APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1997, p. 1185–1188 Vol. 63, No. 3
0099-2240/97/$04.00+0

Assessment of Fecal Bacteria with Bile Acid 7α-Dehydroxylating Activity for the Presence of *bai*-Like Genes

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Received 18 July 1996/Accepted 9 December 1996

***Eubacterium* sp. strain VPI 12708 has several bile acid-inducible (*bai*) genes which encode enzymes in the bile acid 7α-dehydroxylation (7αDeOH) pathway. Twelve 7αDeOH-positive intestinal bacterial strains were assayed for 7αDeOH activity, and 13 strains were tested for hybridization with *bai* genes. Cholic acid 7αDeOH activity varied greatly (>100-fold) among these strains. Southern blot experiments showed that DNA prepared from 7 of 13 strains hybridized with at least one of the *bai* genes from *Eubacterium* sp. strain VPI 12708.**

Bile acids are C24 steroids which are synthesized from cholesterol in the liver, conjugated to either glycine or taurine, and secreted into the small intestines via the bile. Most of the conjugated bile acids are actively absorbed in the ileum and returned to the liver via the portal blood (24). However, roughly 5% of the bile acid pool escapes ileal absorption and enters the large intestines each day. Humans synthesize cholic acid and chenodeoxycholic acid (primary bile acids) which are 7α-dehydroxylated by colonic bacteria, yielding deoxycholic acid and lithocholic acid (secondary bile acids), respectively. The deoxycholic acid pool in humans can vary from 0 to more than 40% of the total bile acids (2).

Several studies have shown that high levels of deoxycholic acid in bile are correlated with an increased risk for cholesterol gallstone disease (19, 21), but studies implicating bile acid 7α-dehydroxylating bacteria were lacking until recently. Berr et al. (2) showed that fecal levels of 7α-dehydroxylating bacteria are approximately 1,000-fold higher in a population of cholesterol gallstone patients exhibiting high levels of deoxycholic acid compared to patients with lower deoxycholic acid levels. Treatment of gallstone patients exhibiting high levels of deoxycholic acid with antibiotics resulted in significant decreases in fecal levels of 7α-dehydroxylating bacteria, levels of deoxycholic acid, and the biliary cholesterol saturation index. These results suggest that the levels of intestinal 7α-dehydroxylating bacteria may control the cholesterol saturation index of bile in some patients, a crucial risk factor for cholesterol gallstone disease.

Members of the genera *Clostridium* and *Eubacterium* are the predominant intestinal species exhibiting bile acid 7α-dehydroxylating activity (4, 5, 8, 11–13, 20, 23). *Eubacterium* sp. strain VPI 12708 has been shown to have a multistep bile acid 7α-dehydroxylation pathway with most of the required enzymes encoded in a large *bai* operon (Fig. 1) (3, 14, 18). Detailed studies concerning the physiology and genetics of other bile acid 7α-dehydroxylating intestinal bacteria have not been reported. The development of DNA probes for detecting and quantifying fecal levels of bile acid 7α-dehydroxylating bacteria may be useful in studying the possible role of these bacteria in cholesterol gallstone disease.

Bacterial strains and whole-cell cholic acid 7α-dehydroxylation activity. Several bacterial strains with known bile acid 7α-dehydroxylating activity were used in this study. *Eubacterium* sp. strain VPI 12708 was originally isolated from feces of a colon cancer patient by R. Hammann (Institute für Medizinische Microbiologie und Immunologie der Universitat,

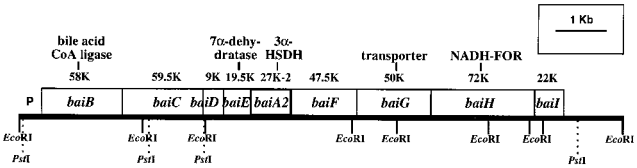

FIG. 1. Partial restriction map and locations of open reading frames in the *bai* operon from *Eubacterium* sp. strain VPI 12708.

TABLE 1. Specific activities of cholic acid 7α-dehydroxylation in selected bacterial strains

Strain	Sp act[a]
Group I	
Eubacterium sp. strain VPI 12708	3.03 ± 0.02
C. scindens ATCC 35704	1.95 ± 0.94
Eubacterium sp. strain Y-1113	5.26 ± 0.12
Eubacterium sp. strain I-10	4.71 ± 2.03
Eubacterium sp. strain M-18	4.70 ± 2.11
Eubacterium sp. strain TH-82	1.92 ± 0.63
Clostridium sp. strain TO-931	7.83 ± 1.11
Clostridium sp. strain HD-17	1.25 ± 0.27
Group II	
C. sordellii ATCC 9714	0.09 ± 0.01
C. sordellii Y-67	0.11 ± 0.019
C. leptum ATCC 29065	0.15 ± 0.06
Clostridium sp. strain TN-271	0.16 ± 0.01
C. bifermentans I-55	0.05 ± 0.001

[a] Conversion of cholic acid into deoxycholic acid (nmol mg^{-1} h^{-1}) by whole cells previously exposed to 100 μM cholic acid. Results are averages ± standard deviations from two or three independent experiments carried out in duplicate.

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TABLE 2. Comparison of bile acid 7α-dehydroxylating bacteria for the presence of *bai* genes originally isolated from *Eubacterium* sp. strain VPI 12708

Strain	*bai* genes used as probes[a]					
	baiB (bile acid/CoA-ligase)	*baiE* (dehydratase)	*baiA2* (3α-HSDH)	*baiG* (bile acid transporter)	*baiH* (NADH/FOR oxidoreductase)	*baiI* (function unknown)
Eubacterium sp. strain VPI 12708	+	+	+	+	+	+
C. scindens ATCC 35074	+	+	+	+	+	+
Eubacterium sp. strain Y-1113	+	+	+	+	+	+
Eubacterium sp. strain 36S	±	±	−	±	−	±
Eubacterium sp. strain I-10		+				
Eubacterium sp. strain M-18		+				
Clostridium sp. strain TO-931	−	−	−	−	−	−
Clostridium sp. strain HD-17		−				
C. sordellii ATCC 9714	−	−	−	−	−	−
C. sordellii Y-67	−	−	−	−	−	−
C. leptum ATCC 29065	−	−	−	−	−	−
Clostridium sp. strain TN-271	−	−	+	+	±	±
Eubacterium sp. strain TH-82	+	+	+	+		
C. bifermentans I-55	−	−	−	−	−	−

[a] +, indicates cross-hybridization occurred; −, indicates cross-hybridization did not occur; ±, indicates weak but detectable hybridization occurred. A blank cell indicates that the strain was not tested with that probe. Abbreviations: CoA, coenzyme A; HSDH, hydroxysteroid dehydrogenase; FOR, flavin oxidoreductase.

Bonn, Germany). *Clostridium scindens* ATCC 35704 was obtained from V. Bokkenheuser (5). *Clostridium sordellii* ATCC 9714 and *Clostridium leptum* ATCC 29065 were obtained from the American Type Culture Collection (Rockville, Md.). *C. sordellii* Y-67, *Eubacterium* sp. strain M-18, *Eubacterium* sp. strain I-10, and *Eubacterium* sp. strain Y-1113 were recently isolated from human feces (23). *Clostridium bifermentans* I-55 and *Clostridium* sp. strain HD-17 were also isolated from human feces (13), and *Clostridium* sp. strain TO-931, *Clostridium* sp. strain TN-271, and *Eubacterium* sp. strain TH-82 were recently isolated from human feces by one of us (F.T.) by using previously described procedures (23). Cholic acid 7α-dehydroxylation activities were measured in whole-cell suspensions for each species essentially as described previously (22). The data presented in Table 1 show that there were two distinct groups of cholic acid 7α-dehydroxylating bacteria with respect to activity, one with relatively high activity (Group I) and the other with low activity (Group II).

Southern blot analysis of *bai* genes. Chromosomal DNA was isolated from each bacterial species, and 2 μg of DNA was digested with either *Eco*RI or *Pst*I at 37°C overnight in the appropriate buffer. DNA fragments were separated electrophoretically, blotted onto MagnaNT nylon membranes (Micron Separations, Inc.), and baked for 1 h at 80°C as previously described (1). Southern blotting was carried out essentially as previously described (10). Cloned *bai* genes from *Eubacterium* sp. strain VPI 12708 encoding various enzymatic activities required for cholic acid 7α-dehydroxylation were used as molecular probes (Fig. 1). Each of these genes is part of a large bile acid-inducible operon (18). The insert containing the *baiE* gene was isolated from pSport1-19K (6). The insert containing the *baiB* gene was isolated from pSport1-58 (16). The insert containing the *baiA2* gene was isolated from pSport1-27K2 (25). The insert containing the *baiG* gene was isolated from pSport1-50K (17). A 0.6-kb *Eco*RI fragment from the 5′ region of the *baiH* gene was subcloned and subsequently isolated (9) for use in the study. The insert containing the *baiI* gene was isolated from pGEM-22K (26).

Southern blotting results showed that DNA from 7 of 13 strains of bile acid 7α-dehydroxylating intestinal bacteria hybridized with at least one *bai* gene probe (Table 2). However, five strains showed no detectable hybridization with any of the six *bai* gene probes used. *Clostridium* sp. strain HD-17 showed no hybridization with the *baiE* gene probe. Chromosomal DNA from *Eubacterium* sp. strain VPI 12708, *C. scindens* ATCC 35704, and *Eubacterium* sp. strain Y-1113 all displayed similar hybridization patterns for each restriction enzyme

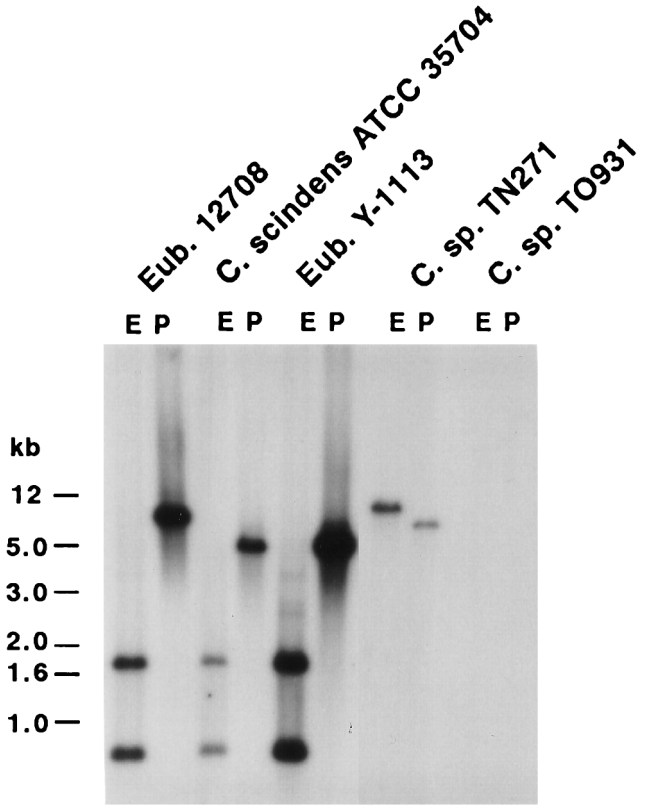

FIG. 2. Autoradiogram of selected 7α-dehydroxylating bacterial chromosomes probed with the *baiG* gene from *Eubacterium* sp. strain VPI 12708. Equivalent quantities of DNA from each strain were digested with *Eco*RI (E) or *Pst*I (P) prior to analysis. Molecular weight markers are indicated on the left. Eub. 12708, *Eubacterium* sp. strain 12708; Eub. Y-1113, *Eubacterium* sp. strain Y-1113; C. sp. TN271, *Clostridium* sp. strain TN271; C. sp. TO931, *Clostridium* sp. strain TO931.

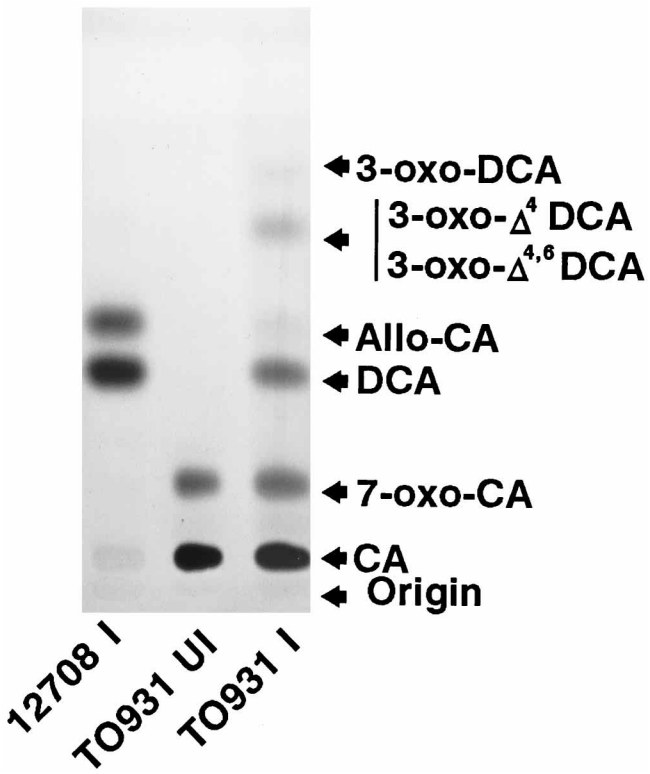

FIG. 3. Thin-layer chromatography autoradiogram of bile acid intermediates from ^{14}C-cholic acid incubated with cell extracts prepared from either *Eubacterium* sp. strain VPI 12708 (12708) or *Clostridium* sp. strain TO-931 (TO931). Cultures were either uninduced (UI) or induced (I) twice with 100 μM cholic acid. The location of various bile acid intermediates in the cholic acid 7α-dehydroxylation pathway are indicated. Abbreviations: CA, cholic acid; 7-oxo-CA, 7-oxo-cholic acid; DCA, deoxycholic acid; allo-CA, allocholic acid; 3-oxo-$\Delta^{4,6}$ DCA, 12α-hydroxy-3-oxo-4,6-choldienoic acid; 3-oxo-Δ^4 DCA, 12α-hydroxy-3-oxo-4-cholenoic acid; 3-oxo-DCA, 3-oxo-deoxycholic acid.

tested for all six probes (Fig. 2). These results suggest that these strains may contain a similar *bai* operon. *Eubacterium* sp. strain TH-82 and *Clostridium* sp. strain TN-271 showed hybridization to four *bai* gene probes (Table 2) but had a different restriction endonuclease digestion pattern than the other strains. Preliminary results from comparative analysis of 16S-rDNA sequences from six 7α-dehydroxylating strains indicate that they are a polyphyletic group within the gram-positive bacteria (7).

Mechanism of cholic acid 7α-dehydroxylation in *Clostridium* sp. strain TO-931. Although *Clostridium* sp. strain TO-931 and *Clostridium* sp. strain HD-17 are both in the high cholic acid 7α-dehydroxylation activity group, neither showed any detectable hybridization to *bai* gene probes (Tables 1 and 2). *Clostridium* sp. strain TO-931 was investigated for evidence of the multistep 7α-dehydroxylation pathway. It was observed that this bacterium had a cholic acid-inducible 7α-dehydroxylation activity that showed the same bile acid intermediates as those in *Eubacterium* sp. strain VPI 12708 (Fig. 3). These results suggest that the bile acid 7α-dehydroxylation pathway in these two bacteria is the same, but the DNA sequences of genes encoding the various enzymes in this pathway may have "drifted" enough to prevent hybridization. Different codon usage for genes within the *bai* operon in *Clostridium* sp. strain TO 931 may be a possible explanation for the lack of hybridization. DNA isolated from members of the genus *Clostridium* generally has a low G plus C content (15). In contrast, the G plus C content of the *bai* operon from *Eubacterium* sp. strain VPI 12708 is approximately 50% (18). However, the *bai* operon in *C. scindens* ATCC 35704 appears to be very similar to that in *Eubacterium* sp. strain VPI 12708 (Table 2), perhaps suggesting lateral transfer of this operon among gram-positive anaerobes. A more detailed explanation for these results will have to await the cloning, sequencing, and analysis of the *bai* genes from *Clostridium* sp. strain TO-931.

In summary, the current data show that intestinal 7α-dehydroxylating bacteria can be divided into high and low activity groups based on whole-cell assays. DNA probes from *Eubacterium* sp. strain VPI 12708 hybridized to DNA of 7 of 13 strains tested. However, in order to design DNA probes to detect all fecal bile acid 7α-dehydroxylating bacteria, the *bai* genes from nonhybridizing strains will have to be isolated and characterized.

This work was supported by grants (PO1 DK38030 and DK40986) from the National Institutes of Health. K.C.D. was supported by a National Research Service Award (5 F32 DK08879) from the NIH. Support for F.T. was provided by a Japan Society for the Promotion of Science (JSPS) Fellowship for Research at Centers of Excellence Abroad.

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