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Isolation of six novel 7-oxo- or urso-type secondary bile acid-producing bacteria from rat cecal contents

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Understanding the dynamics of secondary bile acid (SBA) formation in the gut by SBA-producing bacteria is important for host health, as SBAs have been shown to affect host pathophysiology and gut microbiota composition. However, our knowledge of SBA producers is limited in light of the diversity of gut microbes. Here, we isolated six novel SBA-producing bacteria from rat cecal contents, all of which were members of known species of gut microbes. *Anaerostipes caccae* D10, *Bacteroides nordii* C5, *Clostridioides difficile* D7, and *Clostridium cadaveris* G11 were capable of oxidizing cholic acid and chenodeoxycholic acid into 7-oxo-derivatives with varying yields. *B. nordii* C5 and its type strain JCM 12987^T had the highest molar yield, ~90%. *Clostridium disporicum* F4 and *Clostridium subterminale* C4 epimerized cholic acid into ursocholic acid with yields of ~85%; the corresponding type strains lacked epimerization activity. Furthermore, although not novel as an SBA producer, *Clostridium scindens* G10 that produced deoxycholic acid from cholic acid was isolated for the first time from rodents. These findings will contribute to elucidation of SBA formation in the gut.

[**Key words:** Bile acid; Bile acid transformation; Secondary bile acid; Intestinal bacteria; Deoxycholic acid; 7-Oxo-deoxycholic acid; *Bacteroides nordii*; *Clostridium disporicum*; *Clostridium subterminale*; Ursocholic acid]

Primary bile acids are synthesized in the liver as conjugated forms with taurine or glycine and secreted into the duodenum as the main component of bile. The main primary bile acids are cholic acid (CA; 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid) in humans (1,2), and CA and β-muricholic acid (MCA) (3α,6β,7β-trihydroxy-5β-cholan-24-oic acid) in rodents (3). In the intestine, primary bile acids facilitate fat digestion and absorption; most are reabsorbed and returned to the liver, which we refer to as enterohepatic circulation. Some portion of the bile acids escape enterohepatic circulation and flow into the large intestine (approximately 400–800 mg/day) where they undergo extensive biotransformations such as deconjugation and subsequent conversion into secondary bile acids (SBAs) by gut microbes (4).

Transformation of bile acids generally involves oxidation and epimerization of the hydroxy groups at the C-3, C-7, and C-12 positions (Fig. 1). These reactions are carried out by hydroxysteroid dehydrogenases (HSDHs) of gut microbes. Oxidation reactions are catalyzed by specific α-HSDHs that generate oxo-type bile acid derivatives at the C-3, C-7, and C-12 positions. Epimerization is normally a reversible reaction that changes the stereochemistry of the hydroxy group from α-to a β-configuration. This reaction consists of two sequential reactions: oxidation of CA and CDCA into their oxo-type derivatives by α-HSDHs and subsequent reduction of these oxo groups by β-HSDHs, generating β-hydroxy bile acids (4,5). Among HSDHs, 7α-HSDH catalyzes the oxidation of CA and CDCA into 7-oxo-deoxycholic acid (7-oxo-DCA, 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid) and 7-oxo-lithocholic acid (7-oxo-LCA; 3α-hydroxy-7-oxo-5β-cholan-24-oic acid), respectively (Fig. 1). 7α-HSDH activity is widespread in *Escherichia coli* and among members of genera *Bacteroides* and *Clostridium* (4). In contrast, distribution of 7β-HSDH activity is rather limited. To date, members of the genera *Ruminococcus*, *Clostridium*, and a *Collinsella aerofaciens* strain have been reported to exhibit this activity (6–12). Of these, two *Clostridium* strains, *Clostridium absonum* (13) and *Clostridium limosum* (14), had both 7α- and 7β-HSDH activities, which enabled them to directly epimerize CA and CDCA into ursocholic acid (UCA; 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid) and ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholan-24-oic acid), respectively (Fig. 1). Another common transformation reaction in the gut is a 7α-dehydroxylation, a multi-step reaction that converts CA and CDCA into deoxycholic acid (DCA; 3α,12α-dihydroxy-5β-cholan-24-oic acid) and lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid), respectively (4) (Fig. 1). DCA has been shown to be a strong antimicrobial agent, with approximately 10 times more bactericidal activity than CA due to its hydrophobicity (15).

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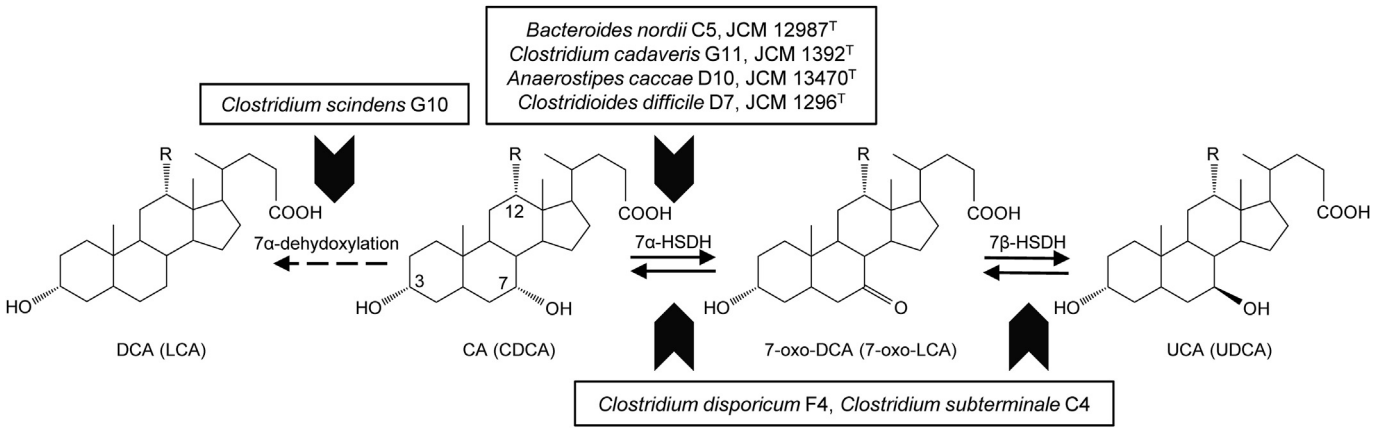

FIG. 1. Biotransformation reactions involved in this study. If R is OH, the bile acids are DCA, CA, 7-oxo-DCA, and UCA. The bile acids in parentheses occur when R is H. 7α-dehydroxylation is a multi-step reaction, represented by the dashed line. The novel strains found to produce 7-oxo- or urso-type derivatives from CA or CDCA are shown. Carbon atom numbers 3, 7, and 12 are shown in the CA molecule.

DCA is implicated as an endogenous etiologic agent in gastrointestinal cancer (16,17). In addition, bile acids have been known to act as signaling molecules to regulate host glucose, lipid, and energy metabolism via interaction with a farnesoid X receptor and/or a G-protein-coupled bile acid receptor, TGR5 (18). Structural variation of each bile acid as the result of biotransformation strongly affects the affinity of the bile acid molecule to these receptors, and thus determines their efficiency as signaling molecules (18,19). In addition, we have recently demonstrated that bile acids are an important factor regulating the composition of the host's gut microbiota due to their bactericidal activities (20) and that they are likely a driving force behind its alteration in response to a high-fat diet (21). Thus, improving our knowledge of bacterial bile acid metabolism and SBA formation in the intestines is essential to host health maintenance.

However, despite this necessity, the number of SBA-producing bacteria that have been characterized in terms of their physiological and biochemical properties is still not sufficient; thus, it is difficult to fully understand the mechanism that determines bile acid composition in the intestine. In this study, we aimed to identify and characterize novel SBA-producing bacteria that produce SBAs common to humans and rodents from rat cecal contents. We used CA as the initial screening substrate for SBA production, because CA is a common and abundant primary bile acid in both humans and rodents (2,3). CDCA was used only to test substrate specificity of the isolated SBA producers for SBA production, as CDCA is abundant primary bile acid in humans but not in rodents (2,3). We also improved the screening protocol in several regards, including inclusion of colistin (also known as polymyxin E) in the isolation media to inhibit the growth of *E. coli*, a known 7-oxo-DCA/7-oxo-LCA-producing bacterium. We successfully isolated many SBA-producing bacteria. Characterization of their taxonomy and bile acid transformation activity resulted in the identification of six novel SBA-producing bacteria belonging to known species of gut microbes.

MATERIALS AND METHODS

Bacterial strains and culture conditions Bacterial strains used and isolates identified in this study are listed in Table 1. *Anaerostipes caccae* JCM 13470^T, *Bacteroides nordii* JCM 12987^T, *Clostridioides difficile* JCM 1296^T, *Clostridium cadaveris* JCM 1392^T, *Clostridium scindens* JCM 6567^T, *Clostridium subterminale* JCM 1417^T, and *Terrisporobacter glycolicus* JCM 1401^T were obtained from the Japan Collection of Microorganisms (JCM; Tsukuba, Ibaraki, Japan). *Clostridium disporicum* DSM 5521^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). All cultures were incubated at 37°C under strict anaerobic conditions using an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA) containing mixed gas (N_2:CO_2:H_2 = 80:10:10). Bacterial isolation from rat cecal contents was conducted on agar media using Eggerth–Gagnon (EG) agar medium (22) and Brucella agar medium (BD BBL Brucella agar, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) both supplemented with defibrinated horse blood (5% at final concentration). Hemin (5 μg/mL) and vitamin K_3 (0.5 μg/mL) were also supplemented in Brucella agar medium. An antibiotic, colistin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), was added to both media at a final concentration of 2 μg/mL. Liquid cultures were grown on Gifu anaerobic medium (GAM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 0.1 M MOPS (Dojindo Laboratories, Mashiki, Kumamoto, Japan; GAM-MOPS). The pH was adjusted to 7.0 with NaOH.

Bile acids Sodium cholate hydrate (CA-Na; 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid sodium salt), sodium chenodeoxycholate (CDCA-Na; 3α,7α-dihydroxy-5β-cholan-24-oic acid sodium salt), DCA, and UDCA were purchased from Sigma–Aldrich (St. Louis, MO, USA). CA-Na and CDCA-Na were used as substrates for bile acid biotransformation experiments. In addition to CA and CDCA, the following bile acids were used as standards for ultra-performance liquid chromatography/electrospray ionization mass spectrometry (UPLC/ESI-

TABLE 1. Bacterial strains used in this study.

Strain	Source	Reference
Anaerostipes caccae		
JCM 13470^T	Human feces	43
D10	Rat cecal contents	This study
Bacteroides nordii		
JCM 12987^T	Human peritoneal fluid	37
C5	Rat cecal contents	This study
Clostridioides difficile		
JCM 1296^T	Healthy infant feces	44
D7	Rat cecal contents	This study
Clostridium cadaveris		
JCM 1392^T	Rabbit	45
G11	Rat cecal contents	This study
Clostridium disporicum		
DSM 5521^T	Rat cecum	46
F4	Rat cecal contents	This study
Clostridium scindens		
JCM 6567^T	Human feces	47
G10	Rat cecal contents	This study
Clostridium subterminale		
JCM 1417^T	–[a]	45
C4	Rat cecal contents	This study
Terrisporobacter glycolicus		
JCM 1401^T	Mud, MD, USA	33
A10	Rat cecal contents	This study

[a] No description.

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