Biotechnologies, Inc., New Haven, Conn.) and the GCG program (University of Wisconsin Biotechnology Center, Madison, Wis.).

RESULTS

Subcloning and expression of the *baiB* gene. The *baiB* gene from *Eubacterium* sp. strain VPI 12708, which had been initially cloned in a 2.4-kb *Eco*RI fragment (20), was amplified by PCR and subcloned into an expression system with plasmid pSPORT 1 and *E. coli* HMS174(DE3). With this system, induction with IPTG allows transcription of the cloned insert from both a *lac* promoter and a T7 promoter (Fig. 2). The plasmid containing the *baiB* gene was designated pSPORT 1-58. Figure 3 shows expression of the 58,000-M_r *baiB* gene product in IPTG-induced *E. coli* HMS174(DE3) containing plasmid pSPORT 1-58.

Partial purification of the bile acid-CoA ligase. The bile acid-CoA ligase from *Eubacterium* sp. strain VPI 12708 was partially purified by HPLC (Fig. 4). A 21-fold purification with a 13.6% final yield was obtained from a representative purification run (Table 1). The enzyme was stable for several months when stored at −20°C in 50% glycerol.

Optimization of bile acid-AMP ligation activity. The bile acid ligation assay is performed by determining the amount

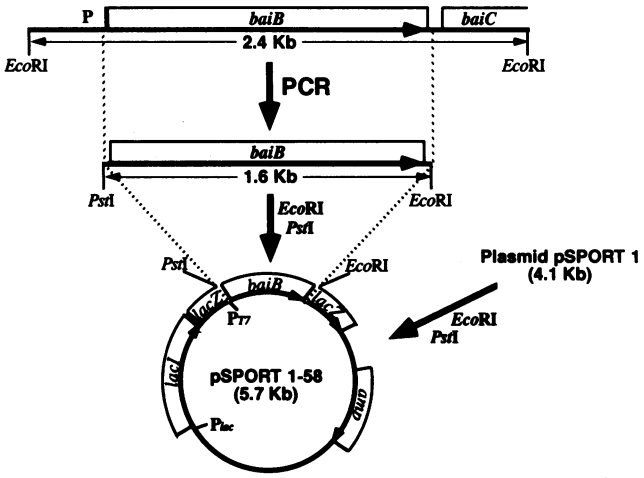

FIG. 2. Construction of an expression vector for the *baiB* gene from *Eubacterium* sp. strain VPI 12708. A 2.4-kb *Eco*RI insert containing the *baiB* gene was used as the starting material for PCR amplification of the *baiB* gene. The amplified material was purified, digested with *Eco*RI and *Pst*I, and ligated into a pSPORT 1 plasmid vector.

of water-soluble radioactive material in an ethyl acetate-extracted reaction mixture containing the ligase and a ^{14}C-labeled bile acid. Initially, the assay was performed for 1 min at 37°C in a phosphate buffer (pH 6.8) with 2ME as previously described (4). However, assays performed with the partially purified *baiB* gene product revealed that ligation activity was greatly enhanced by the addition of ATP and

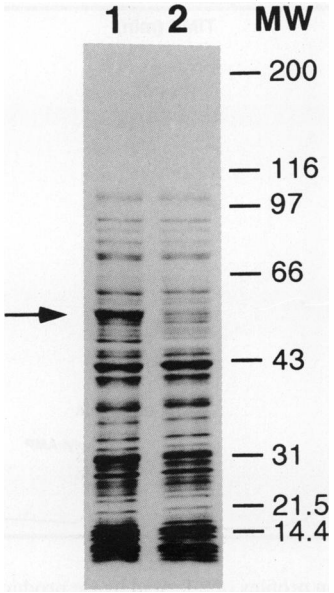

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7 to 20% polyacrylamide gradient) of soluble cell extracts stained with Coomassie blue. Lane 1 shows an extract from IPTG-induced *E. coli* HMS174(DE3) containing plasmid pSPORT 1-58. Lane 2 shows an extract from IPTG-induced strain HMS174(DE3) containing plasmid pSPORT 1 without an insert. The arrow identifies the 58,000-M_r polypeptide encoded by the *baiB* gene. The numbers on the right indicate positions of molecular weight (MW) standards in thousands.

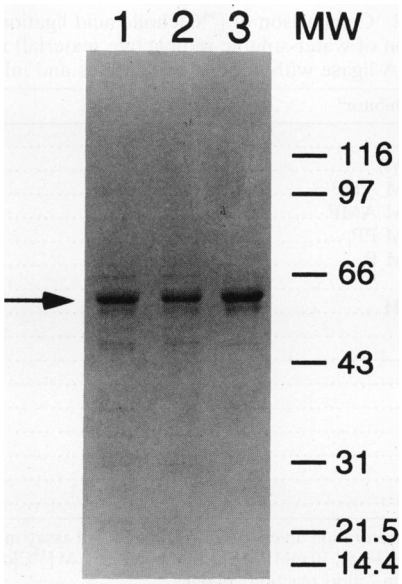

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7 to 20% polyacrylamide gradient) of protein fractions stained with Coomassie blue, showing the purification steps for the 58,000-M_r *baiB* gene product from *E. coli* HMS174(DE3) containing plasmid pSPORT 1-58. Lanes: 1, pooled fractions from DEAE HPLC containing bile acid-CoA ligase activity; 2, pooled fractions from gel filtration HPLC; 3, pooled fractions from Mono-Q HPLC. The arrow identifies the 58,000-M_r polypeptide. The numbers on the right indicate positions of molecular weight (MW) standards in thousands.

Mg^{2+}. Optimum ligation activity was obtained in a MOPS buffer (pH 6.8) with reaction mixtures containing 5 mM $MgCl_2$, 2.5 mM ATP, and 1 mM cholic acid. Increased concentrations of ATP resulted in decreased ligation activity. The ligation reaction had an observed temperature optimum of approximately 50°C and was relatively linear over a period of at least 10 min when 0.05 U of ligase and 5 μM [^{14}C]cholic acid were present in 250-μl reaction mixtures.

Assays with other nucleotides revealed that ADP supported a reduced but detectable amount of apparent ligation activity resulting in water-soluble radioactive reaction products. Both AMP and GTP were inactive in stimulating bile acid ligation activity. Assays performed with potential inhibitors of the ligation reaction revealed that 10 mM pyrophosphate markedly suppressed ligation activity when added to the ATP reaction mixture. Table 2 shows bile acid ligation

TABLE 1. Purification of bile acid-CoA ligase activity

Purification step	Total protein (mg)	Ligase activity (U)[a]	Sp act (U/mg)	Yield (%)	Purification (fold)
Crude extract	353	338	1.0	100	
DEAE	22	167	7.5	49	7.5
Gel filtration	7.3	75	10	22	10
Mono-Q	2.2	46	21	14	21

[a] One unit of activity is defined as the amount of enzyme required to convert 1 nmol of cholic acid per min under standard assay conditions of 10 mM MOPS, 10 mM 2ME, 5 mM $MgCl_2$, 2.5 mM ATP, 2.5 mM CoA, and 5 μM [^{14}C]cholate. Reactions were performed in 250-μl volumes for 5 min at 37°C.

TABLE 2. Comparison of [^{14}C]cholic acid ligation activity (production of water-soluble radioactive material) using bile acid-CoA ligase with various nucleotides and inhibitors

Nucleotide/inhibitor[a]	Activity (%)[b]
ATP	100
ATP (12.5 mM)	80
ATP + 10 mM ADP	60
ATP + 10 mM AMP	117
ATP + 10 mM PP_i	3
ATP + 10 mM P_i	110
ATP + NAD	85
ATP + NADH	85
ATP + CoA	170
CoA	1
ADP	24
AMP	1
GTP	2
NAD	1
NADH	1
None	1

[a] 2.5 mM concentration unless otherwise noted. All assay media contained 10 mM MOPS (pH 6.8), 10 mM 2ME, 5 mM $MgCl_2$, 5 μM [^{14}C]cholic acid, and bile acid ligase in 250-μl reaction volumes.

[b] Compared with activity (production of water-soluble radioactive material) obtained with a reaction mixture containing 2.5 mM ATP. All measurements represent averages of at least two independent assays.

activities determined when various nucleotides and inhibitors were used.

Optimization of bile acid-CoA ligation activity. It was found that adding CoA to standard reaction mixtures enhanced the ligation activity. Addition of CoA without ATP resulted in no stimulation of ligation activity (Table 2), suggesting that CoA reacts with an intermediate product obtained from the ATP-cholic acid reaction. Optimum ligation activity was obtained with 2.5 mM CoA. All other optimum reaction conditions remained the same with or without added CoA, except for the temperature optimum, which was reduced to approximately 42°C.

Ligation reactions using other ^{14}C-labeled bile acids revealed that deoxycholic acid and chenodeoxycholic acid were also effective substrates for the bile acid-CoA ligase (data not shown). The apparent K_m values for cholic acid and ATP were 22 and 200 μM, respectively. A K_m for CoA could not be obtained by using these assay conditions.

Ligation reaction product identification. Products from cholic acid ligation reactions were analyzed by reverse-phase HPLC. This analysis revealed the presence of different reaction products depending on the presence or absence of CoA in the reaction mixture (Fig. 5). Synthetic cholyl-CoA comigrated with a product obtained from the reaction mixture containing CoA and ATP but not with the product of a ligation reaction containing only ATP. Both of these products contained ^{14}C-labeled bile acids, as determined by liquid scintillation counting. A product comigrating with AMP was also obtained from the reaction mixture containing CoA and ATP (Fig. 6). A UV absorption spectrum of the HPLC-purified ligation product that comigrated with cholyl-CoA revealed readings identical to those with authentic cholyl-CoA, including an adenine peak at 260 nm and a thioester bond peak at 230 nm (data not shown).

The bile acid component of the water-soluble ligation reaction products was identified by TLC as described in Materials and Methods. This analysis verified that a product comigrating with cholic acid could be obtained from both the reaction mixture containing ATP and CoA and the reaction

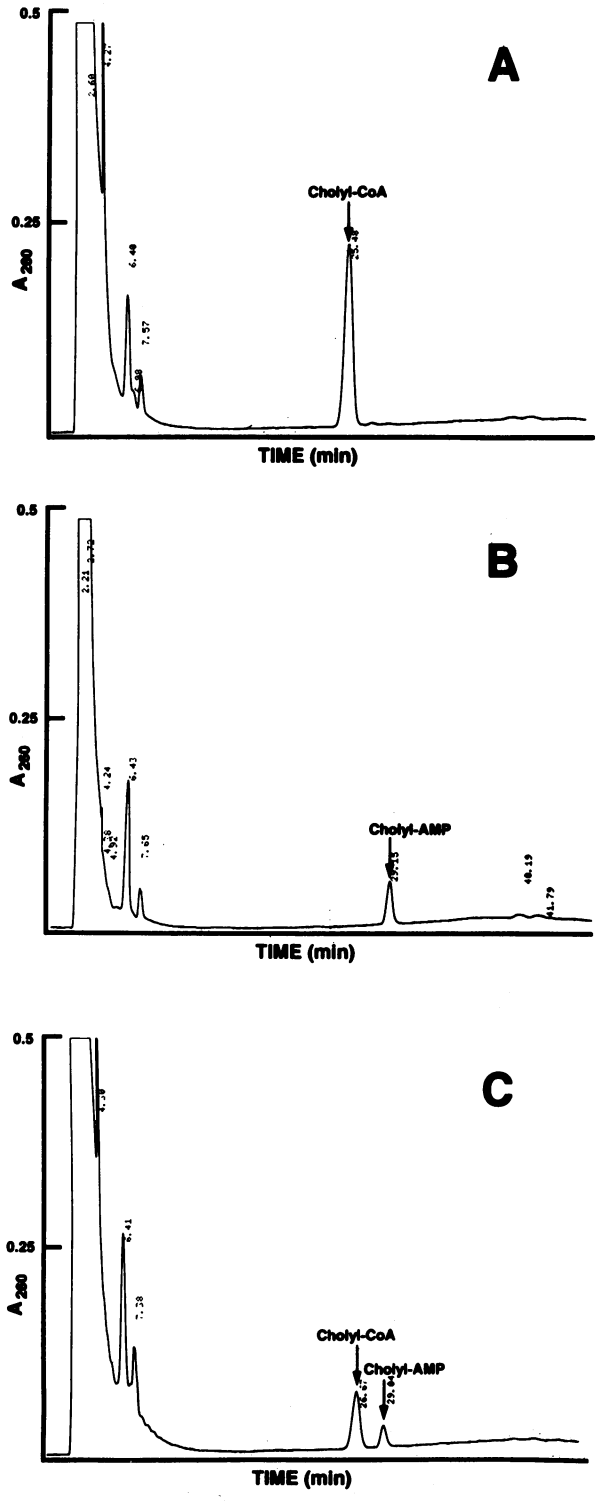

FIG. 5. Elution profiles of bile acid ligase products from reverse-phase HPLC runs using a 5 to 40% isopropanol gradient in 20 mM ammonium bicarbonate buffer (4). Gradients were run over a period of 40 min with a 1-ml/min flow rate. Shown are elution profiles of products from cholic acid ligation reactions using standard reaction mixtures with added 1 mM cholate for 30 min at 37°C with (A) and without (B) CoA. (C) Elution profile obtained when reaction products from experiments whose results are shown in panels A and B were mixed before injection. The peak in panel A comigrates with purified cholyl-CoA.

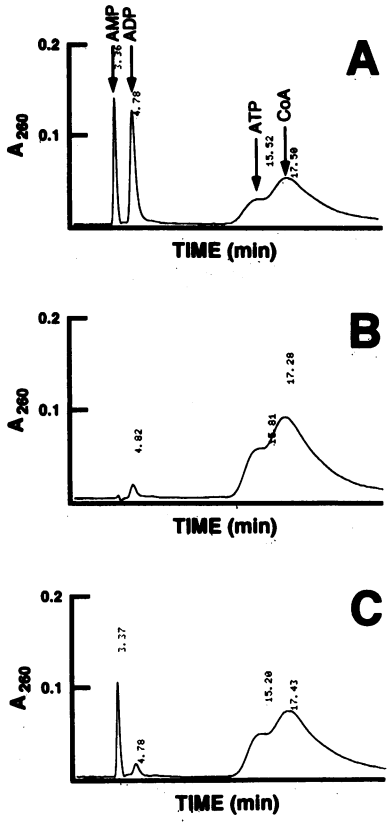

FIG. 6. Elution profiles of bile acid ligase products from reverse-phase HPLC runs using a solvent system containing 19% acetonitrile in 0.03 M potassium phosphate buffer (pH 2.65) with 0.01 M tetrabutylammonium phosphate (4). The flow rate was 1 ml/min. (A) Elution profiles of AMP, ADP, ATP, and CoA standards. Elution profiles of products from cholic acid ligation reactions (30 min at 37°C) using standard reaction mixtures containing ATP, CoA, and added 1 mM cholate without (B) and with (C) ligase are shown.

mixture containing ATP but not CoA. No other bile acid metabolites could be detected.

Data bank searches for sequence similarity. The 58,000-M_r polypeptide encoded by the *baiB* gene has amino acid sequence similarity to several other polypeptides involved in reacting cyclic carboxylated compounds with ATP or CoA to form activated pathway intermediates. Several of the homologous polypeptides have M_rs similar to that of the bile acid-CoA ligase (58,000). These include firefly luciferase (9, 21), 4-coumarate:CoA ligase from parsley and rice (19, 41), and 2,3-dihydroxybenzoate-AMP ligase (enterobactin synthetase component E) from *E. coli* (25, 30). All of these polypeptides show a low degree of sequence identity over the entire length of the polypeptide chains. Table 3 compares several of these related polypeptides. The tyrocidine and gramicidin S synthetase 1 polypeptides from *Bacillus brevis* have reported M_rs of approximately 120,000 (14, 17, 35). The N-terminal halves of these two polypeptides contain the sequences homologous to the bile acid-CoA ligase. One region showing a particularly high degree of amino acid sequence similarity among several of the polypeptides in Table 3, as well as an acetyl-CoA synthetase from *Aspergillus nidulans*, is shown in Fig. 7.

```
                    10         20         30         40         50
Fire  414  IDEEGWLHTG DIGYYDEEKH FFIVDRLKSL IKYKGYQVPP AELESVLLQH
Pars  411  IDEEGWLHTG DIGFIDDDDE LFIVDRLKEI IKYKGFQVAP AELEALLLTH
Asp   509  QVYKGYYFTG DGAGRDHEGY YWIRGRVDDV VNVSGHRLST AEIEAALIEH
Ecol  405  FDANGFYCSG DLISIDPEGY ITVQGREKDQ INRGGEKIAA EEIENLLLRH
Eub   391  VKEGGFRSVG DIGYVDEQGY LYFSDRRSDM LVSGGENMFA TEVETALLRY
Bbre  403  VPGEKLYKTG DQARWLSDGN IEYLGRIDNQ VKIRGHRMEL EEVESILLKH

                    60         70         80         90        100
Fire  464  PSIFDAGVAG VPDPVAGELP GAVVVLESGK NMTEKEVMDY VASQVSNAKR
Pars  461  PTISDAAVVP MIDEKAGEVP VAFVVRTNGF TTTEEEIKQF VSKQVVFYKR
Asp   559  PMVAEAAVVG IADELTGQAV NAFVSLKEGN ETNDQVRKDL ILQVRKSIGP
Ecol  455  PAVIYAALVS MEDELMGEKS CAYLVVKEPL RAVQ..VRRF LREQ..GIAE
Eub   441  KDILDAVVVG IPDEDLGRRL HAVIL..ETGK EIPAEELKTF LRK...YLTP
Bbre  453  MYLSETAVSV HKDHQEQPYL CAYFV..SEK HIPLEQLRQF SSEE...LPT

                   110        120        128
Fire  514  LRGG..VRFV DEVPKGLTGK IDGRAIRE  539
Pars  511  IF.R..VFFV DAIPKSPSGK ILRKDLRA  535
Asp   609  FAAPKAVFVV DDLPKTRSGK IMRRILRK  636
Ecol  501  FKLPDRVECV DSLPLTAVGK VDKKQLRQ  528
Eub   486  YKIPKTFEFV RSIRRGDNGK ADRKRILE  513
Bbre  498  YMIPSYFIQL DKMPLTSNGK IDRKQLPE  525
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FIG. 7. Region of amino acid sequence similarity among luciferase from Japanese fireflies (Fire) (21), 4-coumarate:CoA ligase from parsley (Pars) (19), acetyl-CoA synthetase from *A. nidulans* (Asp) (7), 2,3-dihydroxybenzoate-AMP ligase from *E. coli* (Ecol) (30), bile acid-CoA ligase from *Eubacterium* sp. strain VPI 12708 (Eub), and gramicidin S synthetase 1 from *B. brevis* (Bbre) (14, 17). The numbers before and after the sequences indicate the residue numbers. The boxed residues are regions of sequence identity or conservative basic or hydrophobic substitutions.

DISCUSSION

It has been previously proposed that the formation of a bile acid-adenosine nucleotide is the initial step in the bile acid 7α-dehydroxylation pathway in *Eubacterium* sp. strain VPI 12708 (4, 6). In this article, we report that a bile acid-CoA ligation activity is encoded by the *baiB* gene from *Eubacterium* sp. strain VPI 12708 (Fig. 8). The *baiB* gene had been previously reported to encode a putative 58,000-M_r polypeptide and to be part of a large bile acid-inducible

TABLE 3. Comparison of polypeptides showing amino acid sequence similarity[a] to the bile acid-CoA ligase from *Eubacterium* sp. strain VPI 12708

Organism	Enzyme	Gene	Mol wt	Subunits	% Identity	% Similarity	Reference(s)
Eubacterium sp. strain VPI 12708	Bile acid-CoA ligase	*baiB*	58,294	α2	–	–	20
E. coli	2,3-Dihydroxybenzoate-AMP ligase	*entE*	59,292	α2	23.5	48.5	25, 30
Oryza sativa (rice)	4-Coumarate:CoA ligase		60,841	1	21.5	48.5	41
Petroselinum crispum (parsley)	4-Coumarate:CoA ligase		59,825	1	24.0	46.4	19
B. brevis	Tyrocidine synthetase 1	*tycA*	122,587	1	21.4	44.8	35
B. brevis	Gramicidin S synthetase 1	*grsA*	126,548	1	24.4	47.4	14, 17
Photinus pyralis (North American firefly)	Luciferase		60,745	1	24.6	49.3	9
Luciola cruciata (Japanese firefly)	Luciferase		60,017	1	25.4	50.9	21

[a] As determined with the GCG GAPS program.

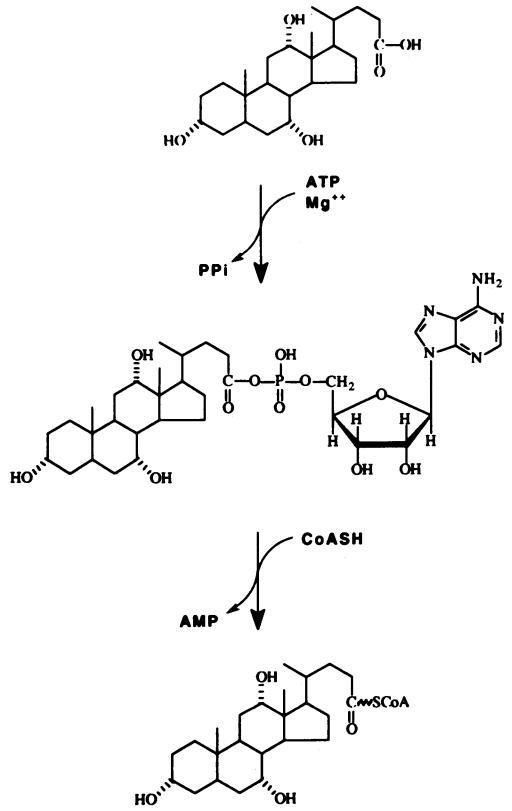

FIG. 8. Proposed reaction mechanism for the bile acid-CoA ligase from *Eubacterium* sp. strain VPI 12708.

operon thought to encode enzymes involved in bile acid 7α-dehydroxylation (20). Therefore, it is now proposed that the bile acid-CoA ligase activity catalyzes the initial step in the bile acid 7α-dehydroxylation pathway. To our knowledge, this is the first report of a bile acid-CoA ligase from a bacterium.

The bile acid-CoA ligase requires ATP and produces AMP and pyrophosphate in addition to the bile acid-CoA thioester. A water soluble intermediate that is presumably a bile acid-AMP conjugate could also be detected when CoA was omitted from the reaction mixture (Fig. 5). Water-soluble radioactive material indicating cholic acid ligation activity was also obtained with ADP, although at a reduced rate compared with ATP. Further studies are necessary to determine the products and significance of this ADP-utilizing reaction.

The bile acid-CoA ligase was shown to have activity with cholic acid, deoxycholic acid, and chenodeoxycholic acid. Previous data (4) suggest that the ligase is active with a large number of C-24 bile acids possessing unhindered C-24 carboxyl groups.

The 58,000-M_r polypeptide encoded by the *baiB* gene has sequence similarity to several other polypeptides from divergent sources (Table 3). These polypeptides share the activity of ATP-dependent linking of cyclic carboxylated compounds to AMP or CoA. Several of the homologous polypeptides have M_rs in the 58,000-to-61,000 range and have sequence similarity over the entire length of the polypeptides. Analysis of the active enzymes suggests that the bile acid-CoA ligase and the 2,3-dihydroxybenzoate-AMP ligase exist as α2 dimers (4, 25). Benzoate-CoA ligases from *Rhodopseudomonas palustris* and a *Pseudomonas* sp. have also been shown to have monomer molecular weights of about 60,000 (1, 12). The *Pseudomonas* sp. enzyme is reported to be an α2 dimer (1).

The data in Fig. 7 show a relatively highly conserved amino acid sequence among several of the polypeptides listed in Table 3 as well as an acetyl-CoA synthetase from *A. nidulans*. All of the enzymes represented in Fig. 7 catalyze either ATP-dependent substrate-AMP ligation reactions or ATP-dependent substrate-CoA ligation reactions. All of the enzymes catalyzing the AMP ligation reactions also exhibit additional activities. The firefly luciferase exhibits an oxidative decarboxylation reaction resulting in light emission. The tyrocidine and gramicidin S synthetase enzymes attach the aminoacyladenylate intermediates to specific thiol groups on the enzyme (18), and the 2,3-dihydroxybenzoate-AMP ligase has been suggested to produce an enzyme-bound product (25). On the basis of the common activities of these enzymes, we hypothesize that the region of similarity in Fig. 8 may represent an ATP binding domain and/or function in positioning the adenylated product for subsequent reactions. No consensus ATP binding domains (10, 34) could be identified in this group of enzymes.

It is possible that transport of bile acids into *Eubacterium* sp. strain VPI 12708 is achieved through group translocation driven by thioesterification of the substrate, as has been described for the uptake of fatty acids in *E. coli* (23, 32). A similar mechanism has been suggested for the anaerobic uptake of benzoate and related aromatic compounds by *R. palustris* (12, 22). As with the *E. coli* and *R. palustris* CoA-thioester synthetases, the bile acid-CoA ligase from *Eubacterium* sp. strain VPI 12708 is found in the soluble fraction of cell lysates. However, the possibility of some association with the cytoplasmic membrane cannot be discounted. The enzymes catalyzing the synthesis of di- and trihydroxycholestanoyl-CoA and C-24 bile acid-CoA thioesters in the liver are also membrane associated and require treatment with detergents for solubilization (16, 27).

To our knowledge, the bile acid-CoA ligase from liver has not been purified or sequenced, so detailed comparisons with the *Eubacterium* bile acid-CoA ligase cannot be made at this time. However, studies using crude liver extracts suggest that the liver enzyme, like the *Eubacterium* bile acid-CoA ligase, is inhibited by pyrophosphate and high concentrations of ATP (27, 33).

It is not known whether bile acids remain as CoA thioesters during the various biotransformation steps of the 7α-dehydroxylation pathway in *Eubacterium* sp. strain VPI 12708. However, the occurrence of bile acid-CoA conjugates other than cholyl-CoA (4) suggests that CoA-linked intermediates may be substrates in subsequent steps of this pathway. Thioesters are common in the anaerobic bacterial degradation of benzoate and related aromatic compounds, and degradation of these compounds is thought to proceed through a series of CoA-thioester intermediates (1, 12, 22). Further studies with *Eubacterium* sp. strain VPI 12708 are necessary to resolve this question.

Finally, the cloned bile acid-CoA ligase could provide a useful alternative to synthetic production of bile acid-CoA thioesters. Our studies indicate that most or all C-24 bile acids with free carboxyl groups are substrates for this enzyme.

ACKNOWLEDGMENTS

We thank William H. Elliott for his generous gift of cholyl-CoA.

This work was supported by research grants DK40986 and P01DK38030 from the National Institutes of Health. D.H.M. was supported by postdoctoral training grant T32-CA-0956404 from the National Institutes of Health.

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