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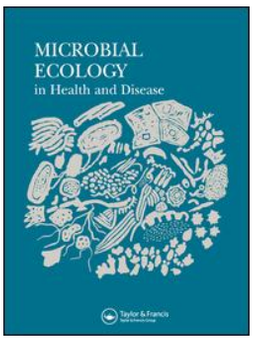

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ORIGINAL ARTICLE

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Isolates from Normal Human Intestinal Flora but not Lactic Acid Bacteria Exhibit 7α- and 7β-Hydroxysteroid Dehydrogenase Activities

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Ursodeoxycholic acid (UDCA)-producing bacteria are of clinical and industrial interest due to the multiple beneficial effects of this bile acid on human health. UDCA is the 7β-OH epimer of the primary (i.e. synthesized by the liver) bile acid chenodeoxycholic acid (CDCA). Epimerization proceeds in two subsequent and reversible steps, catalysed by a 7α- and a 7β-hydroxysteroid dehydrogenase (7α- and 7β-HSDH), with 7oxo-lithocholic acid (7oxo-LCA) as the intermediate product. The aim of this study was to test the 7α- and 7β-HSDH activities of anaerobic whole cell cultures of a number of lactic acid bacteria and human intestinal isolates, using CDCA, UDCA and 7oxo-LCA as the substrates. Among 140 strains tested, 21 exhibited at least one of both 7-HSDH activities. 7α-HSDH activity was detected in six strains, 7β-HSDH in nine strains, and both activities in six other strains. All active strains were isolated from normal human and infant faeces. They belonged to the genera *Clostridium*, *Eubacterium* and *Ruminococcus*, whereas no strain of *Lactobacillus*, *Bifidobacterium* or *Streptococcus* was found to be active under our study conditions. The present study therefore revealed, for the first time, a number of normal human intestinal isolates supporting the epimerization of CDCA to UDCA, and further extended our knowledge of those intestinal bacteria which are responsible for 7α- or 7β-HSDH activity. *Key words:* screening, bile acids, epimerization, intestinal microflora, lactic acid bacteria, probiotics.

INTRODUCTION

In the liver, bile acids are synthesized from cholesterol and conjugated to an amino acid (glycine or taurine) before secretion into the biliary tract and then into the duodenum. In the small bowel, conjugated bile acids contribute to the emulsification, digestion and absorption of lipids. Bile acids are reabsorbed all along the intestine, by means of carrier-mediated transports and passive diffusion. Those bile acids which escape absorption in the small bowel can be further absorbed in the colon by passive diffusion, after they have been extensively transformed by the abundant and highly diversified indigenous microbiota which colonizes the large intestine and the most distal part of the small bowel. Bacterial hydrolysis of the amide bond of conjugated bile acids, resulting in the release of their unconjugated homologues and the amino acid moiety, would be a prerequisite to further transformations of the steroid moiety (1), consisting of dehydroxylations and oxydo-reductions at C-3, C-6, C-7 and C-12.

Ursodeoxycholic acid (UDCA, 3α,7β-dihydroxy-5β-cholan-24-oic acid) is a minor bile acid in human, resulting from the bacterial 7-epimerization of chenodeoxycholic acid (CDCA, 3α,7α-dihydroxy-5β-cholan-24-oic acid). UDCA is widely used for the treatment of cholesterol cholelithiasis (2) and of primary biliary cirrhosis (3). Anti-inflammatory properties have been demonstrated in humans (4) and more recently, UDCA has been shown to inhibit chemically induced tumour development (5) and to reduce hepatocarcinogenesis in rats (6).

Epimerization of CDCA to UDCA is catalysed by two distinct enzymes, a 7α-hydroxysteroid dehydrogenase (7α-HSDH) which oxidizes CDCA into 7oxo-lithocholic acid (7oxo-LCA, 3α-hydroxy,7oxo-5β-cholan-24-oic acid) and a 7β-hydroxysteroid dehydrogenase (7β-HSDH) which reduces 7oxo-LCA to UDCA (Fig. 1). Both enzymes can operate in the oxidative and the reductive pathway so that CDCA can be produced from 7oxo-LCA and 7oxo-LCA can be produced from UDCA (1, 7). The intestinal bacteria

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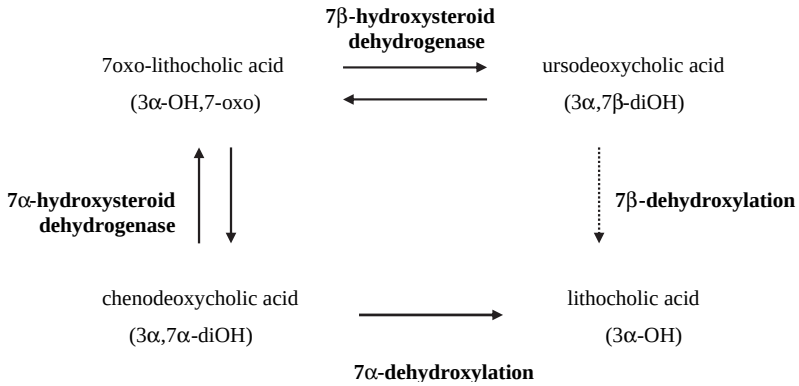

Fig. 1. Possible transformations occurring at position 7 of steroid nucleus, dehydrogenation-hydrogenation, epimerization and dehydroxylation.

responsible for those sequential reactions are poorly known. Six strains of lecithinase-lipase-negative clostridia have been isolated from stools of patients with colon cancer (8). Two soil isolates, *Clostridium absonum* (9) and *Clostridium limosum* (1), as well as the aerobic opportunistic pathogen *Stenotrophomonas* (formerly *Pseudomonas* and *Xanthomonas*) *maltophilia* (7), are also known to epimerize CDCA to UDCA. Indeed, formation of UDCA in the human intestine can result from the association of two kinds of microorganisms, each carrying one of both 7-HSDH (10). The 7α-HSDH-carrying bacteria are widely represented in the intestinal human flora. *Escherichia coli* and members of the genera *Bacteroides*, *Clostridium*, *Bifidobacterium* (11), *Eubacterium* (12) and *Pseudomonas* (13) were shown to be active in pure cultures. The gene encoding for 7α-HSDH has been cloned and sequenced, and the expressed enzyme has been characterized from *E. coli* HB101 and *Clostridium scindens* (formerly *Eubacterium* sp. VPI 12708) (14, 15). In contrast, apart from the 7-epimerizing bacteria previously indicated, only three bacteria, identified as *Ruminococcus* sp. (16), *Eubacterium aerofaciens* (10) and *Peptostreptococcus productus* (17), are known to carry 7β-HSDH. The enzyme was partially purified (16, 18) and its encoding gene remains unknown.

The main purpose of the present study was to test the 7α- and 7β-HSDH activities of whole cell cultures of a number of lactic acid bacteria and human intestinal isolates, using CDCA, UDCA and 7oxo-LCA as the substrates. In the human bowel, however, the major product of CDCA microbial transformation is lithocholic acid (LCA, 3α-hydroxy-5β-cholan-24-oic acid), which is suspected of being involved in colon inflammation and carcinogenesis (19). LCA results from the 7α-dehydroxylation of CDCA by a 7α-dehydroxylase, which is known to be expressed by many bacteria of the intestinal human flora (20). LCA can also be formed from UDCA in the presence of a 7β-dehydroxylase activity (Fig. 1), but formation of LCA appears to be much slower from UDCA than from CDCA (21). *C. scindens* (22) and *Eubacterium* sp. (23) are the only two bacteria known to dehydroxylate UDCA to LCA so far. We therefore also looked for LCA formation in the reported assays.

MATERIALS AND METHODS

Strains

A total of 140 strains was assayed. Forty-one strains of *Lactobacillus* belonging to the species *L. casei* (8 strains), *L. rhamnosum* (4 strains), *L. acidophilus* (5 strains), *L. plantarum* (10 strains), *L. bulgaricus* (9 strains), *L. kefir* (2 strains), *L. salivarius* (1 strain), *L. lactis* (1 strain) and *L. helveticus* (1 strain), plus six strains of *Bifidobacterium* belonging to the species *B. animalis* (1 strain), *B. adolescentis* (1 strain), *B. longum* (4 strains) 10 strains of *Streptococcus thermophilus* and 3 clostridia (*C. butyricum*, *C. innocuum*, *C. bifermentans*) were provided by Danone Vitapole. These bacteria were initially isolated from fermented products (the three *Clostridium* strains were contaminants occasionally formed during beer fermentation) or infant microflora for *Bifidobacterium longum*.

Nine bifidobacteria were kindly donated by Dr F. Gavini (INRA, Lille, France): *B. breve* ATCC 15701, DSM 20091 and NCFB 2257, *B. breve* 89181 and 89246, isolated from normal infant faeces, *B. catenulatum* ATCC 27539, *B. longum* ATCC 15707 and *B. dentium* ATCC 15423 and ATCC 27534.

All other strains were from the UEPSD collection, belonging to the genera *Peptostreptococcus* (26 strains), *Ruminococcus* (15 strains), *Clostridium* (10 strains), *Eubacterium* (7 strains), *Bifidobacterium* (10 strains) and *Pseudomonas* (1 strain). They had been previously isolated from normal human or infant faeces and characterized by biochemical characters and/or16S ribosomal DNA sequencing.

Lastly, *Clostridium absonum* ATCC 27 555 was kindly donated by Dr J-P. Carlier (Institut Pasteur, Paris, France), and *Eubacterium aerofaciens* ATCC 25 986 was purchased from the American Type Culture Collection. Both these strains were used as positive controls for 7-epimerizing and 7β-HSDH activities respectively.

Culture

Microorganisms stored in 20% glycerol at −80°C or lyophilized, were subcultured twice in their appropriate

medium for 24 h at 37°C and under anaerobic conditions before use. Clostridia, eubacteria, ruminococci, peptostreptococci and streptococci were cultivated in brain heart infusion-yeast extract broth (BHI-YE). It was composed of brain heart infusion (37 g/l) (Difco Laboratories, Detroit, MI, USA), yeast extract (5 g/l) (Difco), L-cysteine (0.5) (Sigma, St Quentin Fallavier, France) and 10 ml/l of 0.1 (w/v) haemin (Sigma) solution in water. The pH was adjusted to 7.4. Man Rogosa and Sharp broth (MRS) was used for cultivation of lactic bacteria. It was composed of Man Rogosa and Sharp broth 55 g/l (Difco) supplemented with L-cysteine (0.5 g/l). The pH was adjusted to 6.2. Both media were prepared and sterilized under anaerobic conditions (N_2 atmosphere), according to the Hungate technique (24). They were fractionated to 10 ml in Batch tubes (16×150 mm) and sterilized under N_2.

7-HSDH assays

Sodium salts of CDCA and UDCA were purchased from Sigma-Aldrich Chimie, and sodium salts of 7oxo-LCA and LCA were from Steraloids (Newport, RI, USA). Media for the tests were supplemented with the relevant bile acid, either CDCA or UDCA or 7oxo-LCA, to a final concentration of 10^{-4} m, according to Macdonald et al. (9).

Cultures were 10% inoculated in test tubes containing the appropriate medium (BHI-YE or MRS) supplemented with 10^{-4} m bile acid and grown 48 h at 37°C under anaerobic conditions. Each strain was assayed in triplicate on the three bile acids separately, so that 7α- and 7β-HSDH activities could be independently tested in both the reductive and the oxidative pathways.

Extraction of bile acids

Cultures were acidified to pH 2·0 with 1 N HCl and bile acids were extracted three times with diethyl ether (3×5 ml). The combined solvent fractions were evaporated to dryness and the bile acid extract was reconstituted with 2 ml of methanol and stored at −20°C until analysis (25).

Thin-layer chromatography (TLC) analysis

One hundred µl of bile acid extract were methylated with 200 µl of diazomethane and the solution was allowed to stand at room temperature overnight. The methylated bile acids were evaporated to dryness under N_2 and the residue was reconstituted with 50 µl of dichloromethane. The plate LK6DF silica gel 60 Å (Whatman International, Maidstone, UK) was activated for 1–2 h at 110°C. The totality of the methylated sample was spotted on a plate, and chromatographed in a solvent system: chloroform-acetone-methanol 70:25:5 (v:v:v). After migration, the plate was dried and sprayed with 10% sulphuric acid in ethanol and heated at 160°C for 5 min. Identification of bands was done by comparison with bile acid standards spotted on the same plate (26).

Gas liquid chromatography (GLC) analysis

GLC was carried out for those samples that were found to contain bile acid metabolites by TLC. Fifty µl of bile acid extract were taken and 5 µl of the external standard 5α-cholestane were added from a stock solution of 0.5 mg of 5α-cholestane per ml of hexane. Methylation was done by the addition of 200 µl of diazomethane as previously indicated for thin-layer analysis. After evaporation to dryness, the samples were silylated by the addition of BSTFA and TMSI (Supelco, St Quentin Fallavier, France) in dichloromethane. Trimethylsilyl derivates were analysed using a gas chromatograph (Peri 2000, Perichrom, Saulx-les-Chartreux, France) equipped with a Ros injector, a flame ionization detector and an OV-1701 column measuring 30 m × 0.32 mm × 0.25 µm (Perichrom, Saulx-les-Chartreux, France). The chromatograph was used under isothermal conditions at 250°C. Helium was used as the carrier gas. Individual GLC peaks were identified by relative retention time in relation to the external standard 5α-cholestane. A pool of standards was analysed at the same time for bile acid identification.

RESULTS AND DISCUSSION

Results are summarized in Table I. As expected, we found that *C. absonum* epimerized CDCA to UDCA and that *E. aerofaciens* reduced 7oxo-LCA to UDCA only, which is in agreement with previous studies demonstrating both 7-HSDH activities in *C. absonum* (9) and 7β-HSDH activity alone in *E. aerofaciens* (10).

Among the 140 strains never tested before for the relevant bioconversions, 17 exhibited at least one of both 7-HSDH activities. Six strains exhibited both 7α- and 7β-HSDH activities as inferred from the formation of UDCA starting from CDCA (5 strains) or from the formation of UDCA starting from CDCA (1 strain). In five other strains, only 7α-HSDH activity was detected through the conversion of 7oxo-LCA to CDCA (all five strains) or CDCA to 7oxo-LCA (three among the five previous ones). Finally, the six remaining strains exhibited 7β-HSDH activity only, and operated in the reductive pathway alone, as inferred from the formation of UDCA starting from 7oxo-LCA and the absence of conversion of UDCA to 7oxo-LCA. All active strains were isolated from normal human or infant faeces. They belonged to the genera *Clostridium*, *Eubacterium* and *Ruminococcus*, whereas no strain of *Lactobacillus*, *Bifidobacterium* or *Streptococcus* was found to be active. In our study, lactic acid bacteria did not form LCA either. Nevertheless, the ability of bifidobacteria to dehydrogenate the 3α-, 7α- or 12α-OH group of cholic acid and dehydroxylate the 7α-OH group (11) of cholic acid has been reported but,

Table I

Transformation of chenodeoxycholic acid (CDCA), 7oxo-lithocholic acid (7oxo-LCA) and ursodeoxycholic acid (UDCA) at 10^{-4}M by whole cell cultures of bacteria

	Bile acids metabolites from								
	CDCA			7oxo-LCA			UDCA		
	7oxo-LCA	UDCA	LCA	CDCA	UDCA	LCA	CDCA	7oxo-LCA	LCA
Clostridium absonum ATCC 27555	+	+	−	+	+	−	−	−	−
Clostridium clostridioforme B12	+	−	−	+	−	−	−	−	−
Clostridium clostridioforme B36	−	−	−	−	+	−	−	−	−
Clostridium innocuum Cl2	−	+	−	−	+	−	−	−	−
Clostridium nexile B04	−	+	+	−	+	−	−	−	−
Clostridium nexile V73	−	−	−	−	+	−	−	−	−
Eubacterium aerofaciens ATCC 25986	−	−	−	−	+	−	−	−	−
Eubacterium sp. V61	+	+	+	+	−	−	−	−	−
Ruminococcus gnavus B07	+	+	−	+	+	−	−	−	−
Ruminococcus gnavus B09	+	+	−	−	+	−	−	+	−
Ruminococcus gnavus B94	−	−	+	−	+	−	+	−	−
Ruminococcus gnavus G25	−	−	+	−	+	−	−	−	−
Ruminococcus gnavus V12	+	−	−	+	−	−	−	−	−
Ruminococcus gnavus V62	−	−	−	−	+	−	−	−	−
Ruminococcus gnavus V63	−	−	+	−	+	−	−	−	−
Ruminococcus gnavus V95	−	−	−	−	+	−	−	−	−
Ruminococcus gnavus V99	−	−	−	+	−	−	−	−	−
Ruminococcus hansenii V98	−	−	−	+	−	−	−	−	−
Ruminococcus obeum B14	+	−	−	+	−	−	−	−	−

LCA, lithocholic acid.

to our knowledge, this was never investigated using CDCA as the substrate. Indeed, hydrolysis of the amide bond of the naturally occurring taurine and glycine conjugates of bile acids is the only activity of lactic acid bacteria on bile acids that is well known at this time (27).

7-Epimerizing bacteria

Our study provides the first demonstration of CDCA to UDCA epimerization by anaerobic non-*Clostridium* strains (*Eubacterium* sp. V61, *R. gnavus* B07, B09 and B94), although we also found two new epimerizing clostridia (*Clostridium innocuum* Cl2 and *Clostridium nexile* B04). Other related strains were previously shown to exhibit HSDH activities: 3β-HSDH for *C. innocuum* (28), 7β-HSDH for *E. aerofaciens* (10), and 3β-HSDH (29) and 7β-HSDH (16, 29) for *Ruminococcus* sp., but none of these strains could be shown to carry 7-epimerizing activity.

As for *C. absonum*, the intermediate product 7oxo-LCA was observed in whole cell cultures of *Eubacterium* sp. and *R. gnavus* B07 and B09, whereas it could not be detected in cultures of *C. innocuum*, *C. nexile* and *R. gnavus* B94. However, the three latter strains did reduce 7oxo-LCA to UDCA, suggesting that epimerization would proceed through 7oxo-LCA for these strains too.

With the exception of *R. gnavus* B94, epimerization proceeded from CDCA to UDCA, just like *C. absonum*, whereas the reverse transformation could not be obtained under our study conditions. Macdonald and Roach (30) showed that 7α- and 7β-HSDH of *C. absonum* were induced by the addition of CDCA or, to a lesser extent, of 7oxo-LCA in the culture medium, but were repressed by UDCA. Our assays with whole cultures of *C. absonum*, *C. innocuum*, *C. nexile*, *Eubacterium* sp. and *R. gnavus* B07 coincided with this figure, as those strains epimerized CDCA to UDCA, formed UDCA and/or CDCA from 7oxo-LCA, but failed to transform UDCA. In the particular case of *R. gnavus* B09, however, UDCA did not repress 7β-HSDH, as revealed by UDCA to 7oxo-LCA oxidation, but 7oxo-LCA was not further reduced to CDCA. When starting from 7oxo-LCA, *R. gnavus* B09 did not form CDCA either. However, this does not make it possible to draw conclusions about the state of induction of 7α-HSDH, because conditions of whole cell cultures could have impaired its activity, even if it would have been induced otherwise.

R. gnavus B94 was the only organism to carry out 7-epimerization of UDCA to CDCA, but not the reverse. This has been previously observed in *C. perfringens*, which epimerizes 3β,7α-dihydroxy-5β-cholanoic acid in its 3α-homologue (31). This does not coincide with the previously advanced hypothesis that epimerization would represent a detoxifying mechanism for bacterial cells which would metabolize highly toxic bile acids such as CDCA into far less toxic ones such as UDCA (32). Moreover, under our

study conditions, *R. gnavus* B94 metabolized CDCA to LCA (but not to UDCA). The figure obtained with *C. nexile* and *Eubacterium* sp. was different, as both these strains 7α-dehydroxylated and 7-epimerized CDCA in the same culture batch. Up until now, some species of clostridia have been described as carrying both 7α-dehydroxylation and 7α-dehydrogenation activities (11). This study provides the first demonstration of bacteria capable of 7-epimerizing and 7α-dehydroxylating CDCA.

7α-Dehydrogenating bacteria

7α-HSDH activity was detected in five strains, two strains of *R. gnavus* (V12 and V99), one strain of *R. obeum*, one strain of *R. hansenii* and one strain of *Clostridium clostridioforme*. The reaction was reversible for *C. clostridioforme* B12, *R. gnavus* V12 and *R. obeum* B14, as shown by CDCA to 7oxo-LCA oxidation and 7oxo-LCA to CDCA reduction. It only operated in the reductive pathway for the three remaining strains. No 7α-dehydroxylation activity was shown for these 7α-dehydrogenating bacteria. 7α-Dehydrogenating bacteria are widely represented in the large intestine, this activity has been described for the genus *Clostridium* (20) but no member of the genus *Ruminococcus* has so far revealed 7α-HSDH activity. However, one *Ruminococcus* sp. from the human intestine is known for its 3β- and 7β-HSDH activities (33).

7β-Dehydrogenating bacteria

7β-HSDH activity was detected in six strains, four strains of *R. gnavus* (G25, V62, V63 and V95), one strain of *C. clostridioforme* (B36) and one strain of *C. nexile* (V73). For the six strains, just like *E. aerofaciens* ATCC 25986 taken as a positive control in our study, the reaction proceeded in the reductive pathway only: 7oxo-LCA was reduced to UDCA, whereas UDCA was never oxidized to 7oxo-LCA. Two of these 7β-dehydrogenating bacteria (two strains of *R. gnavus*) also 7α-dehydroxylated CDCA to LCA. Bacteria known to carry 7β-HSDH activity are far less numerous than those with 7α-HSDH activity. They were identified as *Ruminococcus* sp. (16), *E. aerofaciens* (10) and *Peptostreptococcus* (17, 18). This study was therefore able to reveal new 7β-dehydrogenating bacteria, belonging to the genus *Clostridium*.

This study revealed a number of normal human intestinal isolates supporting the epimerization of CDCA to UDCA for the first time, and further extended our knowledge of those intestinal bacteria which are responsible for 7α- and 7β-HSDH activity. All active strains were isolated from the dominant human intestinal microbiota, which is thought to be the most relevant from a functional point of view. Yet, UDCA is a naturally low-occurring bile acid in man, normally representing $<4\%$ of total biliary, blood and faecal bile acids (34). Formation of UDCA, rather than its intestinal absorption and/or hepatic uptake, does appear to be the rate-limiting step of the UDCA accumulation in enterohepatic circulation in man. Indeed, UDCA becomes the major circulating bile acid in patients receiving oral doses of UDCA of 13–15 mg/kg/day for 2 months to 2 years, leading to the conclusion that both intestinal absorption and hepatic uptake of UDCA are very efficient in humans (34–36). One study further suggested that intestinal absorption of UDCA might involve the entire small bowel and the colon (37). In fact, 7α-dehydroxylation is usually the major bacterial transformation of CDCA in the human hindgut, generating LCA (21) and therefore limiting the formation of UDCA. In other species such as the rabbit, UDCA does not form under physiological conditions, whereas epimerizing bacteria might be present and would be active following nearly complete caecectomy with appendectomy (38). Indeed, small amounts of UDCA could be detected in the bile of the operated animals, and substantial amounts of this bile acid were present in the caecal contents and bile (representing up to 32% of total biliary bile acids) of these animals when they were fed with CDCA on a daily basis. This reinforces the idea that intestinal bioavailability of absorbable bile acids from endogenous or exogenous origin is a key element for determining the bile acid pattern in the enterohepatic circulation, whereas intestinal absorption and hepatic uptake would not be rate-limiting under physiological conditions (39, 40). It would therefore have been promising to find safe food microorganisms carrying 7α- and 7β-HSDH activities, able to epimerize CDCA to UDCA in the small intestine, before CDCA was exposed to the indigenous 7α-dehydroxylating bacteria. Unfortunately, we could not find any *Lactobacillus*, *Bifidobacterium* or *Streptococcus* strain exhibiting these activities under our study conditions, whereas bile salt hydrolase activity is widespread among bifidobacteria and lactobacilli (41), and can operate during the transit of these probiotics in the intestine (42, 43).

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