Contribution of the 7β-hydroxysteroid dehydrogenase from *Ruminococcus gnavus* N53 to ursodeoxycholic acid formation in the human colon[S]

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Abstract Bile acid composition in the colon is determined by bile acid flow in the intestines, the population of bile acid-converting bacteria, and the properties of the responsible bacterial enzymes. Ursodeoxycholic acid (UDCA) is regarded as a chemopreventive beneficial bile acid due to its low hydrophobicity. However, it is a minor constituent of human bile acids. Here, we characterized an UDCA-producing bacterium, N53, isolated from human feces. 16S rDNA sequence analysis identified this isolate as *Ruminococcus gnavus*, a novel UDCA-producer. The forward reaction that produces UDCA from 7-oxo-lithocholic acid was observed to have a growth-dependent conversion rate of 90–100% after culture in GAM broth containing 1 mM 7-oxo-lithocholic acid, while the reverse reaction was undetectable. The gene encoding 7β-hydroxysteroid dehydrogenase (7β-HSDH), which facilitates the UDCA-producing reaction, was cloned and overexpressed in *Escherichia coli*. Characterization of the purified 7β-HSDH revealed that the k_{cat}/K_m value was about 55-fold higher for the forward reaction than for the reverse reaction, indicating that the enzyme favors the UDCA-producing reaction. As *R. gnavus* is a common, core bacterium of the human gut microbiota, these results suggest that this bacterium plays a pivotal role in UDCA formation in the colon.—Lee, J-Y., H. Arai, Y. Nakamura, S. Fukiya, M. Wada, and A. Yokota. **Contribution of the 7β-hydroxysteroid dehydrogenase from *Ruminococcus gnavus* N53 to ursodeoxycholic acid formation in the human colon. *J. Lipid Res.* 2013.** 54: **3062–3069.**

Supplementary key words secondary bile acids • bile acid conversion • epimerization • intestinal bacteria

The dynamic balance of bile acid composition in the colon is influenced by bile acid flow in the intestines, the population of bile acid-converting bacteria, and the properties of the responsible bacterial enzymes (1–3). However, our knowledge is still far from being able to predict changes in the dynamic balance because we have a limited catalog of bile acid-converting bacteria and little information about the enzymes involved in bile acid conversion.

Primary bile acids are synthesized from cholesterol in the liver and secreted into the duodenum as the main component of bile (2–4). While bile acids contribute to the emulsification, digestion, and absorption of dietary lipids, some secondary bile acids formed from the primary bile acids by bacterial biotransformation (1–4); [e.g., deoxycholic acid (DCA; 3α, 12α-dihydroxy-5β-cholan-24-oic acid) from cholic acid (CA; 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid)] are regarded as carcinogens due to their DNA-damaging effects and induction of apoptosis (5–9). In contrast, hydrophilic ursodeoxycholic acid (UDCA; 3α, 7β-dihydroxy-5β-cholan-24-oic acid) is regarded as a chemopreventive bile acid (9, 10), because it protects hepatocytes and bile duct epithelial cells against necrosis and apoptosis induced by more hydrophobic secondary bile acids (11–13) (e.g., DCA). Another characteristic of bile acids is their strong antimicrobial activity (2, 14). Recently, we showed that bile acid regulates the rat cecal microbiota composition, as feeding CA to rats resulted in a shift in the relative abundance of two major gut microbiota phyla; such feeding increased Firmicutes and decreased Bacteroidetes (15). Interestingly, this is similar to that reported in the fecal microbiota of a mouse model fed a high-fat diet, and such imbalanced bacterial populations have been argued to trigger the development of metabolic syndrome (16–23). As a high-fat diet enhances bile secretion to facilitate lipid digestion (3), bile acid may be responsible for the alteration in the gut microbiota composition in response to a high-fat diet. Thus, bile acid is likely involved in development of metabolic syndrome by altering the gut microbiota composition during high-fat diet intake. Gaining an understanding of the regulatory mechanism(s) of the bile acid pool through characterization

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Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; HSDH, hydroxysteroid dehydrogenase; LCA, lithocholic acid; 7oxo-LCA, 7oxo-lithocholic acid; RT, retention time; SDR, short-chain dehydrogenase/reductase superfamily; UDCA, ursodeoxycholic acid.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two tables.

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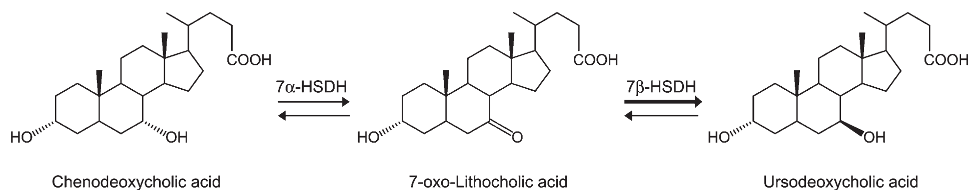

Fig. 1. Epimerization reaction from CDCA to UDCA, which is catalyzed by the 7α-HSDH and 7β-HSDH enzymes. The 7β-HSDH from *R. gnavus* N53 preferentially catalyzes the UDCA-forming reaction (reductive reaction) as compared with the 7-oxo-LCA-forming reaction (oxidative reaction), which is denoted by the thickness of the arrow lines.

of the bacteria involved in bile acid conversion is important for host health.

Accordingly, we focused on UDCA, which is a beneficial secondary bile acid, although it represents less than 2% of total biliary and fecal bile acids in humans (1, 12). UDCA is produced from chenodeoxycholic acid (CDCA; 3α, 7α-dihydroxy-5β-cholan-24-oic acid) by successive reactions catalyzed by 7α- and 7β-hydroxysteroid dehydrogenases (HSDH) of intestinal bacteria (24–26), with 7-oxo-lithocholic acid (7-oxo-LCA; 3α-hydroxy-7oxo-5β-cholan-24-oic acid) as an intermediate (27) (**Fig. 1**). In the present study, we screened and identified a novel UDCA-producing bacterium, *Ruminococcus gnavus* N53, from human feces. As *R. gnavus* has been identified as a common core species of the human intestinal microbiota (28), it is important to investigate UDCA production by this bacterium to understand formation of the UDCA pool in the human colon. Thus, we investigated the bile acid conversion reaction mediated by this bacterium and conducted a functional characterization of recombinant 7β-HSDH.

MATERIALS AND METHODS

Bacterial strains, culture media, and culture conditions

UDCA-producing *R. gnavus* N53 was isolated from human feces and characterized in our laboratory. *R. gnavus* ATCC 29149^T, a reference strain, and *Collinsella aerofaciens* ATCC 25986^T, another UDCA-producing bacterium (29, 30), were obtained from the Japan Collection of Microorganisms (JCM; Tsukuba, Ibaraki, Japan). The strains were grown at 37°C in Gifu anaerobic medium broth (GAM broth; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2) in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI). When necessary, 15 g/l agar was added to solidify the medium. The media were kept in the anaerobic chamber at least for 24 h after autoclaving and before use. Luria-Bertani (LB) medium (plate/broth) and M9 liquid medium containing ampicillin (100 μg/ml) were used to culture *Escherichia coli* JM109 transformants. Strains were cultured aerobically at 37°C unless otherwise specified.

Chemicals

UDCA was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Sodium UDCA and 7-oxo-LCA were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Sodium 7-oxo-LCA was prepared by neutralizing free 7-oxo-LCA with NaOH using a method described previously (31). Sodium 7-oxo-LCA was used as the substrate for measuring enzyme activity because it dissolves easily in water, while free 7-oxo-LCA does not.

Analysis of bile acids

Bile acids in culture broths or enzyme reaction mixtures were characterized either by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), or gas chromatography-mass spectrometry (GC-MS), as described previously (32). Methods for the extraction of bile acids and sample preparation were as described by Fukiya et al. (32).

Screening and identification of UDCA-producing bacteria from human feces

UDCA-producing bacteria were screened using methods described previously (32). Fecal samples were provided by a healthy Japanese adult male. Samples were homogenized, diluted, and plated on 1/4 GAM agar medium. Colonies on the plates were picked and cultured in 200 μl GAM broth containing either 0.1 mM 7-oxo-LCA or UDCA (both as free acids) in 96-well microtiter plates for 48 h. Detection of bile acid conversion in the culture broths was carried out by TLC. Culture samples showing corresponding spots with either 7-oxo-LCA or UDCA by TLC were further analyzed by GC-MS. Identification of the candidate strain by 16S rDNA sequencing and its biochemical characterization using the API 20A and Rapid ID 32A kits (bioMérieux SA, Marcy-l'Etoile, France) were performed as described previously (32).

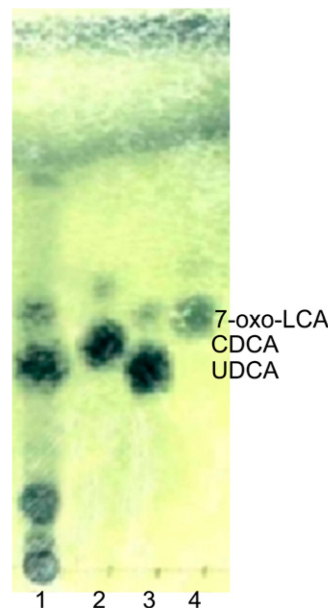

Fig. 2. TLC analysis of biotransformation by *R. gnavus* N53. The bacterial strain was cultured anaerobically in GAM broth with 0.1 mM 7-oxo-LCA for 48 h (lane 1). Lanes 2, 3, and 4 are authentic, CDCA, UDCA, and 7-oxo-LCA, respectively.

Conversion of 7-oxo-LCA into UDCA by *R. gnavus* N53 and other bacteria in GAM broth

R. gnavus N53 and its type strain ATCC 29149^T, as well as *C. aerofaciens* ATCC 25986^T, another UDCA-producing bacterium (29, 30), were precultured in 5 ml GAM broth in screw-capped vials at 37°C in an anaerobic chamber until stationary phase. The cultures were then inoculated into 50 ml GAM broth in 200 ml screw-cap bottles supplemented with 1 mM 7-oxo-LCA at an initial optical density at 660 nm (OD_{660}) of 0.01 and cultured under the same conditions. During culture, 5 ml of the culture broth was withdrawn periodically. Growth was measured at OD_{660} using a photometer (MiniPhoto518R; TAITEC Corp., Koshigaya, Saitama, Japan), and bile acid concentration was determined by HPLC.

Measurement of 7β-HSDH activity

Cells were harvested by centrifugation at 7,000 *g* for 10 min. Pellets (0.2 g wet cells) were resuspended in 1 ml extraction buffer containing 0.1 M Tris, 3 mM EDTA, 1 mM dithiothreitol (DTT; Wako Pure Chemical Industries Ltd., Osaka, Japan), and 10% (v/v) glycerol (adjusted to pH 8.0 with HCl). Cell suspensions were disrupted under cooling conditions by sonication with 1 min pulses, followed by 1 min breaks repeatedly for 15 min using a Bioruptor UCD-200T (COSMO BIO Co. Ltd., Tokyo, Japan). Cell debris was removed by centrifugation (46,000 *g*, 4°C) for 40 min, and the supernatant was used as the crude enzyme solution. The assay mixture for the UDCA-forming reaction (forward reaction) contained, in a total volume of 1 ml, 10 mM Tris-HCl buffer (pH 6.0), 250 μM NADPH, 1 mM sodium 7-oxo-LCA, and 10 μl enzyme solution, while that for the 7-oxo-LCA-forming reaction (reverse reaction) contained 10 mM glycine-NaOH buffer (pH 10.0), 1 mM $NADP^+$, 2 mM sodium UDCA, and 10 μl enzyme solution. The reactions were carried out at 37°C with addition of the substrate. Reaction mixtures without the substrate served as controls. The 7β-HSDH activity was determined by the change in NADP(H) concentration by monitoring absorbance at 340 nm with a spectrophotometer (DU800; Beckman Coulter Inc., Brea, CA). Enzyme activity was calculated using a molar extinction coefficient of 6.22 $mM^{-1} \times cm^{-1}$ for NADPH. The protein concentration was determined by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard. Enzyme activity was expressed as micromoles of NADPH oxidized or $NADP^+$ reduced per minute per milligram of protein.

Cloning of the gene encoding 7β-HSDH

Total genomic DNA was extracted from both *R. gnavus* N53 and ATCC 29149^T using ISOPLANT II (Nippon Gene Co. Ltd., Tokyo, Japan) and used as the template for PCR amplification. The gene putatively encoding 7β-HSDH (hypothetical protein RUMGNA_02585; accession number, ZP_02041813; GenBank database, <www.ncbi.nlm.nih.gov/Genbank>) was amplified from the genomic DNA of *R. gnavus* strains by PCR using synthetic primers: forward (5′-ttaaGCATGCATGACATTGAGAGAAAAATA-3′) and reverse (5′-taatCTGCAGTTATTCTTGATAGAAAGATC-3′). The underlined bases denote the *Sph*I and *Pst*I restriction sites, respectively. The PCR conditions were as follows: initial 2 min denaturation at 94°C followed by 30 cycles of amplification at 98°C for 10 s, 50°C for 30 s, and 68°C for 1 min, and then elongation at 68°C for 7 min. The PCR product from N53 was further purified using the MinElute PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). The PCR product and vector pQE30 (ampicillin resistance; QIAGEN) containing an N-terminal six-His tag were double-digested with *Sph*I and *Pst*I, and then purified using the MinElute Reaction Cleanup Kit (QIAGEN). The digested pQE30 was dephosphorylated and ligated with the PCR product using Ligation high Ver.2 (Toyobo Co. Ltd., Osaka, Japan). The plasmids were transformed into *E. coli* JM109 and were purified from colonies on LB plates containing ampicillin. The insert DNA was sequenced using standard primers. The resulting plasmid was named pQE30-7β-HSDH and was also used for the expression of 7β-HSDH for enzyme purification.

Purification of recombinant 7β-HSDH from *R. gnavus* N53

E. coli JM109 containing pQE30-7β-HSDH was precultured overnight in 5 ml LB broth containing ampicillin. The preculture was inoculated into 50 ml M9 medium containing ampicillin at an initial OD_{660} of 0.01. 7β-HSDH gene expression was induced by adding isopropyl β-D-Thiogalactoside at a final concentration of 0.1 mM to exponentially growing cells at an OD_{660} of 0.4, at which time the culture temperature was shifted from 37°C to 25°C. After 22 h of culture at 25°C, the cells were collected by centrifugation at 7,000 *g* for 10 min at 4°C. Cell-free extract for

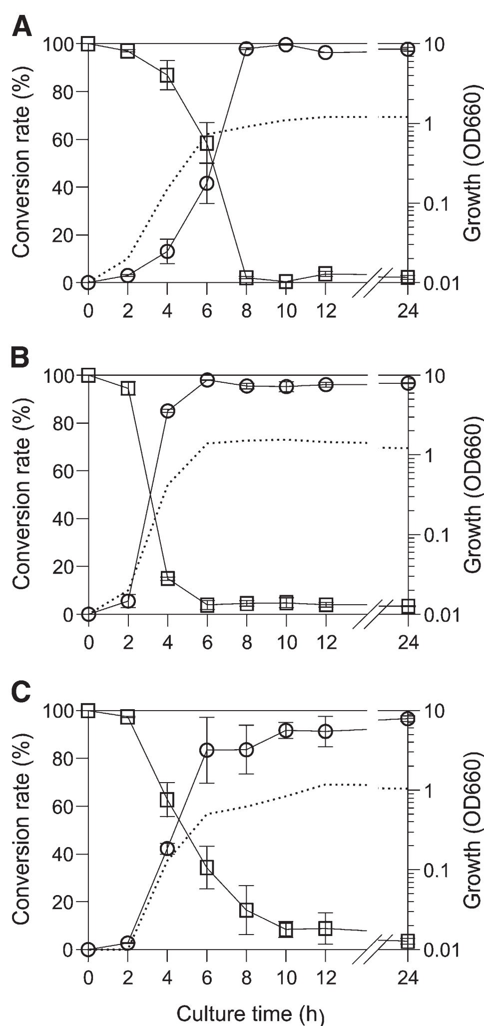

Fig. 3. Time course of UDCA formation from 7-oxo-LCA by three bacterial strains: (A) *R. gnavus* N53, (B) *C. aerofaciens* ATCC 25986^T, and (C) *R. gnavus* ATCC 29149^T. Bacteria were cultured anaerobically in GAM broth supplemented with 1 mM 7-oxo-LCA. Data are the means of at least three independent experiments. Bars represent the means ± SD. Dashed line, growth expressed as optical density at 660 nm (OD_{660}); open square, residual 7-oxo-LCA in the culture broth; open circle, UDCA in the culture broth formed from 7-oxo-LCA.

7β-HSDH purification was prepared using the method described above. The cell-free extract, after membrane filtration (Minisart, 0.20 μm; Sartorius Stedim Biotech GmbH, Goettingen, Germany), was applied to a TALON Single Step Column (Clontech Laboratories Inc., Mountain View, CA) equilibrated with equilibration/wash buffer (50 mM sodium phosphate buffer at pH 8.0, 300 mM NaCl). Nonabsorbed materials were removed by washing the column with two column bed volumes of equilibration/wash buffer. Weakly bound proteins were removed by washing with two column bed volumes of wash-2 buffer (50 mM sodium phosphate buffer at pH 8.0, 300 mM NaCl, 7.5 mM imidazole). His-tagged 7β-HSDH protein was eluted with 1 ml elution buffer (50 mM sodium phosphate buffer at pH 8.0, 300 mM NaCl, 150 mM imidazole). The eluate was dialyzed in a dialysis tube (TOR-3K; Nippon Genetics Co. Ltd., Tokyo, Japan) with a molecular weight cutoff of 3.5 kDa against 1 l extraction buffer containing 0.1 M Tris, 3 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol (adjusted to pH 8.0 with HCl). The solution was then concentrated by Microcon (YM-10; Millipore, Billerica, MA), resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by staining with Coomassie Brilliant Blue R-250 (Wako Pure Chemical Industries) followed by destaining in methanol:acetic acid:water (40:7:53).

Amino acid sequence analysis

Partial N-terminal amino acid sequencing was performed. The purified enzyme was subjected to SDS-PAGE and electrically transferred to a polyvinylidene difluoride membrane (Bio-Rad) using transfer buffer containing 10 mM CAPS and 10% methanol (adjusted to pH 11.0 with NaOH). Transferred protein was stained with 0.25% Coomassie Brilliant Blue R-250. The band was excised and sequenced by the Instrumental Analysis Division, Equipment Management Center, Creative Research Institution at Hokkaido University.

RESULTS

Screening and identification of UDCA-producing bacteria from a human fecal sample

As a result of screening for UDCA-producing bacteria from human feces, 1,482 colonies were isolated; these were evaluated for their ability to convert 7-oxo-LCA to UDCA by TLC. Consequently, we isolated strain N53, which gave a spot corresponding to UDCA by TLC after culture in GAM broth containing 7-oxo-LCA (**Fig. 2**). The bile acids extracted from the culture medium were characterized by GC-MS; their retention times (RT) corresponded to 7-oxo-LCA (RT, 12.2 min) and UDCA (RT, 12.6 min), respectively. The *m/z* values for the fragment ions corresponding to the conversion products (213, 255, 355, 370, and 460) were identical to authentic UDCA. 16S rDNA sequencing showed that the strain N53 was closely related to *R. gnavus* ATCC 29149^T (99.85% sequence homology). 7β-HSDH activity has been reported for several intestinal bacteria, such as *Collinsella aerofaciens* 25986^T (29, 30), *Ruminococcus productus* b-52 (29, 30, 33), *Ruminococcus* sp. PO1-3 (34), and *Clostridium baratii* (26), but not for *R. gnavus*. Biochemical tests of strain N53 and the *R. gnavus* type strain ATCC 29149^T with API 20 and Rapid API ID 32A showed a few differences in sugar assimilation (i.e., saccharose; supplementary Table I) and enzyme activity (α-fucosidase, leucine arylamidase, pyroglutamic acid arylamidase, and alanine arylamidase; supplementary Table II) between the two strains. From these phylogenetic and biochemical data, strain N53 was identified as *R. gnavus*. Thus, we identified the *R. gnavus* N53 isolate as a novel UDCA producer.

Conversion of 7-oxo-LCA to UDCA

To identify the conversion reaction catalyzed by 7β-HSDH, *R. gnavus* N53 was cultured in GAM broth supplemented with 7-oxo-LCA (**Fig. 3A**). The type strain ATCC 29149^T (Fig. 3C) and the known UDCA-producing bacterium *C. aerofaciens* ATCC 25986^T (Fig. 3B) were also cultured for comparison. The three bacterial strains nearly reached stationary phase 6 h after inoculation. All of these strains were found to convert 7-oxo-LCA to UDCA, and their conversion rates peaked (>90%) during stationary phase, suggesting that the reductive conversion reaction was growth-dependent. In contrast, when *R. gnavus* N53 was cultured in the presence of UDCA, 7-oxo-LCA was not detected (data not shown), indicating that the oxidative reaction with whole

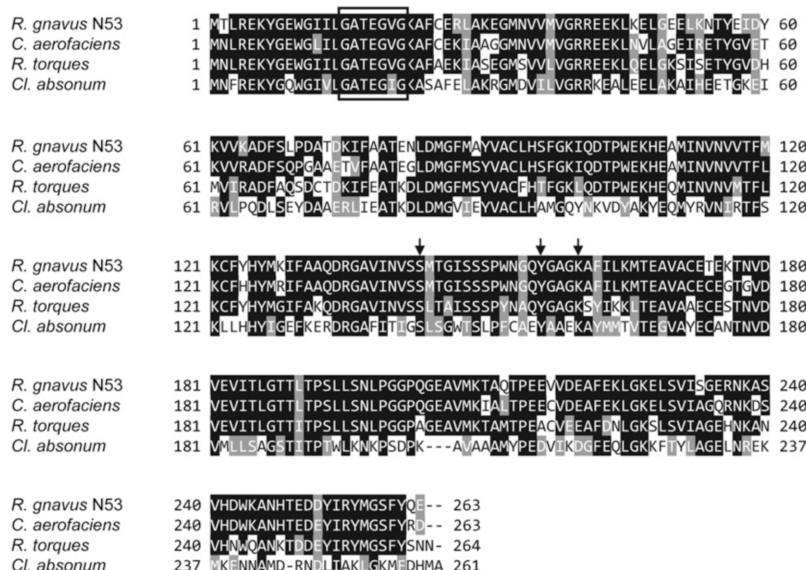

Fig. 4. Amino acid sequence alignment of 7β-HSDH from *R. gnavus* N53 and selected other HSDH proteins, including those from *R. gnavus* ATCC 29149^T (GenBank accession number, ZP_02041813; not shown as it has the identical amino acid sequence to that from *R. gnavus* N53), *C. aerofaciens* ATCC 25986^T (GenBank, ZP_01773061), a putative short-chain dehydrogenase from *R. torques* L2-14 (GenBank, CBL26204), and 7β-HSDH from *Cl. absonum* DSM 599^T (GenBank, AET80684). Residues inside the box indicate the N-terminal cofactor-binding site. Catalytic triad residues are indicated by arrows.

cells was unable to proceed. Measurement of the 7β-HSDH activity of the cells cultured for 12 h revealed that *R. gnavus* N53 showed the highest specific activity (0.023 μmol·min^{-1}·mg^{-1} protein), followed by *C. aerofaciens* ATCC 25986^T (0.013 μmol·min^{-1}·mg^{-1} protein), then *R. gnavus* ATCC 29149^T (0.007 μmol·min^{-1}·mg^{-1} protein). These results confirmed that *R. gnavus* is a novel UDCA producer that has a UDCA productivity comparable to *C. aerofaciens*.

Prediction of the 7β-HSDH gene in *R. gnavus* ATCC 29149^T genome

Several 7β-HSDH enzymes from intestinal bacteria have been purified and characterized. However, the gene encoding this enzyme has not been sequenced. Recently, the genes encoding NADPH-dependent 7β-HSDH enzymes were identified and cloned from an intestinal bacterium, *C. aerofaciens* ATCC 25986^T (35), and a soil bacterium, *Cl. absonum* DSM 599^T (36), by using genome information. HSDHs in general belong to the short-chain dehydrogenase/reductase superfamily (SDR) (37). From BLAST analysis using both sequence information of the 7β-HSDH gene from *C. aerofaciens* and an N-terminal cofactor-binding motif of SDR proteins, an open reading frame (ORF) named RUMGNA_02585 (formerly a hypothetical protein) in *R. gnavus* ATCC 29149^T, the whole genome of which has been sequenced, was predicted to encode the gene for 7β-HSDH. The ORF consisted of 789 nucleotides encoding 263 amino acids, including the N-terminal cofactor-binding motif (Gly-X-X-X-Gly-X-Gly) (38). Amino acid sequence alignment indicated high sequence identity of RUMGNA_02585 with 7β-HSDH COLAER_02088 from *C. aerofaciens* ATCC 25986^T (GenBank accession number, ZP_01773061; homology, 84%) and with short-chain dehydrogenases of various substrate specificities [e.g., CBL26204 from *R. torques* L2-14 (GenBank accession number, CBL26204; homology, 76%)], whereas relatively low homology with 7β-HSDH from *Cl. absonum* DSM 599^T was observed (GenBank accession number, AET80684; homology, 40%). Moreover, these proteins contain three amino acids, Ser-143, Tyr-156, and Lys-160, which are putative active site residues that may comprise the catalytic triad of SDRs (37) (**Fig. 4**).

Cloning, expression, and identification of the 7β-HSDH gene

To determine whether RUMGNA_02585 encodes 7β-HSDH, PCR amplification was conducted using both *R. gnavus* ATCC 29149^T and *R. gnavus* N53 genomic DNA as templates with primers designed for RUMGNA_02585 of

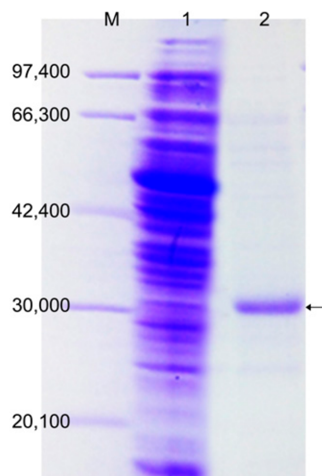

Fig. 5. SDS-PAGE. M, marker; Lane 1, crude enzyme; Lane 2, purified recombinant protein (indicated by the arrow). The marker standards were phosphorylase (97,400 Da), BSA (66,300 Da), aldolase (42,400 Da), carbonic anhydrase (30,000 Da), and trypsin inhibitor (20,100 Da).

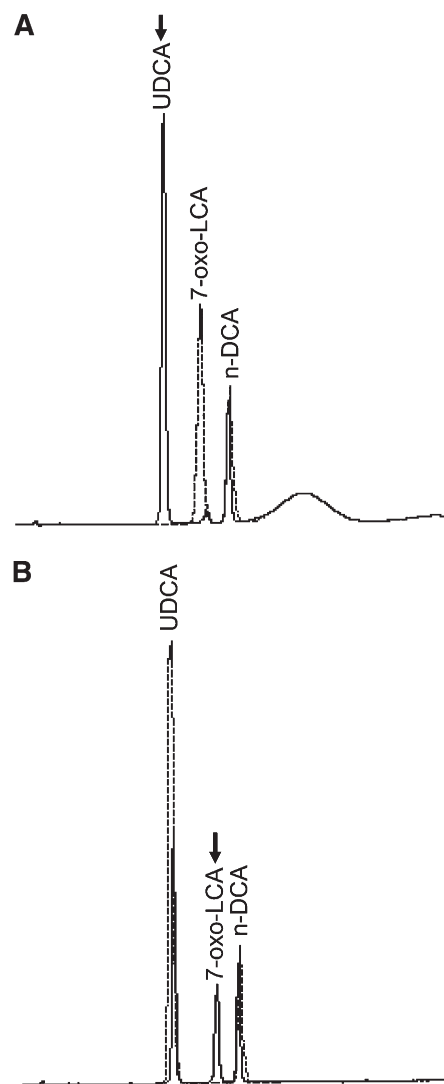

Fig. 6. Reversibility of the 7β-HSDH reaction as assessed using purified enzyme. HPLC chromatogram of the mixture for the (A) reductive reaction and (B) oxidative reaction. The reductive reaction mixture contained 200 μM sodium 7-oxo-LCA, 400 μM NADPH, 50 μl (0.6 μmol·min^{-1}·ml^{-1}) enzyme, and 100 mM MES (pH 6.0) in a final volume of 0.5 ml. The oxidative reaction mixture contained 1 mM sodium UDCA, 1 mM $NADP^+$, 50 μl enzyme, and 100 mM glycine-NaOH (pH 10.0). Reaction mixtures without the enzyme served as controls. All reaction mixtures, after incubation at 37°C for 1 h, were analyzed by HPLC as described previously (32). Solid line, reaction in the presence of 7β-HSDH; dashed line, control reaction mixture without 7β-HSDH. Arrows indicate the conversion product of each reaction.

TABLE 1. Kinetic parameters of 7β-HSDHs purified from UDCA-producing bacteria

Strains	Substrate	K_m (μM)	V_{max} (μmol·min^{-1}·mg^{-1})	k_{cat} (μmol·μmol^{-1}·min^{-1})	k_{cat}/K_m	Ref.
Ruminococcus gnavus N53	7-oxo-LCA	38.8	23.8	700	18	This study
	UDCA	974	11.1	326	0.33	
Collinsella aerofaciens ATCC 25986^T	7-oxo-LCA	5.20	30.8	951	183	(35)
	UDCA	6.23	38.2	1,180	189	
Clostridium absonum DSM 599^T	7-oxo-LCA	2,650	80.1*	3.83 x 10^7*	14,500*	(36)
	UDCA	3,060	55.8*	2.58 x 10^7*	8,430*	

*In the case of *Clostridium absonum* DSM 599^T, the unit used in the Table 2 in reference 36 for V_{max} and k_{cat} did not include enzyme amount information. Thus, these values are just apparent ones.

R. gnavus ATCC 29149^T. The results of DNA sequencing of the PCR products from both *R. gnavus* ATCC 29149^T and N53 were identical. The PCR product from *R. gnavus* N53 was cloned into the pQE30 expression vector. The His-tagged expressed protein purified by cobalt affinity chromatography showed high 7β-HSDH activity of 25.2 μmol·min^{-1}·mg^{-1} protein, as assayed in the reductive reaction, with a purification fold of 1,100 based on the activity of crude extract from *R. gnavus* N53. By SDS-PAGE, the molecular weight of the enzyme was revealed to be 30 kDa (**Fig. 5**). Edman degradation of the purified enzyme revealed a 20 N-terminal amino acid sequence, which matched the N-terminal amino acid sequence of RUMGNA_02585, according to the database. To identify the bile acid conversion products for the reductive and oxidative reactions using the purified enzyme, the reaction mixtures were subjected to TLC and HPLC. Both the reduction and oxidation reaction products, which correspond to UDCA and 7-oxo-LCA, respectively, were detected by TLC (data not shown). HPLC (**Fig. 6**) indicated interconversion between 7-oxo-LCA (RT, ~14 min) and UDCA (RT, ~11 min), with strong preference for the reductive reaction. These results clearly demonstrated that the RUMGNA_02585 gene from *R. gnavus* encodes 7β-HSDH.

Characterization of 7β-HSDH from *R. gnavus* N53

All reactions in this section were carried out using purified 7β-HSDH under the conditions described in Materials and Methods, unless otherwise stated. To identify cofactor specificity, enzyme activities for the reduction and oxidation reactions were measured using NADPH/NADP$^+$ or NADH/NAD$^+$. The activity observed with NADH/NAD$^+$ was less than 0.5% of that assayed using NADPH/NADP$^+$ as cofactor in both directions (data not shown). Thus, it was confirmed that 7β-HSDH from *R. gnavus* N53 is an NADP(H)-dependent enzyme.

TABLE 2. Directions of 7α/7β-HSDH reactions from UDCA-producing bacteria.

Organism	Direction of reactions as observed with: Whole cell 7α / 7β	Purified enzyme 7α / 7β	Reference
Ruminococcus gnavus N53	ND / →	ND / ⇄ (dashed ←)	This study
Ruminococcus productus b-52	ND / ⇄	ND / ←	29, 30, 33
Ruminococcus PO1-3	ND / ⇄	ND / ⇄	34
Collinsella aerofaciens ATCC 25986^T	ND / ⇄	ND / ⇄	30, 35
Clostridium baratii	→ / →	ND	26
Clostridium absonum DSM 599^T	⇄ / ⇄	⇄ / ⇄	39-41

ND, not determined. Directions of 7α/β-HSDH reactions are the same as those shown in Fig. 1. Dashed line denotes a weak reaction.

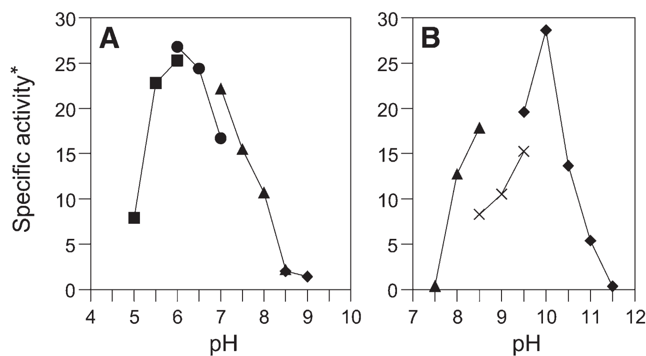

Fig. 7. Effect of pH on the activity of 7β-HSDH from *R. gnavus* N53 in (A) reductive and (B) oxidative reactions. The activity of 7β-HSDH was assayed at a pH range from 5.0 to 11.5 using the following buffers, each at 100 mM and 37°C: sodium acetate (filled square); MES (filled circle); Tris-HCl (filled triangle); glycine-NaOH (filled diamond); and *N*-tris[hydroxymethyl]methyl-4-aminobutane-sulfonic acid (TABS) (cross). *micromoles of NADP(H) per minute per milligram of protein.

To identify the optimal reaction temperature, the reductive reaction was conducted at temperatures between 20°C and 55°C. The highest activity was observed at 37°C. Raising or lowering the temperature caused a decrease in enzyme activity. To determine thermal stability, the purified enzyme was incubated at temperatures between 20°C and 60°C for 30 min in 100 mM 2-(*N*-Morpholino)ethanesulfonic acid (MES) buffer at pH 6.0. The residual enzyme activity measured in the reductive reaction decreased to ~50% of the original value at 50°C within 30 min, and the enzyme appeared mostly inactivated at 60°C. At a pH range of 5.0–11.5, maximal enzyme activities for the reduction (**Fig. 7A**) and oxidation (Fig. 7B) reactions were observed at pH 6.0 and 10.0, respectively. The kinetic parameters of the enzyme in both directions were determined by Lineweaver-Burk plots. The enzyme showed relatively lower K_m and higher maximum velocity values for the reductive reaction than for the oxidative reaction at each optimal pH value (Table 1). These results indicate that this enzyme catalyzes reductive reactions more readily than oxidative reactions.

DISCUSSION

Bacterial 7β-HSDHs catalyze the conversion reactions between 7-oxo-LCA and UDCA, which have been reported in relatively few bacterial species, including *C. aerofaciens* 25986^T (29, 30, 35), *R. productus* b-52 (29, 30, 33), *Ruminococcus* sp. PO1-3 (34), and *Cl. baratii* (26), all from the human intestine, and in the soil bacterium *Cl. absonum* (36, 39–41). Table 2 summarizes the favored directions of the 7α/7β-HSDH reactions as assessed using whole cells under anaerobic conditions or purified enzymes. In general, while reversible reactions were observed with whole cells, purified enzyme from *R. productus* b-52 (30) showed a unidirectional reaction yielding 7-oxo-LCA from UDCA. This discrepancy may be explained either by differences in cofactor (NADPH/NADP$^+$ balance) availability between the two conditions or by the possible existence of a 7β-HSDH isozyme(s) that can produce UDCA or an unknown metabolic pathway(s) yielding UDCA from 7-oxo-LCA. Under these conditions, it is interesting to note that strain *R. gnavus* N53 was found to have a unique 7β-HSDH that primarily catalyzed the reductive reaction (forward reaction) to yield UDCA (Fig. 6).

Functional characterization of purified 7β-HSDH from *R. gnavus* N53 showed properties similar to those of 7β-HSDHs from other bacterial strains. In terms of cofactor requirements, the enzyme showed specificity toward NADPH/NADP$^+$ but not NADH/NAD$^+$. Namely, 7β-HSDHs from *C. aerofaciens* ATCC 25986^T (29, 30), *R. productus* b-52 (29, 30, 33), *Ruminococcus* sp. PO1-3 (34), and *Cl. baratii* (26) have been shown to be NADP(H)-dependent. The optimum pH range of the enzyme was similar to already known 7β-HSDHs: an acidic range for the forward reaction producing UDCA and an alkaline range for the reverse reaction producing 7-oxo-LCA (33–35) (Fig. 7). Determination of the K_m value of the 7β-HSDH from *R. gnavus* N53 revealed a 25-fold higher affinity toward 7-oxo-LCA than that toward UDCA, whereas those of 7β-HSDHs from other bacteria, such as *C. aerofaciens* ATCC 25986^T and *Cl. absonum* DSM 599^T, were similar toward both substrates (Table 1). As a result, the k_{cat}/K_m value for the UDCA-producing reaction of 7β-HSDH from *R. gnavus* N53 was about 55-fold higher than that for the reverse reaction, while the ratios of the k_{cat}/K_m values for both reactions in *C. aerofaciens* ATCC 25986^T (35) and *Cl. absonum* DSM 599^T (36) were in the range 1 to 2 (Table 1). These results indicate that the 7β-HSDH from *R. gnavus* N53 preferentially catalyzes the UDCA-producing reaction (Fig. 1), reflecting the results obtained in growth experiments in which only the UDCA-producing reaction (Fig. 3A), but not the reverse reaction, was detected in *R. gnavus* N53. In contrast, *C. aerofaciens* ATCC 25986^T (30, 35) and *Cl. absonum* DSM 599^T (39–41) showed interconversion between 7-oxo-LCA and UDCA in whole-cell reactions (Table 2). These results indicate that the reaction direction of 7β-HSDH is strain-specific.

Despite the presence of 7α- and 7β-HSDH-positive bacteria in the intestinal microbiota, UDCA is a minor bile acid component of human feces (2% of total bile acid pool) as compared with DCA (34%) and LCA (29%) (1, 12). The bile acid composition of the human intestine is determined by multiple factors, including the characteristics of the bioconversion reactions, the abundances of the bacterial taxa involved in the reaction, and the availability of substrates/cofactors in the intestinal environment. Recently, *R. gnavus* has been identified as a common core member of the human intestinal microbiota (28). Therefore, our data suggest that *R. gnavus* contributes to the formation of UDCA in the human intestine.

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