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Characterization and Regulation of the NADP-Linked 7α-Hydroxysteroid Dehydrogenase Gene from *Clostridium sordellii*

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A bile acid-inducible NADP-linked 7α-hydroxysteroid dehydrogenase (7α-HSDH) from *Clostridium sordellii* ATCC 9714 was purified 310-fold by ion-exchange, gel filtration, and dye-ligand affinity chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the purified enzyme showed one predominant peptide band (30,000 Da). The N-terminal sequence was determined, and the corresponding oligonucleotides were synthesized and used to screen *Eco*RI and *Hin*dIII genomic digests of *C. sordellii*. Two separate fragments (4,500 bp, *Eco*RI; 3,200 bp, *Hin*dIII) were subsequently cloned by ligation to pUC19 and transformation into *Escherichia coli* DH5α-MCR. The *Eco*RI fragment was shown to contain a truncated 7α-HSDH gene, while the *Hin*dIII fragment contained the entire coding region. *E. coli* clones containing the *Hin*dIII insert expressed high levels of an NADP-linked 7α-HSDH. Nucleotide sequence analyses suggest that the 7α-HSDH is encoded by a monocistronic transcriptional unit, with DNA sequence elements resembling rho-independent terminators located in both the upstream and downstream flanking regions. The transcriptional start site was located by primer extension analysis. Northern (RNA) blot analysis indicated that induction is mediated at the transcriptional level in response to the presence of bile acid in the growth medium. In addition, growth-phase-dependent expression is observed in uninduced cultures. Analysis of the predicted protein sequence indicates that the enzyme can be classified in the short-chain dehydrogenase group.

During their enterohepatic circulation, the primary bile acids of humans, cholic and chenodeoxycholic acids and their taurine and glycine conjugates, are susceptible to a variety of transformations by the intestinal microflora. These transformations include the hydrolysis of the amide linkage of the conjugated bile acids, hydrolysis of their sulfate esters, dehydrogenation of ring hydroxy groups, epimerization of ring hydroxy groups, and dehydroxylation of ring hydroxy groups (31).

The 7-dehydroxylation of bile acids is considered to be the most important of these reactions in a qualitative sense, because of the potential toxicity of the secondary bile acid products. Although the reaction products (deoxycholic acid and lithocholic acid) may constitute a significant proportion of the circulating bile acid pool, the ability to carry out the reaction is apparently restricted to a few species, mainly in the genera of *Clostridium*, *Eubacterium*, and possibly *Bacteroides* (14, 27, 29, 31). On the other hand, the presence of bile acid and steroid dehydrogenases appears to be widespread among intestinal as well as soil microflora, having been reported in *Escherichia coli* (40) and species of *Alcaligenes* (48), *Bacteroides* (14, 32, 41, 46, 54), *Bifidobacterium* (18), *Clostridium* (11, 15, 16, 34), *Enterococcus* (6), *Eubacterium* (19, 28, 37), *Peptostreptococcus* (17, 28), *Pseudomonas* (43, 55, 56), *Ruminococcus* (1, 2), and *Streptomyces* (36).

The dehydrogenation of bile acids and steroids displays several interesting features. The majority of those bacterial steroid and bile acid dehydrogenases which have been characterized at the sequence level are related as members of the short-chain alcohol dehydrogenase family (8, 49, 60). As additional enzymes active on a variety of substrates are identified as members of this family, investigators will have available a unique data set which can be used for studies on the role of specific residues and domains on enzyme conformation, substrate specificity, and reaction stereospecificity.

The regulation of expression of these enzymes varies depending on the enzyme and the source organism. In organisms such as *E. coli* and a *Eubacterium* sp., for example, the 7α-hydroxysteroid dehydrogenase (7α-HSDH) is expressed constitutively (8, 19, 40), while two 3α-HSDHs from the *Eubacterium* sp. are inducible by bile acids containing a 7α-hydroxy group (13, 23, 58). The 7α- and 7β-HSDHs of *Clostridium limosum* and *Clostridium absonum* are induced by chenodeoxycholic acid and repressed by ursodeoxycholic acid (34, 38, 39, 57). In *Bacteroides* spp., the appearance of 7α-HSDH activity is both growth phase dependent and bile acid inducible (32, 54). Despite these observations of bile acid-mediated regulation of gene expression, little is known about the molecular mechanisms responsible for this phenomenon.

Clostridium sordellii ATCC 9714 (formerly *Clostridium bifermentans*) has previously been demonstrated to express inducible 7α-dehydrogenase as well as 7α-dehydroxylase and conjugated bile acid hydrolase activities (3–5, 26) (Fig. 1). The presence of all three of these activities in one organism, the fact that they are bile acid inducible, and the availability of a related *Clostridium* sp. which can be genetically transformed (33) make this organism an attractive model system to begin genetic studies on the regulation of these enzyme systems by bile acids. To begin to address these studies, we have cloned and sequenced the NADP-linked 7α-HSDH gene from *C.*

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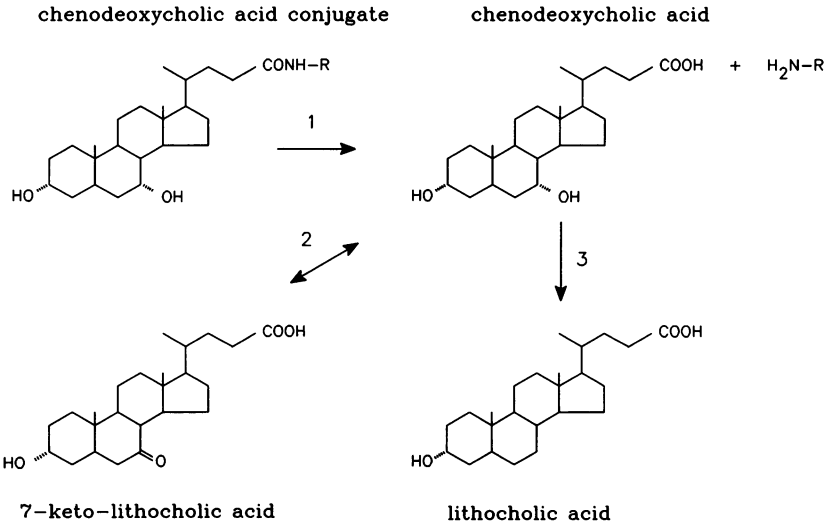

FIG. 1. Bile acid transformation activities of *C. sordellii*. Reactions previously demonstrated to exist are as follows: 1, conjugated bile acid hydrolase (R, CH_2COOH [glycine conjugate] or $CH_2CH_2SO_3H$ [taurine conjugate] [3]); 2, 7α-HSDH (26); and 3, bile acid 7α-dehydroxylase (4, 5, 26).

sordellii and have analyzed its regulatory properties as described below.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. sordellii* ATCC 9714 was obtained from P. B. Hylemon, Medical College of Virginia, Virginia Commonwealth University. *E. coli* DH5α-MCR (GIBCO-BRL, Gaithersburg, Md.) was used as a host strain for cloning and subcloning manipulations. *C. sordellii* was grown on brain heart infusion medium (Difco Laboratories, Detroit, Mich.) supplemented with yeast extract (10 g/liter), fructose (2 g/liter) VPI mineral salts (30) (40 ml/liter), hemin (2 mg/liter, added from 1,000× stock in 10 mM NaOH), cysteine (0.5 g/liter), and sodium carbonate (4 g/liter). Medium was maintained under anaerobic conditions after autoclaving by cooling under positive pressure with nitrogen gas. Cells were grown at 37°C under nitrogen gas. Chenodeoxycholic acid (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 0.4 mM when cells had reached an optical density at 600 nm of 0.2, and cells were harvested in the early stationary phase of growth (optical density at 600 nm = 1.4 to 1.5). *E. coli* strains were grown in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) when appropriate.

Purification of 7α-HSDH. All 7α-HSDH purification steps were performed at 4°C unless otherwise indicated. Bacteria were harvested by centrifugation at 6,000 × *g* for 15 min. The harvested pellet (20 g) was suspended in 60 ml of 100 mM sodium phosphate–1 mM EDTA–10 mM 2-mercaptoethanol, pH 7.0, and ruptured by a single passage through a French pressure cell at 12,000 lb/in^2. After addition of approximately 2 mg of DNase, the extract was centrifuged at 12,000 × *g* for 90 min. The supernatant was decanted and adjusted to 40% saturation with solid ammonium sulfate. The supernatant obtained after centrifugation at 12,000 × *g* for 30 min was adjusted to 65% saturation with addition ammonium sulfate, and after centrifugation as described above, the pellet was recovered and resuspended in 20 ml of 20 mM sodium phosphate–1 mM EDTA–10 mM 2-mercaptoethanol, pH 7.0 (buffer A). The suspension was dialyzed overnight against 4 liters of buffer A and applied at 0.5 ml/min to a DEAE-Sepharose CL-6B column (Pharmacia, Piscataway, N.J.) (5 by 12 cm) equilibrated with buffer A. The column was then washed with buffer A (approximately 100 ml) until a stable baseline was obtained, as measured by monitoring the optical density of the eluent at 280 nm. Proteins were then eluted at 1 ml/min with a linear gradient of 0 to 1 M sodium chloride in buffer A. Fractions (10 ml) were collected, and a portion of each was monitored for 7α-HSDH activity. Active fractions were precipitated by the addition of solid ammonium sulfate to 70% saturation and centrifuged at 12,000 × *g* for 30 min. The pellet was resuspended in 6 ml of 20 mM sodium phosphate–100 mM sodium chloride–1 mM EDTA–10 mM 2-mercaptoethanol, pH 7.0 (buffer B), and applied at a flow rate of 0.5 ml/min to a Sephacryl S-300 column (1.5 by 85 cm). Proteins were eluted with buffer B at 0.5 ml/min, and 5-ml fractions were collected and assayed for 7α-HSDH activity. Active fractions were combined and concentrated by ultrafiltration through an Amicon YM-10 membrane. The concentrated active fractions were diluted 10-fold in 50 mM sodium phosphate–1 mM EDTA–5 mM 2-mercaptoethanol–0.1% Triton X-100–10% glycerol, pH 7.0 (buffer C), and then applied to a 10-ml column of reactive red 120-agarose (Sigma Chemical Co.) equilibrated with buffer C. The column was washed with 15 ml of 50 mM NaCl in buffer C, and 7α-HSDH was then eluted with 1 M NaCl in buffer C. Peak activity fractions were combined and dialyzed against 4 liters (twice) of distilled water containing 0.01% sodium dodecyl sulfate (SDS). The final dialyzed preparation was lyophilized to dryness, and approximately 30 μg was subjected to N-terminal amino acid sequence analysis.

Protein sequencing. N-terminal amino acid sequence analysis was performed by the Genetic Engineering Facility at the University of Illinois Biotechnology Center, Urbana-Champaign, on an Applied Biosystems model 470A protein sequencer.

Protein and enzyme assays. Protein was assayed by the dye-binding method of Bradford (10) using commercially available reagents (Bio-Rad Laboratories, Hercules, Calif.). The standard enzyme assay mixture for 7α-HSDH contained, in a final volume of 1 ml, 300 μmol of glycine-NaOH (pH 10.5),

1.0 μmol of NADP (or NAD for *E. coli*), and protein. After establishment of a stable baseline at 340 nm, 1.0 μmol of sodium chenodeoxycholate was added and the increase in absorbance was monitored. One unit of activity is defined as the amount of enzyme which reduces 1 μmol of NADP per min under the assay conditions described above. All calculations were based on an extinction coefficient for NADPH of 6.20 mM^{-1} cm^{-1} at 340 nm. Measurements were performed at 37°C on a Gilford model 250 spectrophotometer with a water-jacketed cuvette holder. Potential interference by the action of a 3α-HSDH acting on the 3α-hydroxy group of chenodeoxycholic acid was not considered to be a problem because of the absence of 3α-HSDHs in *C. sordellii*.

Protein electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out in 0.75-mm-thick gradient gels (7 to 20% T; 2.7% C) by the discontinuous-buffer system of Laemmli (35). Low-molecular-weight protein standards (Bio-Rad Laboratories) were used as size markers. Proteins were stained with Coomassie brilliant blue R-250. Relative staining intensities were determined by scanning laser densitometry. Nondenaturing pore gradient PAGE was carried out in 0.75-mm-thick gradient gels (5 to 15% or 10 to 20% T; 2.7% C) by a Tris-borate buffer system (90 mM Tris, 80 mM boric acid, 2.5 mM EDTA, pH 8.4). Electrophoresis was conducted at 4°C for 15 h at a 150-V constant voltage. Gels were stained either for protein as described above or for enzyme activity by a modification of the procedure described by Grell et al. (25). The reaction mixture contained, in a final volume of 10 ml, 200 μmol of sodium phosphate (pH 7.5), 5 μmol of NAD or NADP, 6 μmol of nitroblue tetrazolium, 0.6 μmol of phenazine methosulfate, and 10 μmol of sodium chenodeoxycholate. Gels were incubated in the dark in the mixture until bands appeared (<30 min). Dehydrogenase activity appeared as purple bands against a clear background. Gels were then rinsed with deionized water and stored in 5% acetic acid.

Recombinant DNA techniques. Plasmid DNA was isolated from *E. coli* by the Ish-Horowitz modification of the method of Birnboim and Doly (42). Large-scale preparations were further purified by CsCl-ethidium bromide gradient centrifugation. Chromosomal DNA was isolated from *C. sordellii* by the procedure of Marmur (45), with the inclusion of the lysozyme pretreatment. Restriction endonuclease digestions were carried out as recommended by the suppliers. Electrophoresis of DNA, ligation reactions, sucrose gradient centrifugation, and other nucleic acid manipulations were performed as described by Maniatis et al. (42).

Southern blotting and colony hybridizations. DNA restriction fragments were transferred to nitrocellulose as described by Maniatis et al. (42). Transformed colonies on agar plates were transferred to nitrocellulose disks and lysed in situ as described by Berent et al. (9). Filters were treated at 70°C for 2 h in vacuo and then washed, prehybridized, and hybridized as described by Woods (59). Oligonucleotides were labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear Corp.) and T4 polynucleotide kinase as described by Maniatis et al. (42). Unincorporated label was removed with Nensorb 20 cartridges (New England Nuclear Corp.) according to the manufacturer's instructions. Hybridizations were performed at 37°C with probes at a final concentration of approximately 10^6 cpm/ml (10^9 cpm/μg).

DNA sequencing. DNA was sequenced by the dideoxynucleotide chain-terminating method (52) with alkali-denatured double-stranded plasmid templates, according to the procedure supplied with the Sequenase (version 2.0) sequencing reagent kit (U.S. Biochemical, Cleveland, Ohio). Labeling reactions were performed using [α-^{35}S]ATP (1,325 Ci/mmol; DuPont-NEN, Boston, Mass.). Universal M13/pUC forward and reverse primers were used for sequencing those regions flanking vector sequences, while internal regions were sequenced with specific primers based on observed DNA sequences. All primers were synthesized by the DNA Synthesis Core Laboratory at the School of Medicine Biotechnology Program, East Carolina University.

Analysis of sequence data. Nucleic acid and protein sequence analyses were performed with the sequence analysis software package of Genetics Computer Group, Inc. (GCG; Madison, Wis.). Free energies for stem-loops and Shine-Dalgarno sequences were calculated as described by Freier et al. (20). Sequence information was submitted to GenBank with AUTHORIN (GenBank, Intelligenetics, Inc., Mountain View, Calif.).

Induction experiments. *C. sordellii* cultures (500 ml) were sampled at various time points before and after addition of sodium chenodeoxycholate to a final concentration of 0.1 mM. Samples (41 ml) were placed in polypropylene centrifuge tubes containing chloramphenicol (final concentration = 125 μg/ml). The samples were then cooled immediately on ice, a 1-ml aliquot was removed for measurement of the optical density at 600 nm, and the remainder was centrifuged at 8,000 × *g* for 15 min in two aliquots, one 10 ml (for enzymatic assay) and one 30 ml (for RNA extraction). The 10-ml pellet was resuspended in 2 ml of 30 mM Tris-HCl–100 mM NaCl–3 mM EDTA, pH 7.4 (buffer R), containing 1 mM dithiothreitol, while the 30-ml pellet was suspended in 1 ml of buffer R containing 1% SDS and 100 μg of proteinase K per ml. Samples were then frozen at −70°C until processed further. Cell suspensions for enzymatic measurements were disrupted by thawing and sonicating for 1 min with a Branson 450 Sonifier at 50% duty cycle. The lysates were then centrifuged at 100,000 × *g* for 1 h before aliquots were assayed for protein and 7α-HSDH activity.

RNA isolation and analysis. RNA was isolated from *C. sordellii* as described by Ausubel et al. (7). Cell samples from the 30-ml cultures described above were thawed on ice and sonicated at 50% duty cycle for 1 min. The lysates were then heated at 37°C for 1 h before being extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol) and once with chloroform-isoamyl alcohol (24:1, vol/vol). Nucleic acids were precipitated by addition of 5 M NaCl to a final concentration of 0.18 M and 2 volumes of cold 100% ethanol. After centrifuging and washing once with 70% ethanol, the pellets were dissolved in 200 μl of formamide. Nucleic acids were quantitated by diluting an aliquot of the formamide solution into distilled water and measuring the A_{260}. For Northern (RNA) blot analyses, RNA samples were loaded on 1% agarose gels made up in 20 mM morpholinepropanesulfonic acid (MOPS)–5 mM sodium acetate–0.5 mM EDTA–0.66 M formaldehyde, pH 7.0. Sample buffer contained 10 μg of RNA, 50% formamide, 20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA, and 0.66 M formaldehyde, pH 7.0. After heating at 65°C for 15 min, bromophenol blue and ethidium bromide were added to final concentrations of 0.005% and 20 μg/ml, respectively. After electrophoresis at 80 V for 2 to 4 h, gels were soaked in diethylpyrocarbonate-treated water for 20 min and then photographed. RNA was transferred overnight to GeneScreen membranes (DuPont-NEN) with 20× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) as the capillary transfer buffer. Formaldehyde residue was removed, and nucleic acids were fixed to the membrane by baking at 80°C for 2 h. Membranes were prehybridized in 6× SSPE–1× Denhardt's solution–0.1% SDS, pH 7.0, at 52°C for 1 h. Hybridizations were then carried out in identical buffer containing 100 μg of yeast tRNA per ml and 2 × 10^6 cpm of

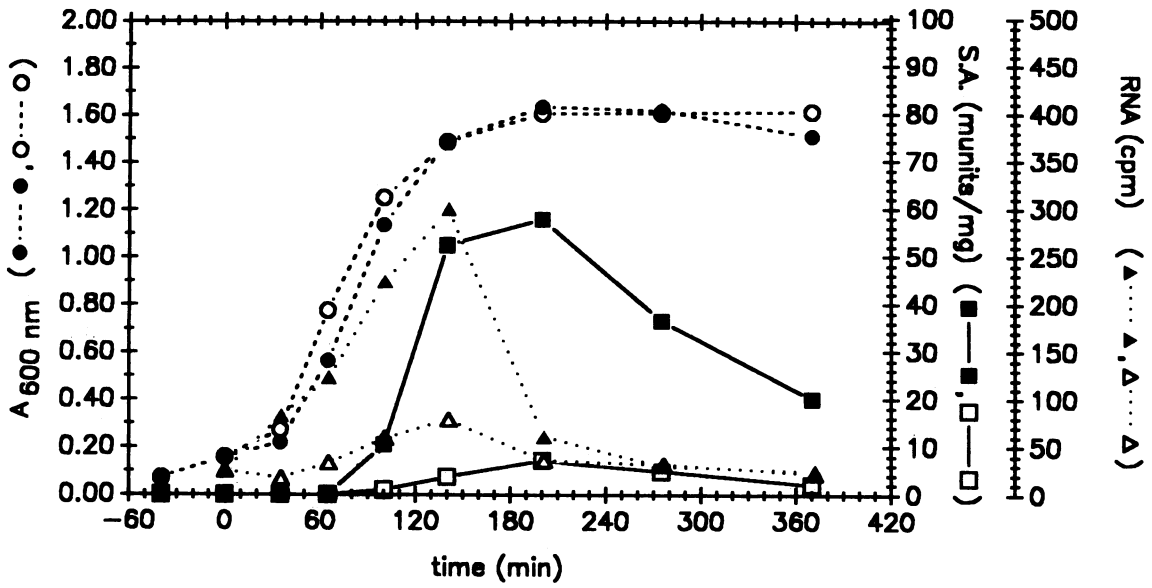

FIG. 2. Induction of 7α-HSDH during growth of *C. sordellii*. Open symbols, minus bile acid; filled symbols, plus bile acid. Cells were grown as described in Materials and Methods. Sodium chenodeoxycholate was added to one culture at time zero to a final concentration of 0.2 mM. Cellular growth was monitored by measuring the A_{600} of samples at different times. S.A., 7α-HSDH SA of sonicated and ultracentrifuged cell extracts; RNA cpm, measured amount of radioactivity in bands excised from the Northern blot described in the text, as determined by liquid scintillation counting.

^{32}P-labeled probe per ml at 52°C overnight. Washes were performed with 6× SSPE–0.1% SDS twice at room temperature for 5 min and once at 52°C for 15 min. Following autoradiography, radioactive counts in individual bands were quantitated by excising the bands and subjecting them to liquid scintillation counting using Ready Safe liquid scintillation cocktail (Beckman Instruments, Inc., Fullerton, Calif.).

For primer extension analysis, 10 to 20 μg of RNA and 500,000 cpm of ^{32}P-labeled primer were coprecipitated and suspended in 15 μl of a hybridization buffer containing 10 mM Tris-HCl, 150 mM KCl, and 1 mM EDTA, pH 8.3. The samples were heated at 65°C for 90 min then slowly cooled to 42°C. To each sample was added 30 μl of reaction mix containing 30 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 8 mM dithiothreitol, 225 μg of actinomycin D per ml, 250 μM deoxynucleoside triphosphates, and 5 U avian myeloblastosis virus reverse transcriptase. Reaction mixes were incubated at 42°C for 1 h, then reactions were stopped with EDTA (25 mM final concentration), and mixes were treated with 10 μg of DNase-free RNase per ml for 30 min at 37°C. Samples were then diluted with 150 μl of 10 mM Tris-HCl–1 mM EDTA (pH 8.0), extracted once with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol), and ethanol precipitated. Pellets were dissolved in 10 μl of 1× DNA sequencing loading buffer and electrophoresed on DNA sequencing gels alongside DNA sequencing reactions run using the same primer.

Expression of 7α-HSDH in *E. coli*. *E. coli* strains were grown to late logarithmic phase in Luria-Bertani medium and harvested by centrifugation. Crude extracts were prepared by suspending the pellet in 2 volumes of 50 mM sodium phosphate, pH 7.0, and rupturing the suspension in a French pressure cell at 12,000 lb/in^2. Crude lysates were then centrifuged at 100,000 × *g* for 1 h, following which the supernatants were dialyzed overnight against 1 liter of 50 mM sodium phosphate–1 mM dithiothreitol, pH 7.0. Extracts were assayed spectrophotometrically and also following polyacrylamide gel electrophoresis for both NAD- and NADP-linked 7α-HSDH activity by the assays described above.

Nucleotide sequence accession number. The nucleotide sequence of the DNA fragment containing the 7α-HSDH gene has been submitted to GenBank with the accession number L12058.

RESULTS AND DISCUSSION

Induction of 7α-HSDH. As shown in Fig. 2, 7α-HSDH activity is expressed in *C. sordellii* in both the absence (open symbols) and presence (closed symbols) of bile acid in the growth medium, reaching maximal levels in both cases during the early stationary phase of growth. However, cells grown in the presence of bile acid synthesize 7α-HSDH at levels approximately 10-fold higher than in uninduced cells. This observation suggests that at least two levels of control are operative in the regulation of 7α-HSDH, one responsive to the growth phase of the cell and the other responsive to the presence of bile acid in the growth medium. Transcriptional analyses (see below) are also included in Fig. 2 and show a similar pattern of growth-phase induction with increased expression when bile acid is present in the growth medium. Previous studies on dehydroxylation and dehydrogenation of bile acids by *C. sordellii* demonstrate that these activities are also present during growth on cholic acid (4, 5). In addition, we have demonstrated 7α-HSDH induction in *C. sordellii* by growth in the presence of 0.1 mM deoxycholic acid. This finding is noteworthy since deoxycholic acid lacks a 7-hydroxy group. A similar response to deoxycholic acid has been observed with the inducible bile acid 7α- and 7β-dehydrogenases of *C. absonum* (38). Although unlikely, the possibility that the induction seen in these cases is due to the presence of trace 7-hydroxylated contaminants in the commercial deoxycholic acid has not been excluded.

Purification and amino acid sequence analysis of 7α-HSDH. Large-scale batches (two of 8 liters) of *C. sordellii* were grown to stationary phase in the presence of chenodeoxycholic acid. From 20 g of wet cells, the 7α-HSDH was purified 310-fold from crude extracts (specific activity [SA] = 1.1 U/mg) by

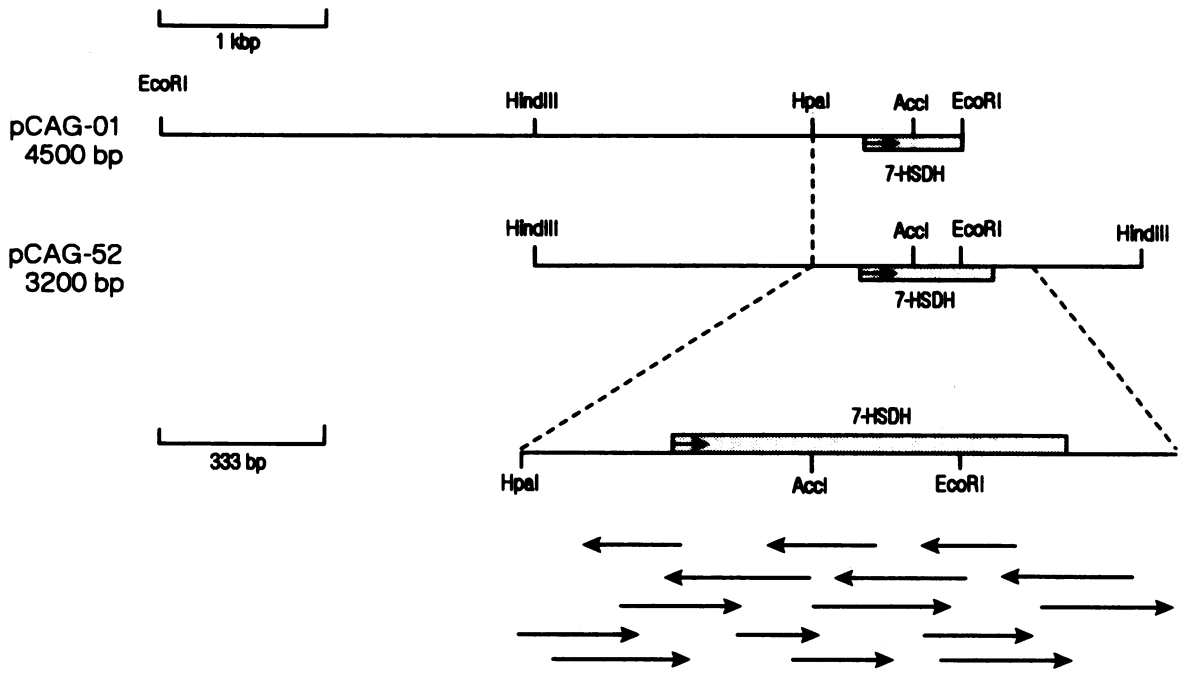

FIG. 3. Restriction map and sequencing strategy of 7α-HSDH clones. The 4.5-kbp *Eco*RI fragment and the 3.2-kbp *Hin*dIII fragment are aligned to indicate the overlap regions. The 7α-HSDH ORF is indicated by the open box, with the direction of translation indicated by the arrow. The individual sequencing reactions used to compile the entire sequence of the region shown below the *Eco*RI and *Hin*dIII maps are indicated by the arrows at the bottom of the figure.

ammonium sulfate fractionation (SA = 1.6 U/mg in 40 to 60% fraction; 90% yield), anion-exchange chromatography (SA = 3.0 U/mg; 35% yield), gel filtration chromatography (SA = 7.0 U/mg; 18% yield), and dye-ligand chromatography (SA = 340 U/mg; 10% yield). SDS-PAGE analysis indicated that the final preparation contained a predominant polypeptide with a relative molecular weight of 30,000. The level of purity was estimated to be greater than 85% by densitometric analysis of the Coomassie blue-stained gel (data not shown). The observed subunit size is similar to that seen for the 7α-HSDHs purified from a *Eubacterium* sp. (32 kDa) (19) and from *E. coli* (28 kDa) (61). In addition, nondenaturing pore gradient PAGE analysis demonstrated that the *C. sordellii* 7α-HSDH migrated in a manner similar to that of the 7α-HSDHs from *E. coli* and the *Eubacterium* sp. (data not shown). This suggests that the *C. sordellii* 7α-HSDH also exists as a tetramer in its native state. Approximately 30 μg of the purified protein was subjected to N-terminal amino acid sequence analysis. The sequence for residues 2 through 18 was determined (NH_2?NK LENKVALVTSATRGI). This sequence was 58.8% identical (10 matches in 17 residues) to the corresponding region of the NADP-linked 7α-HSDH from *Eubacterium* sp. strain VPI 12708 (8).

Cloning of 7α-HSDH. Based on the N-terminal amino acid sequence, a set of degenerate oligonucleotides was synthesized for use in identifying the 7α-HSDH gene in restriction fragments of DNA from *C. sordellii*. Amino acids 2 through 8 were judged to be the best sequence to use to design probes with the least degeneracy. Probe I [AA(TC)AA(AG)TT(AG)GA(AG) AA(TC)AA(AG)GT] had a 64-fold degeneracy, and probe II [AA(TC)AA(AG)CT(ATGC)GA(AG)AA(TC)AA(AG)GT] had a 128-fold degeneracy. Southern blot analysis of *C. sordellii* genomic DNA digested to completion with various restriction enzymes was performed using each ^{32}P-labeled probe. Strong signals were observed with probe I but not with probe II (data not shown). Therefore, probe I was used for subsequent screening experiments. *Eco*RI digestion gave a well-defined hybridization band at approximately 4.5 kb, so this enzyme was utilized for the initial cloning experiments. *Eco*RI-digested genomic DNA from *C. sordellii* was fractionated by sucrose density gradient centrifugation, and fractions showing maximal hybridization to probe I were ligated into pUC19 and transformed into *E. coli* DH5α-MCR. Colony lifts and hybridizations were performed on approximately 250 colonies with plasmid inserts. Of these, six colonies which hybridized strongly to probe I were identified. These were subsequently shown to have identical restriction patterns and therefore were assumed to contain common inserts in identical orientations. One representative of this group was selected and designated pCAG-01 (Fig. 3). Initial mapping, hybridizations, and sequence analysis indicated that the 7α-HSDH gene was located on a terminal portion of the 4.5-kb *Eco*RI insert and that the gene was incomplete. With the preliminary restriction map it was predicted that the 3.2-kb *Hin*dIII fragment would contain the entire gene. This *Hin*dIII fragment was cloned in a fashion identical to that of the *Eco*RI fragment and designated pCAG-52 (Fig. 3). Cell extracts prepared from *E. coli* transformants containing this plasmid produced NADP-linked 7α-HSDH activity at levels approximately 200-fold higher than did *C. sordellii*. This activity was electrophoretically distinct from the endogenous NAD-linked 7α-HSDH found in *E. coli* (data not shown).

DNA sequence analysis. The 1,314-bp nucleic acid segment extending from the *Hpa*I restriction site to a point 402 bases downstream from the *Eco*RI site was sequenced in both strands, and the sequence (Fig. 4) was analyzed for information content. An 804-base open reading frame (ORF) was found extending from base 322 through base 1125. This

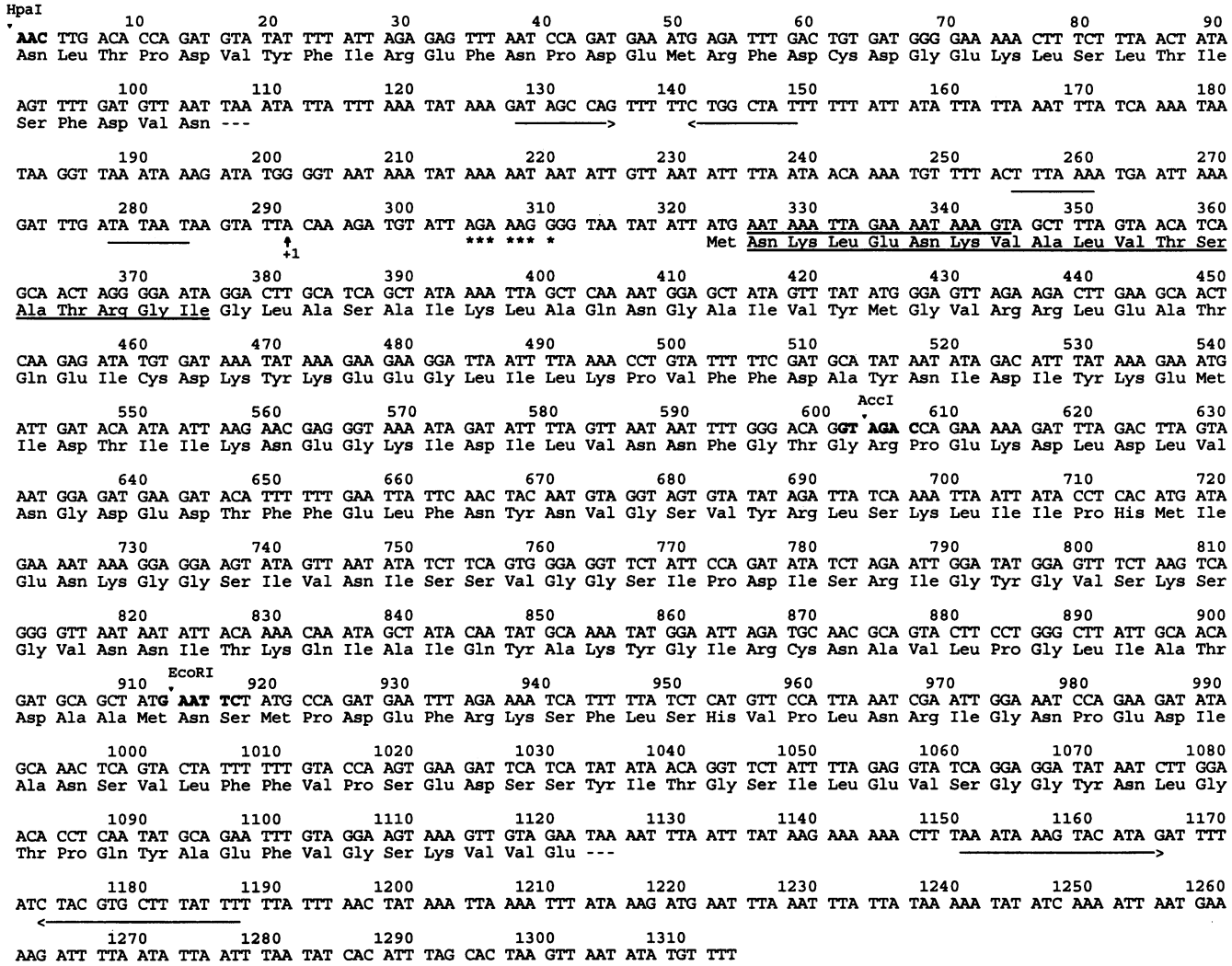

FIG. 4. Nucleotide and amino acid sequences of the 7α-HSDH gene. Restriction recognition and cleavage sites for *Hpa*I, *Acc*I, and *Eco*RI are indicated by boldface type and arrowheads, respectively. Possible rho-independent terminator regions are indicated by horizontal arrow pairs below the sequence. Possible promoter −35 and −10 regions are indicated by the horizontal bars below the sequence. The putative Shine-Dalgarno region complementary to the 3′ end of *C. perfringens* 16S rRNA (21) is indicated by asterisks. The amino acid sequence corresponding to that determined experimentally by N-terminal analysis is underlined. The nucleotide sequence at the N terminus which is a subpopulation of the probe I mixture is also underlined.

reading frame included codons for the identical amino acid residues determined by the N-terminal amino acid analysis of the purified 7α-HSDH and the corresponding sequence contained in a subpopulation of probe I (bases 325 to 344). Downstream (25 bp) from the termination codon of this ORF was a sequence resembling a rho-independent terminator ($\Delta G_{37°C}$ = −11.9 kcal [ca. −49.8 kJ]/mol). In the upstream region, the presence of another rho-independent terminator-like structure ($\Delta G_{37°C}$ = −9.1 kcal [ca. −38 kJ]/mol) suggests that the ORF extending through base 108 encodes the carboxyl terminus of a peptide. The region from the end of this putative upstream terminator (base 153) to the start codon for the 804-base reading frame is characterized by an extremely low G+C composition (14% G+C compared to 28 and 29% for the upstream and downstream ORFs, respectively). This base composition pattern is also seen in the region downstream from the 804-base ORF, with a G+C composition of 13%. The entire sequenced region has an overall base composition of 25% G+C.

The region upstream from the 804-base ORF contains sequences with homology to previously recognized promoter sequences from gram-negative and gram-positive organisms (24, 47, 51). These include a sequence (TATAAT) identical to the consensus −10 region from gram-positive and enteric gram-negative organisms. The calculated frequency of random occurrence of the sequence TATAAT is high (approximately once per 175 bases) in a DNA region with the observed low G+C composition. However, the presence of additional residues shown to be highly conserved in prokaryotic promoters, such as the TG 1 base upstream from the −10-like hexameric sequence, lends support to the function of this region as the promoter. Further upstream lies a sequence (TTTAAA) which matches the consensus −35 sequence (TTGACA) from *E. coli* and gram-positive organisms in 4 to 6 bases. The spacing

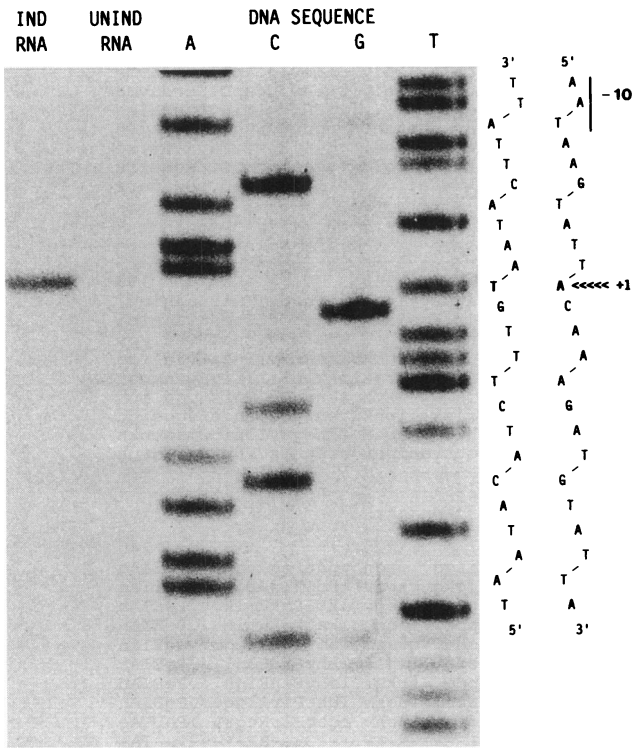

FIG. 5. Primer extension mapping of the 5′ end of the 7α-HSDH transcript. The 5′ end of the 7α-HSDH message (140-min sample) is shown (IND RNA) alongside a DNA sequencing ladder with the same primer. The DNA sequence which can be read from this region is shown to the right. The vertical bar indicates the position of the last three bases of the consensus −10 hexamer just upstream from this transcriptional start site. Uninduced RNA (UNIND RNA) from the same time point is also included.

between these putative −35 and −10 sequences is 17 bases, which is considered the optimum distance. The results of primer extension analyses (see below) also strongly suggest that this region functions as the promoter. Downstream (20 bases) from this promoter region lies a potential ribosomal binding sequence consisting of a heptanucleotide ($\Delta G_{37°C}$ = −10.4 kcal [ca. −43.5 kJ]/mol) with 100% complementary homology to the 3′ end region of clostridial 16S rRNA (21). This putative Shine-Dalgarno sequence precedes the start codon by 11 bases.

Transcript analysis. Total RNA was isolated from *C. sordellii* at time intervals throughout the logarithmic and stationary growth phases under both inducing (0.2 mM chenodeoxycholic acid) and noninducing conditions. Samples of this RNA were subjected to Northern blot analysis using a 22-mer oligonucleotide probe (5′-GCTGATGCAAGTCCTATTCCC C-3′) based on the N-terminal coding region of the 7α-HSDH gene (bases 368 to 389). The 7α-HSDH bands observed after autoradiography were excised and quantitated by liquid scintillation counting. The results clearly show a dramatic elevation of 7α-HSDH mRNA levels in response to the presence of bile acid in the growth medium (Fig. 2). Also noteworthy and in agreement with measured enzyme levels is the apparent growth phase-dependent elevation of 7α-HSDH mRNA levels in the absence of bile acid. The mechanisms of both the bile acid-dependent and growth phase-dependent increases in 7α-HSDH mRNA levels are currently under investigation.

The size of the 7α-HSDH band seen (<1,000 bases) is also consistent with the prediction based on the DNA sequence analysis that the gene is located on a monocistronic operon.

The 5′ end of the 7α-HSDH RNA (from bile acid-induced, late-log-phase cells) was mapped by primer extension analysis using the same 22-mer oligonucleotide used for the Northern analysis (Fig. 5). The 5′ end of the transcript was localized to an A residue corresponding to position 291 of the DNA sequence (Fig. 4). This base is located 7 bases downstream from the consensus −10 sequence (bases 278 to 283) described above.

Amino acid sequence comparisons. The predicted amino acid sequence encoded on the 804-base ORF (267 residues; 29,175 Da) shows significant homology to enzymes in the short-chain dehydrogenase family. The closest relative among previously described enzymes is the NADP-linked 7α-HSDH from *Eubacterium* sp. strain VPI 12708 (8) (48% identity; 65% similarity), followed by the NAD-linked 7α-HSDH from *E. coli* (61) (34% identity; 58% similarity). Table 1 summarizes the identity scores between the *C. sordellii* 7α-HSDH and six other bacterial HSDHs as well as two other closely related proteins, the 3-oxoacyl-(acyl carrier protein) reductase from *E. coli* (involved in fatty acid biosynthesis) (50) and the putative 3-oxoacyl reductase from *Streptomyces violaceoruber* (involved in polyketide antibiotic biosynthesis) (53). The relationship of the *C. sordellii* and *Eubacterium* NADP-linked 7α-HSDHs to the *E. coli* NAD-linked 7α-HSDH and the *E. coli* NADP-linked 3-oxoacyl reductase is noteworthy in that the two NADP-linked HSDHs appear to be as closely related to the NADP-linked 3-oxoacyl reductase as they are to the NAD-linked HSDH from *E. coli* (see below).

TABLE 1. Pairwise identity scores for short-chain dehydrogenases similar to the *C. sordellii* 7α-HSDH

Enzyme[a]	% Identity[b] *with:*							
	CTE3DH	EUB3DH1	EUB3DH2	ECO3KR	EUB7DH	CSO7DH	ECO7DH	STV3KR
STH20DH	36	32	33	36	35	29	31	32
CTE3DH	100	29	30	28	30	30	31	30
EUB3DH1		100	90	33	30	29	27	26
EUB3DH2			100	34	30	29	27	25
ECO3KR				100	35	34	31	28
EUB7DH					100	48	34	33
CSO7DH						100	34	33
ECO7DH							100	29

[a] See Fig. 6 legend for sequence abbreviations.

[b] The GCG DISTANCES program was used to calculate scores based on a cutoff value of 1.5 (residue identity) and the alignment shown in Fig. 6. The total number of matches for each pair is divided by the length of the shortest member of that pair to obtain the identity score, expressed as a percentage.

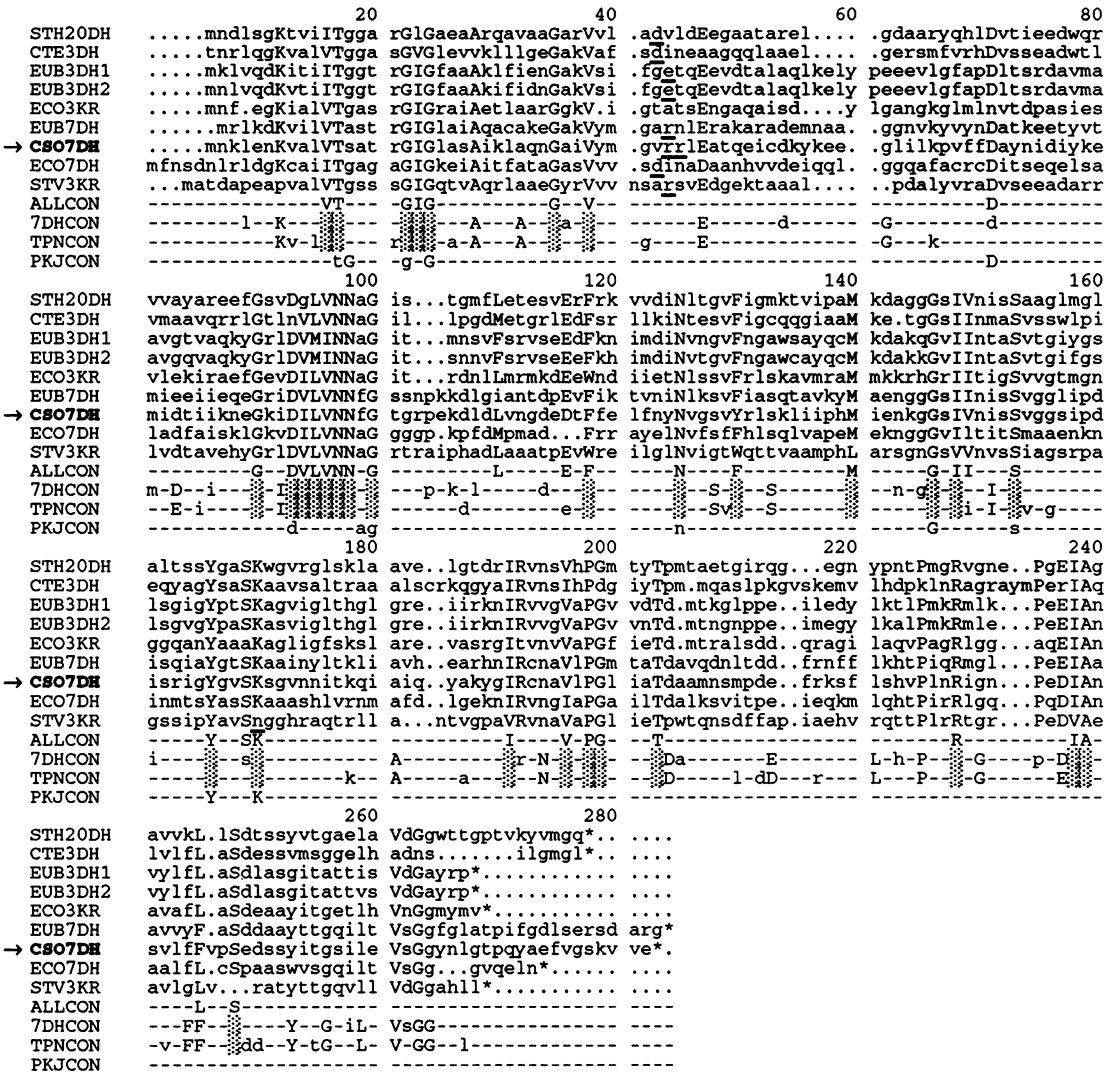

FIG. 6. Amino acid sequence homologies between nine bacterial HSDHs and closely related short-chain dehydrogenases aligned with the GCG PILEUP program. Below the actual protein sequences are listed various consensus sequences generated with the GCG DISTANCES program. ALLCON, nine members; 7DHCON-3, residues conserved in the three 7α-HSDHs (CSO7DH, EUB7DH, and ECO7DH); TPNCON, residues conserved in the most closely related NADP-linked enzymes (CSO7DH, EUB7DH, and ECO3KR). For 7DHCON and TPNCON, residues unique to either are noted by lowercase letters. PJKCON, the 13 residues which are identical in all (uppercase) or most (lowercase) of the 20 short-chain dehydrogenases examined by Persson et al. (49). For actual sequences, highly conserved or identical residues are indicated in uppercase letters. The *C. sordellii* sequence is marked by the horizontal arrow. Underlined residues around position 40 are polar residues which may be involved in cofactor specificity. The Asn residue of STV3KR in the otherwise highly conserved -Y---K- region is also underlined. Sequence abbreviations: STH20DH, *Streptomyces hydrogenans* 3α,20β-HSDH (44); CTE3DH, *Comamonas testosteroni* 3β-HSDH (60); EUB3DH1 and EUB3DH2, *Eubacterium* sp. strain VPI 12708 3α-HSDHs involved in bile acid 7-dehydroxylation (23); CSO7DH, *C. sordellii* 7α-HSDH (this work); EUB7DH, *Eubacterium* sp. strain VPI 12708 7α-HSDH (8); ECO7DH, *E. coli* HB101 7α-HSDH (61); ECO3KR, *E. coli* 3-keto-(acyl carrier protein) reductase (fatty acid biosynthesis) (50); STV3KR, *S. violaceoruber* putative 3-keto reductase (polyketide biosynthesis) (53).

The nine HSDHs and closely related proteins are shown in alignment in Fig. 6. The consensus sequence ALLCON listed below the alignments indicates that five of the six highly conserved residues (PKJCON) of the short-chain dehydrogenase family (49) are also found in the *C. sordellii* 7α-HSDH and its close relatives. Exceptions are found in that this group of sequences lacks the conserved Gly-18. In addition, the highly conserved Lys-170 is apparently replaced by Asn-170 in the case of the *S. violaceoruber* 3-oxoacyl reductase.

The consensus sequences 7DHCON and TPNCON show additional residues which are conserved in particular sequence subsets exclusive of those residues conserved throughout the entire group. TPNCON shows the residues which are conserved between the two NADP-linked 7α-HSDHs and the NADP-linked 7α-HSDHs and the NADP-linked *E. coli* 3-keto reductase. 7DHCON shows those residues conserved in the three 7α-HSDHs (NADP or NAD linked). Although definitive conclusions cannot be drawn from such a small number of sequences, the differences between 7DHCON and TPNCON may be indicative of residues which play a role as cofactor determinants (TPNCON) versus residues which play a role as 7α-hydroxy group determinants (7DHCON). There also appears to be some correlation between the polarity of charged amino acid residues in the region around amino acid 37

(STH20DH numbering) and the cofactor specificity. Several of the enzymes known to be NAD linked (STH20DH, CTE3DH, and ECO7DH) have aspartic residues in this region, whereas the majority of the NADP-linked members (EUB7DH, CSO7DH, and STV3KR) have arginine residues in the same region. This region has previously been suggested to be involved in cofactor specificity via H bonding to the adenosine ribose C_2-hydroxy group of NAD (acidic or uncharged residues) or the adenosine ribose C_2-phosphate group of NADP (basic residues). The cofactor specificity of *Drosophila* alcohol dehydrogenase (a short-chain dehydrogenase) has actually been altered by mutation of Asp-38 to Asn-38 (12). However, three-dimensional structural predictions for the 3α,20β-HSDH (STH20DH) instead suggest that this region (Asp-37) is involved in H bonding to the carboxamide group of the nicotinamide moiety rather than the adenosine ribose hydroxyl positions (22).

Our research activities are now directed toward elucidating the mechanism of bile acid and growth phase regulation of the 7α-HSDH gene in *C. sordellii*.

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