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Biotransformation of Bile Acids by *Bacteroides* sp. Strain T-40 Isolated from Human Microflora

Yoshio Ogura, Tsuyoshi Takei, Takao Suzuki*, Nobuo Yamaga, Kikuji Itoh†, Kazuo Yamada and Kiyohisa Uchida

*Division of Medical Biochemistry, Department of Pathophysiological and Therapeutic Science, School of Medicine, Tottori University Faculty of Medicine and *Division of Functional Radiation Science, Research Center for BioScience and Technology, Tottori University, Yonago 683-8503, and †Laboratory of Veterinary Public Health, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657 Japan*

The effects of *Bacteroides* sp. strain T-40 isolated from human feces on the biotransformation of bile acids were examined in an anaerobic culture system. *Bacteroides* sp. T-40 oxidized cholic acid (CA) and chenodeoxycholic acid (CDCA) to 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid and 3α-hydroxy-7-oxo-5β-cholanoic acid, and reduced these oxo-bile acids to CA and CDCA, respectively. However, the reduction activities were lower than the oxidation activities. Hyocholic acid was dehydrogenated, but to a lesser extent than CA or CDCA. On the other hand, α-muricholic acid, which has a hydroxyl group at the position of 7α, was not dehydrogenated. Glycocholic acid was converted to free 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid but any glycine conjugated 7-oxo product was not detected. These data indicate that *Bacteroides* sp. T-40 possesses bile acid hydrolase and 7α-hydroxysteroid dehydrogenase, by which conjugated bile acids are initially deconjugated, and then undergo oxidization of the 7α-hydroxy group.

Key words: *Bacteroides;* bile acid; biotransformation; dehydrogenase; hydrolase

Ursodeoxycholic acid (UDCA), which is present in small amounts in human bile and intestine, is a useful therapeutic agent for dissolution of cholesterol gallstones (Sugata and Shimizu, 1974) and for treatment and prevention of hepatic diseases (Angulo et al., 1999). It is speculated that UDCA is generated from chenodeoxycholic acid (CDCA) through two-step reaction processes via 3α-hydroxy-7-oxo-5β-cholanoic acid (7=O-LCA) (by microbial biotransformation reactions) in human intestine as shown in Fig. 1. The first step, oxidation of the 7α-hydroxy group of CDCA, is carried out by 7α-hydroxysteroid dehydrogenase (7α-HSDH), and the second step, reduction of the 7-oxo group of 7=O-LCA, by 7β-hydroxysteroid dehydrogenase (7β-HSDH). In a previous paper, we reported the oxidation/reduction characteristics of 7α-hydroxyl bile acids by *Eschericha coli*, a facultative anaerobe, under aerobic and anaerobic conditions (Ogura et al., 2005). Meanwhile,

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; GLC, gas-liquid chromatography; G-βα, glycine-conjugated 7β,12α-dihydroxy-5β-cholanoic acid; HCA, hyocholic acid; HSDH, hydroxysteroid dehydrogenase; LCA, lithocholic acid; α-MCA, α-muricholic acid; β-MCA, β-muricholic acid; Me-DMES, methyl ester dimethylethylsilyl ether; MPYG, modified peptone yeast extract glucose; 7=O-DCA, 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (7-oxodeoxycholic acid); 7=O-LCA, 3α-hydroxy-7-oxo-5β-cholanoic acid (7-oxolithocholic acid); PHP GEL, piperidinohydroxypropyl dextran gel; T-αβ, taurine-conjugated 7α,12β-dihydroxy-5β-cholanoic acid; UDCA, ursodeoxycholic acid; ββ, 7β,12β-dihydroxy-5β-cholanoic acid

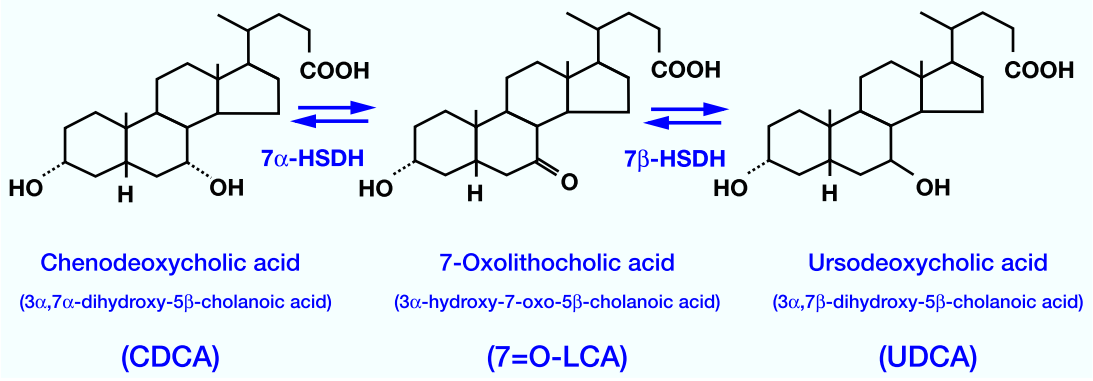

Fig. 1. Reaction sequences of chenodeoxycholic acid (CDCA), 3α-hydroxy-7-oxo-5β-cholanoic acid (7=O-LCA) and ursodeoxycholic acid (UDCA). 7α-HSDH, 7α-hydroxysteroid dehydrogenase; 7β-HSDH, 7β-hydroxysteroid dehydrogenase.

one strain of *Bacteroides* (*Bacteroides* sp. strain T-40) was isolated from healthy human feces, in which a relatively high amount of UDCA was detected, and this bacterium was found to have the highest 7α-HSDH activity among bacteria isolated from the feces. In the present experiments, we examined the effect of *Bacteroides* sp. T-40, an obligate anaerobe, on bile acid biotransformation (the first step for production of UDCA) in an anaerobic culture system.

Materials and Methods

Chemicals

CA, CDCA, hyocholic acid (HCA), and glycocholic acid (GCA) were purchased from Sigma Chemical, St. Louis, MO. α-Muricholic acid (α-MCA) and β-muricholic acid (β-MCA) were synthesized according to the methods reported by Iida et al. (1989). 3α,12α-Dihydroxy-7-oxo-5β-cholanoic acid (7=O-DCA) and 7=O-LCA were prepared by the oxidation of CA and CDCA with *N*-bromosuccinimide (Fieser and Rajagopalan, 1949), respectively. 7β,12β-Dihydroxy-5β-cholanoic acid (ββ), glycine-conjugated 7β,12α-dihydroxy-5β-cholanoic acid (G-βα), and taurine-conjugated 7α,12β-dihydroxy-5β-cholanoic acid (T-αβ) were synthesized as described previously (Arimoto et al., 1982; Yamaga et al., 1987), and used as internal standards for analysis of bile acids by capillary gas-liquid chromatography (GLC). These bile acids were estimated to be more than 96% pure so far examined by capillary GLC. Dimethylethylimidazole was purchased from Tokyo Kasei Kogyo, Tokyo, Japan. Piperidinohydroxypropyl dextran gel (PHP GEL) was purchased from Shimadzu, Kyoto, Japan. The other reagents and solvents of analytical grade were obtained from Wako Pure Chemical Industries, Osaka, Japan. If not otherwise stated, the solvents were distilled once before use.

Bacteroiological procedures

Eighty-four strains (*E. coli*: 4, *Enterococcus*: 12, Bacteroidaceae 38, *Bifidobacterium*: 8, *Eubacterium*: 5, *Clostridium*: 1 and unidentified anaerobes: 6) were isolated from a human fecal sample using 3 non-selective and 7 selective agar plates (Narushima et al., 2006) and characterized on bases of colony form, Gram stain, morphology, growth in air and spore formation (Mitsuoka et al., 1965). Each strain was cultured anaerobically for 3 days in a modified peptone yeast extract glucose (MPYG) medium (Hirano et al., 1981; Takahashi and Morotomi, 1994) containing CA and the bile acids transformed from CA were examined by thin-layer chromatography (Van den

Ende et al., 1982) and GLC (Ogura et al., 2003). The colony (*Bacteroides* sp. T-40) which showed the highest production of 7=O-DCA was selected for the present experiment. Strain T-40 was finally identified by approximately 1,500 bp of the 16S rDNA gene according to methods published previously (Miyamoto and Itoh, 2000). Although this sequence suggested strong similarity for the species in *Bacteroides* cluster, the sequence homology between strain T-40 and type strains in the *Bacteroides* cluster was less than 95%. *E. coli* K-12 which possesses 7α-HSDH (Ogura et al., 2003) was obtained from the American Type Culture Collection, Manassas, VA. Both bacteria, the strain T-40 and *E. coli* K-12, were precultured anaerobically in MPYG medium (Hirano et al., 1981; Takahashi and Morotomi, 1994) at 37˚C for 1 day by the gas-pack method (BBL Gas Pak Anaerobic System; Becton Dikinson, Sparks, MD). Aliquots of the growth of the strain T-40 (8×10^7/10 μL) and K-12 (3×10^7/10 μL) were added to 10 mL of MPYG medium containing 0.5 mM bile acid and cultured anaerobically at 37˚C for 4 days.

Analytical methods

Bile acids were extracted from the medium as previously described (Ogura et al., 2003). A portion of the cultured medium, 100 μL, was treated with 8 volumes of ethanol by warming for 10 min and then filtered. The filtrate was evaporated to dryness under a stream of nitrogen and the residue was hydrolyzed in alkaline solution. After hydrolysis, the reaction mixture was acidified with diluted hydrochloric acid and bile acids were extracted with diethylether. In the PHP GEL analysis, prior to the analysis of bile acids, ββ, G-βα and T-αβ as internal standards were added to 100 μL of the culture medium. The analytical sample with the three internal standards was treated with 8 volumes of ethanol by warming for 10 min and then filtered. The filtrate was evaporated to dryness under a stream of nitrogen and the residue was subjected to PHP GEL column chromatography to separate bile acids into free, glycine-conjugate and taurine-conjugate fractions (Yamaga et al., 1987). Glycine-conjugate fraction was hydrolyzed in alkaline solution (Yamaga et al., 1997). The hydrolysate and free fraction were acidified with diluted hydrochloric acid, and the bile acids were extracted with diethylether. The extracted bile acids were converted into methyl ester dimethylethylsilyl ether (Me-DMES) derivatives as described previously (Yamaga et al., 1987, 1996). The methyl ester of 7=O-LCA was converted to its methoxime derivative as described by Horning et al. (1968), to separately determine this compound and CDCA by GLC.

Capillary GLC

An aliquot of the bile acid derivatives dissolved in n-hexane was injected into a gas chromatograph (Model G14A; Shimadzu) equipped with a flame ionization detector, a solventless injector, and a computerized data system (Model C-R4A; Shimadzu). A Hicap CBP-1 capillary column (25 m × 0.25 mm I.D.; Shimadzu) was used. The column temperature was maintained at 285˚C and helium was used as the carrier gas.

Experiments with cell-free enzyme preparation

Crude 7α-HSDH was prepared from *Bacteroides* sp. T-40 according to the method of Macdonald et al. (1973). The enzyme activity was assayed for bile acid transformation by a slight modification of the method reported by Macdonald et al. (1974, 1975). The reaction was performed at 37˚C for 10 min with 7 mM NAD^+, 0.2 M glycine-NaOH buffer (pH 9.5), 0–4 mM bile acid and 45 μL of the crude enzyme preparation in final volume of 1 mL. One unit of enzyme activity of 7α-dehydrogenation was defined as the amount of enzyme required to yield 1 nmole of NADH per min under the conditions described above.

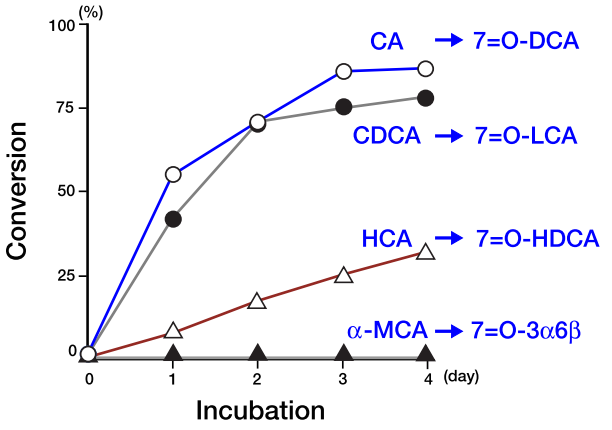

Fig. 2. Dehydrogenation of cholic acid (CA), chenodeoxycholic acid (CDCA), hyocholic acid (HCA) and α-muricholic acid (α-MCA) by *Bacteroides* sp. T-40 in anaerobic culture system. The strain was precultured anaerobically in modified peptone yeast extract glucose (MPYG) medium at 37˚C for 1 day by use of the gas-pack method. The aliquots (10 μL) was added to 10 mL of MPYG medium containing 0.5 mM bile acid and cultured at 37˚C for 4 days. Bile acids were extracted from the medium, and analyzed by capillary gas liquid chromatography. 7=O-DCA, 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid; 7=O-LCA, 3α-hydroxy-7-oxo-5β-cholanoic acid; 7=O-HDCA, 3α,6α-dihydroxy-7-oxo-5β-cholanoic acid; 7=O-3α6β, 3α,6β-dihydroxy-7-oxo-5β-cholanoic acid.

Results

The time course of changes in the 7α-dehydrogenation of cholic acid (CA), chenodeoxycholic acid (CDCA), hyocholic acid (HCA) and α-muricholic acid (α-MCA) by *Bacteroides* sp. T-40 under anaerobic conditions is shown in Fig. 2. CA and CDCA were rapidly oxidized to 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (7=O-DCA) and 3α-hydroxy-7-oxo-5β-cholanoic acid (7=O-LCA), about 80% being transformed in 4 days. In the present experiments, CA was oxidized mostly to 7=O-DCA and little or no further transformations were detected so far examined by GLC. HCA was 7α-dehydrogenated, but to a lesser extent than CA or CDCA. α-MCA has a 7α-hydroxyl group but not 7α-dehydrogenated. On the other hand, β-muricholic acid (β-MCA) and ursodeoxycholic acid (UDCA), possessing a 7β-hydroxy group, were not changed (data not shown). In addition, CA and CDCA were not transformed to deoxy-

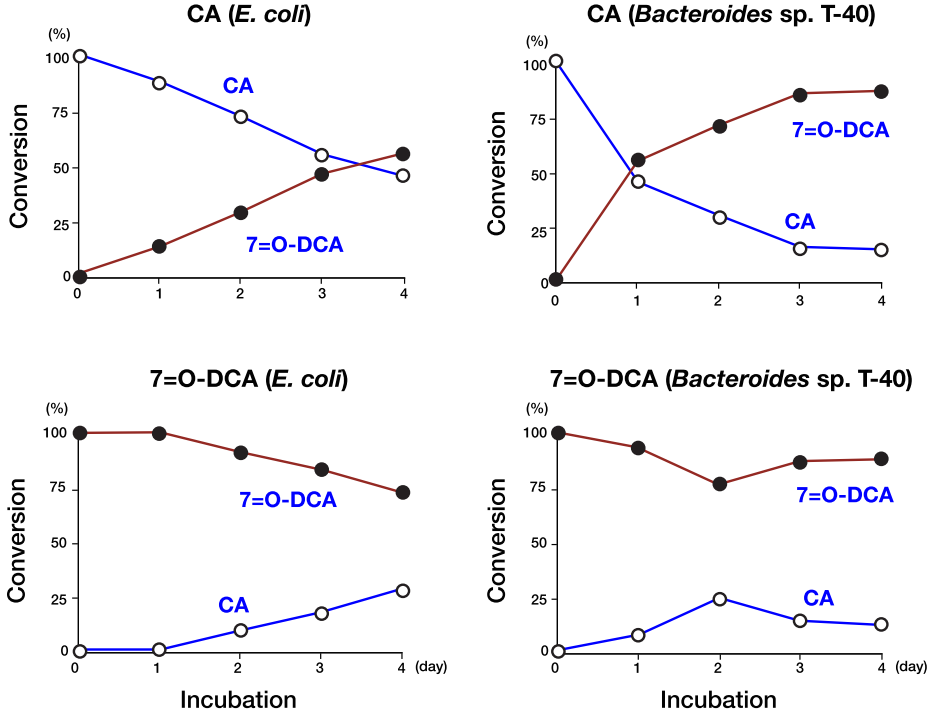

Fig. 3. Oxidation of cholic acid (CA) and reduction of 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (7=O-DCA) by *Bacteroides* sp. T-40 and *E. coli* K-12. The two strains were cultured in anaerobic culture system as described in Fig.2. Bile acids were extracted from the medium, and analyzed by capillary gas-liquid chromatography.

cholic acid (DCA) and lithocholic acid (LCA) in anaerobic culture.

The transforming activities of *Bacteroides* sp. T-40 and *E. coli* K-12 are given in Fig. 3 which shows the time course of changes in the 7α-dehydrogenation of CA, and in the reverse reaction, reduction of 7=O-DCA to CA. Although *E. coli* K-12 oxidized CA to 7=O-DCA about 50% by 4 days, the strain T-40 showed a much higher activity than K-12, oxidizing about 50% in 1 day and about 80% in 4 days. The reduction of 7=O-DCA to CA by these bacteria was slower than the oxidation of CA to 7=O-DCA.

Figure 4 shows the conversion of glycocholic acid (GCA) by the strain T-40. In the present experiment, the samples were hydrolyzed at first and then analyzed by GLC. About 30% of GCA was converted to 7=O-DCA in 4 days.

It was not clear from the data of Fig. 4 whether GCA was oxidized to 7=O-DCA with or without deconjugation. Therefore, the medium cultured with GCA for 4 days was fractionated to taurine-conjugated, glycine-conjugated and

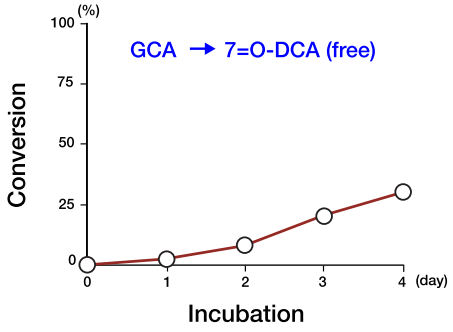

Fig. 4. Conversion of glycocholic acid (GCA) to 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (7=O-DCA) by *Bacteroides* sp. T-40. The strain was cultured in anaerobic culture system as described in Fig. 2. Bile acids were extracted from the medium without the fractionation by a piperidinohydroxypropyl dextran gel (PHP GEL) column, hydrolyzed and analyzed by capillary gas-liquid chromatography.

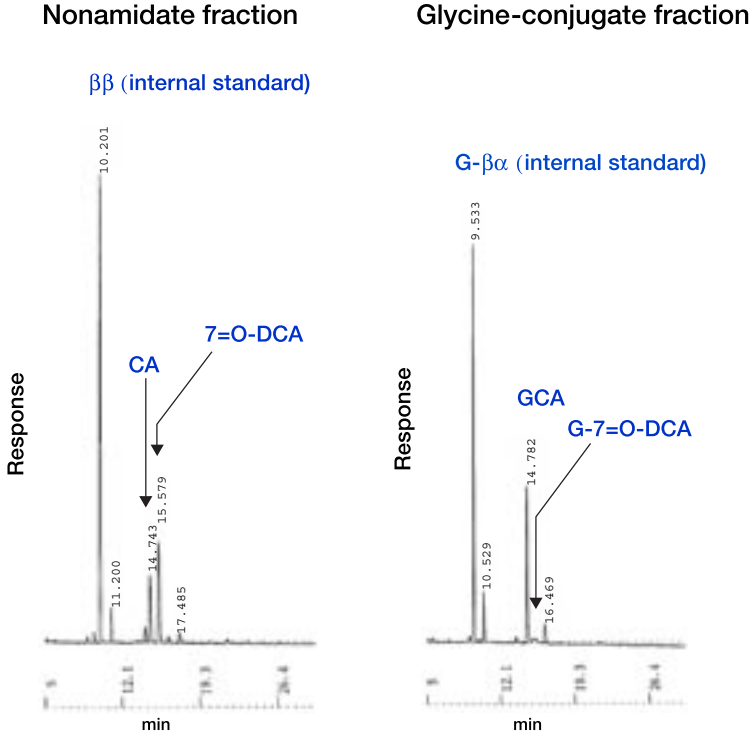

Fig. 5. Gas-liquid chromatograms of bile acids in the nonamidate and glycine-conjugate fractions after a piperidinohydroxypropyl dextran gel (PHP GEL) column. The medium cultured with 0.5 mM glycocholic acid (GCA) for 4 days was added three internal standard compounds, 7β,12β-dihydroxy-5β-cholanoic acid (ββ), glycine-conjugated 7β,12α-dihydroxy-5β-cholanoic acid (G-βα) and taurine-conjugated 7α,12β-dihydroxy-5β-cholanoic acid (T-αβ), and then fractionated by PHP GEL column. Each fraction was hydrolyzed and the bile acids were converted into ME-DMES derivatives. βα, 7β,12α-dihydroxy-5β-cholanoic acid; CA, cholic acid; 7=O-DCA, 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid.

nonamidate bile acid fractions by PHP GEL column chromatography. After that, each fraction was hydrolyzed and subjected to GLC analysis. As shown in Fig. 5, 7β,12α-dihydroxy-5β-cholanoic acid (βα) and CA were detected only in the glycine-conjugated fraction but 7=O-DCA was detected only in the nonamidate bile acid fractions. The bile acids were detected as free bile acids after hydrolysis, but these bile acids found in the glycine-conjugated fraction are shown as G-βα and GCA in Fig. 5.

Subsequently, substrate specificity of the cell-free enzyme preparation of the strain T-40 was examined. CA and CDCA showed the highest responses, GCA showed about a 70% response and α-MCA, though this bile acid possesses a 7α-hydroxyl group, showed no response (Table 1).

Table 1. Dehydrogenation of bile acids in vitro by crude 7α-hydroxysteroid dehydrogenase (7α-HSDH) prepared from *Bacteroides* sp. T-40

Bile acids	7α-HSDH activity (units/mg protein)
CA	107
GCA	73
CDCA	107
HCA	27
α-MCA	0

The reaction was performed at 37˚C for 10 min with 7 mM NAD^+, 0.2 M glycine-NaOH buffer (pH 9.5), 4 mM bile acid and 45 mL of the crude enzyme preparation in final volume of 1 mL. One unit of enzyme 7α-dehydrogenation activity was defined as the amount of enzyme required to yield 1 nmole of NADH per min under the conditions described above.

CA, 3α,7α,12α-trihydroxy-5β-cholanoic acid; GCA, glycine-conjugated 3α,7α,12α-trihydroxy-5β-cholanoic acid; CDCA, 3α,7α-dihydroxy-5β-cholanoic acid; HCA, 3α,6α,7α-trihydroxy-5β-cholanoic acid; α-MCA, 3α,6β,7α-trihydroxy-5β-cholanoic acid.

Discussion

The major conversions of bile acids by intestinal bacteria include deconjugation of taurine- or glycine-conjugated bile acids, oxidation of hydroxyl groups at C-3, C-7 and C-12, and dehydroxylation of 7α- or 7β-hydroxyl group. In the present study, we examined the characteristics of bile acid conversion by *Bacteroides* sp. T-40 that possesses high 7α-hydrogenation activity.

7α-HSDH is a widely distributed enzyme among several types of bacteria, and has been partially purified from intestinal bacteria including *Bacteroides fragile* (Hylemon and Sherroed, 1975) and *E. coil* (Aries and Hill, 1970). Macdonald and Roach (1981) have reported that *Clostridium absonus* has both 7α- and 7β-HSDH activities. 7α-HSDH is a non-inducible intracellular enzyme with substrate specificity for bile acids (both free and conjugated) having a 7α-hydroxyl group (Haselwood and Haslewood, 1976). In a previous paper (Ogura et al., 2005), we reported that *E. coli* K-12, a facultative anaerobe, oxidized CA and CDCA to the corresponding 7-oxo-bile acids, which in turn were reduced to CA and CDCA, respectively.

In the present experiments, the rate of CA conversion by *Bacteroides* sp. T-40 was found to be higher than that by *E. coli* K-12 in anaerobic culture, but α-MCA, which has a 7α-hydroxyl group, was not dehydrogenated. UDCA and β-muricholic acid (β-MCA), both have a 7β-hydroxyl group, could not be substrates for this reaction under the same conditions. In addition, the strain T-40 was unable to convert CA to deoxycholic acid (DCA) and CDCA to lithocholic acid (LCA). These data indicate that *Bacteroides* sp. T-40 has 7α-dehydrogenation activity, but has neither 7β-dehydrogenation nor 7α/β-dehydroxylation activity.

The substrate specificity of 7α-HSDH of the strain T-40 was examined using the cell-free crude enzyme preparation. CA, CDCA, GCA and HCA were 7α-dehyrogenated in the order of CA = CDCA > GCA > HCA, but α-MCA was not. This nature was the same as that obtained with *E. coli* K-12 (Ogura et al., 2005), suggesting that 6α-substitution of the CDCA molecule with a hydroxyl group decreases the activity, and 6β-substitution completely blocks the activity.

7α-Dehydroxylation of bile acids by intestinal bacteria is considered to take place after deconjugation (Aries and Hill, 1970). When GCA was cultured with the strain T-40, 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (7=O-DCA) was not detected in the glycine-conjugated fraction, but was detected in the nonamidate fraction. These data indicate that GCA is deconjugated at first and then dehydrogenated, suggesting that the strain T-40 possesses both enzymes, hydrolase and 7α-dehydrogenase.

In conclusion, our results have shown that *Bacteroides* sp. T-40 possesses both deconjugation activity and 7α-dehydrogenation activity for bile acids having a 7α-hydroxy group except for α-MCA, but possesses neither 7β-dehydrogenation nor 7α/β-dehydroxylation activity. These findings indicate that *Bacteroides* sp. T-40 will be a promising probiotic bacterium for production of UDCA.

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Corresponding author: Yoshio Ogura, PhD