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Isolation of a Rat Intestinal *Clostridium* Strain Producing 5α- and 5β-Bile Salt 3α-Sulfatase Activity

J. ROBBEN, G. PARMENTIER, AND H. EYSSEN*
The Rega Institute, University of Leuven, B-3000 Leuven, Belgium

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An unnamed sporeforming microorganism, termed *Clostridium* sp. strain S_2, possessing bile salt sulfatase activity was isolated from rat intestinal microflora. The microorganism was a strictly anaerobic, nonmotile, gram-negative, asaccharolytic, sporeforming rod requiring CO_2, vitamin K, and taurine; the guanine-plus-cytosine content of the DNA was 40.8 mol% (T_m), and the strain was tentatively classified as an atypical *Clostridium* species. Sulfatase activity was specific for 3α-sulfate esters of 5α- and 5β-bile salts, leaving the 3β-, 7α-, and 12α-sulfates unchanged. Strain S_2 also deconjugated tauro- and glyco-conjugated bile salts and produced 6α- and 6β-hydroxysteroid dehydrogenase. The resulting unconjugated 6-oxo derivatives were partially reduced into the corresponding 6α-hydroxy bile salts. By these reactions, α-muricholate and β-muricholate were more than 80% converted into hyocholate and ω-muricholate, respectively. In addition, strain S_2 produced 12α-hydroxysteroid dehydrogenase converting deoxycholate into 3α-hydroxy-12-oxo-5β-cholanoate. When strain S_2 was associated with gnotobiotic rats, the fecal bile salts were more than 90% desulfated and the fecal excretion of allochenodeoxycholate was five times lower than in control rats.

Sulfate esterification of bile salts occurs in rat liver and kidney (3). Since bile salt sulfates are less readily reabsorbed from the intestine, sulfation should limit their enterohepatic recirculation and promote their fecal elimination (5, 10, 20). However, the microflora in the large intestine is able to hydrolyze bile salt sulfates into the less polar unsulfated compounds (4, 7, 18, 23) which are more readily reabsorbed. In previous studies a bile salt-desulfating *Clostridium* sp., strain S_1, was isolated from rat intestinal microflora (13, 14). Subsequently, the influence of microbial bile salt sulfatase activity upon fecal elimination of bile salts was quantitatively established in gnotobiotic rats associated with this strain (10). In these rats, strain S_1 prolonged the 50% excretion times of [24-^{14}C]taurocholate-3-sulfate and [24-^{14}C]taurolithocholate-3-sulfate by 4.5 and 4 times, respectively, to values in the same range of time as those of unsulfated tauroCA and tauroLCA (10). *Clostridium* sp. strain S_1 desulfated 3α-sulfates of 5β-bile salts and 3β-sulfates of 5α-bile salts, whereas the 3α-sulfates of 5α-bile salts such as allochenodeoxycholate-3-sulfate, the predominant bile salt sulfate in female germfree rats (9), were not desulfated (13).

In this study we now report the isolation from rat intestinal flora of the bile salt-desulfating *Clostridium* sp. strain S_2, capable of hydrolyzing both 5α- and 5β-bile salt-3α-sulfates, including alloCDCA-3-sulfate.

MATERIALS AND METHODS

Bile salts. Trivial names and abbreviations for bile salts are as follows: lithocholate (LCA), 3α-hydroxy-5β-cholanoate; chenodeoxycholate (CDCA), 3α,7α-dihydroxy-5β-cholanoate; ursodeoxycholate, 3α,7β-dihydroxy-5β-cholanoate; hyodeoxycholate (HDCA), 3α,6α-dihydroxy-5β-cholanoate; murocholate (muroCA), 3α,6β-dihydroxy-5β-cholanoate; deoxycholate (DCA), 3α,12α-dihydroxy-5β-cholanoate; cholate (CA), 3α,7α,12α-trihydroxy-5β-cholanoate; hyocholate (HCA), 3α,6α,7α-trihydroxy-5β-cholanoate; ω-muricholate (ω-MCA), 3α,6α,7β-trihydroxy-5β-cholanoate; α-muricholate (α-MCA), 3α,6β,7α-trihydroxy-5β-cholanoate; β-muricholate (β-MCA), 3α,6β,7β-trihydroxy-5β-cholanoate; 23-nordeoxycholate (23-norDCA), 3α,12α-dihydroxy-23-nor-5β-cholanoate. The prefix "allo" refers to a 5α-steroid nucleus; the prefix "iso" refers to a β-orientation of the 3-hydroxyl group. Unless stated otherwise, the bile salts were obtained from Steraloids (Wilton, N.H.).

The muricholates (α-, β-, and ω-MCA) were obtained after hydrolysis of their methyl esters in 5% KOH in methanol at 70°C for 1 h. The preparation of these methyl esters has been described previously (25). The enzymatically deconjugated bile salt fraction of feces from cholesterol-fed germfree rats was used to prepare 3α,7α-dihydroxy-6-oxo-5β-cholanoate (25). Tauro- and glyco-conjugates of LCA, CDCA, DCA, and CA were supplied by Maybridge (Launceston, U.K.). Tauro-β-MCA and glyco-β-MCA were prepared from β-MCA according to Lack et al. (19).

AlloCDCA was prepared from CDCA by a series of reactions involving the following: (i) oxidation of the methyl ester with Ag_2CO_3-celite in toluene to the 3-oxo derivative (28); (ii) hydrolysis followed by dehydrogenation with SeO_2 to 7α-hydroxy-3-oxo-5-cholenoic acid; (iii) reduction with lithium in liquid ammonia, giving the 5α derivative (17); and (iv) reduction of the 3-oxo group with K-selectride in tetrahydrofuran (11), yielding alloCDCA. A small amount of isoalloCDCA was formed during the reduction with lithium in liquid ammonia and was separated from alloCDCA by column chromatography of the methyl esters on silica gel. IsoCDCA was prepared as the methyl ester from methyl CDCA by oxidation with Ag_2CO_3-celite in toluene, followed by reduction of the 3-oxo derivative with K-selectride in tetrahydrofuran.

AlloLCA was prepared by combination of the methods of Kallner (17) and Hiremath and Elliott (11). Methyl 3β-hydroxy-5-cholenoate was hydrogenated in acetic acid with PtO_2 catalyst, yielding methyl isoalloLCA; oxidation with the Jones reagent provided methyl 3-oxo-5α-cholanoate (17). This 3-oxo derivative was reduced with K-selectride, yielding methyl alloLCA.

* Corresponding author.

To prepare the 3-sulfates of alloCDCA, isoCDCA, and isoalloCDCA, the 7α-hydroxyl function of the bile salts was protected by performylation followed by removal of the 3α-formoxyl group by partial deformylation as described by Tserng and Klein (29). The resulting bile acids were sulfated by treatment with chlorosulfonic acid in dry pyridine (24). Finally, the 7α-formoxyl group was removed and the methyl esters were converted into the sodium salts by hydrolysis in 5% NaOH in methanol. All other 3-, 7-, and 12-monosulfated bile salts and their taurine and glycine conjugates were synthesized according to previously described methods (24).

Bacteriology. (i) Media and incubation. Brucella broth, Columbia broth (CB), and tryptic soy broth media were supplied by GIBCO Bio-Cult Ltd. (Paisley, U.K.). Brain heart infusion medium and thioglycolate medium without added dextrose were supplied by BBL Microbiology Systems (Cockeysville, Md.). Reinforced clostridial medium and Todd-Hewitt broth (THB) were obtained from Oxoid (Basingstoke, U.K.). Schaedler broth (bioMérieux, Brussels, Belgium) was also used. When required, media were supplemented with the reducing agent 0.05% L-cysteine hydrochloride or 1,4-dithiothreitol and enriched with 0.0005% hemin (Sigma Chemical Co., Dorset, U.K.) and 0.0001% vitamin K_1 (Konakion; Roche, Basel, Switzerland) or vitamin K_3 (menadione sodium bisulfite; Merck AG, Darmstadt, Federal Republic of Germany). Other supplements used were cooked-meat medium, Lab Lemco powder, neutralized soya peptone (Oxoid), proteose peptone, tryptone and yeast extract (Difco Laboratories, Detroit, Mich.), bio-Trypcase and PolyVitex (bioMérieux), or oxgall (BBL).

Cultures were incubated at 37°C under 90% nitrogen–10% hydrogen in an anaerobic glove box similar to that described by Aranki et al. (1). The autoclaved media were prereduced in the glove box for at least 48 h before inoculation. Inoculated media (5 to 10%, vol/vol) were incubated in the glove box in an anaerobic jar for 4 to 5 days. CO_2 was provided by placing a GasPak disposable H_2 + CO_2 generator envelope (BBL) in the jar. Growth was measured by determination of the optical densities (OD) of the cultures with a Bausch and Lomb Spectronic 20 photometer at 520-nm wavelength and 10-mm pathlength.

Bile salt transformations were studied on medium CB^+, i.e., medium CB supplemented with 0.0005% hemin, 0.0001% vitamin K_1, and 0.1% taurine and containing 100 to 200 μg of bile salt per 6 ml of medium.

(ii) Isolation procedures. Fresh feces from female conventional Wistar rats were immediately transferred into the anaerobic glove box and suspended in 10 ml of culture medium. Portions (10 μl) of serial 10-fold dilutions were streaked onto agar plates. After 3 to 5 days of incubation, single colonies and mixtures of different colony types were subcultured in 6 ml of medium containing 100 μg of alloLCA-3-sulfate and checked for sulfatase activity.

(iii) Identification procedures. Strain S_2 was tentatively identified according to the methods described by Holdeman et al. (12), with the following modifications. The basal medium was CB^+. Reduction of nitrates, indole production, and urease activity were investigated by adding 0.1% KNO_3, 1% tryptone (Difco), or 0.5 to 2% urea, respectively, to medium CB^+. Motility and the presence of flagella were investigated in semisolid basal medium (0.5% agar), by direct phase-contrast microscopy of slides from young cultures (12 to 18 h) prepared in the anaerobic glove box and by electron microscopy. Spores were stained in warm 5% malachite green (5 min), followed by cold contrast staining with safranin (30 s). The guanine-plus-cytosine (G+C) content of DNA was estimated by determination of the thermal denaturation temperature (T_m) in 0.015 M NaCl–1.5 mM sodium citrate, using a double-beam Beckman UV 5230 spectrophotometer at 260 nm with a thermocouple in the sample cuvette for temperature reading. The molar percentage base composition was calculated according to the equation, G+C = (T_m − 53.9)2.44, of Mandel and Marmur (22).

Study of in vitro bile salt transformation. To study transformations of unconjugated unsulfated bile salts, 3 ml of grown culture or uninoculated culture medium was mixed with 3 ml of 20% NaCl solution in H_2O, 1 ml of methanol, and 0.5 ml of internal standard solution containing 50 μg of 23-norDCA. This mixture was acidified to pH < 2 with HCl and extracted twice with 8 ml of diethyl ether. The extract was evaporated and the bile acids were derivatized to methyl ester trimethylsilyl ethers and methyl ester acetates for identification and quantification by gas-liquid chromatography (8). The identification was carried out by comparing relative retention times of the derivatized bile salts with those of reference compounds on columns of 3% OV-1, 3% OV-17, and 1% QF-1.

Deconjugation of tauro- and glyco-conjugated unsulfated bile salts was determined by comparing the amounts of free bile salts and total bile salts in grown cultures with those of the conjugate in uninoculated control medium. Deconjugated bile salts were analyzed as described above. To analyze total bile salts, 3 ml of culture was mixed with 50 μg of 23-norDCA, evaporated to dryness, and subjected to alkaline deconjugation with 1 ml of 20% (wt/vol) KOH in ethylene glycol (8). The hydrolysate was then acidified and further treated as for unconjugated bile salts.

Desulfation of unconjugated bile salt sulfates was determined by comparing the amounts of free and total bile salts in grown cultures with those of the sulfated bile salts in uninoculated culture medium. To analyze free bile salts, 3 ml of culture was extracted with diethyl ether after mixing with 50 μg of 23-norDCA and acidifying to pH 4 with 4 ml of buffered salt solution (10% NaCl plus 10% KH_2PO_4 in H_2O). The extract was washed with 7 ml of H_2O and further treated as for unconjugated bile salts. To analyze total bile salts, 3 ml of culture was desalted on a reverse-phase octadecylsilane bonded silica cartridge (J. T. Baker Chemical Co., Phillipsburg, N.J.); bile salts were then eluted with methanol (27). After addition of 50 μg of 23-norDCA, the eluate was evaporated and solvolyzed for 18 h at 37°C in 10 ml of methanol-acetone (1:9) acidified with 0.1 ml of 6 N HCl. The hydrolysate was neutralized with 20% NaOH, evaporated, and further treated as described above.

To study transformations of conjugated monosulfated bile salts, 6 ml of culture medium was desalted on an octadecylsilane cartridge, and the methanol eluate was mixed with 100 μg of 23-norDCA and divided into three equal parts. The first part was analyzed for deconjugated desulfated bile salts as described above. In the second aliquot the total deconjugated bile salts were determined after solvolysis. The third aliquot was solvolyzed and deconjugated to determine total bile salts.

Study of in vivo bile salt transformations. Germfree and gnotobiotic female inbred Fisher rats about 5 months old were kept in Trexler flexible-film plastic isolators (Standard Safety Equipment, Pallatine, N.J.) and were fed a steam-sterilized L-356-Rega diet (25) and water ad libitum. Feces were collected every 2 or 3 days, combined per group of six rats, homogenized with an equal volume of water, and lyophilized. Fecal bile salts were analyzed by gas-liquid

chromatography and combined gas-liquid chromatography-mass spectrometry as described elsewhere (9, 25).

RESULTS

Isolation. Mixed cultures from female rat feces caused no desulfation of alloLCA-3-sulfate unless under anaerobic conditions. Sulfatase activity (20 to 60% desulfation) was found in primary anaerobic cultures on brucella broth, THB, tryptic soy broth, brain heart infusion, and thioglycolate media, but was absent in cultures on Schaedler broth and reinforced clostridial medium. Serial dilutions of fecal suspensions showed sulfatase activity only in dilutions 10^{-2} (brain heart infusion and brucella broth), 10^{-3} (CB and tryptic soy broth), and 10^{-4} (thioglycolate medium and THB) versus growth in dilutions to 10^{-9}. The sulfatase activity disappeared in the first or second subculture on all media tested, except on THB medium. When brain heart infusion agar plates supplemented with 10% defibrinated horse blood, vitamin K_1, hemin, and yeast extract were inoculated with fecal suspensions, none of the isolates from these plates was sulfatase positive.

Sulfatase activity was still observed in mixed cultures on THB medium prepared from fecal suspensions heated at 70°C for 30 min. Addition of 0.02 to 0.05% dithiothreitol or enrichment of the culture medium with hemin, vitamin K_1, yeast extract, Lab Lemco powder, cooked-meat medium, proteose peptone, neutralized soya peptone, or taurine did not alter the growth ratios in favor of the desulfating species. In subcultures from heated fecal suspensions the initial THB medium (20 to 40% desulfation) could be replaced by CB medium on which a higher percentage (50 to 60%) of alloLCA-3-sulfate was desulfated. In addition, the incubation temperature could be gradually increased from 37 to 44°C, and these cultures were sulfatase positive up to a dilution of 10^{-6}. Single colonies and mixtures of different colony types were subcultured on CB^+ medium. Sulfatase-positive isolates were further purified on this medium until a pure culture of a desulfating strain, termed strain S_2, was obtained.

Identification. On CB^+ medium strain S_2 appeared as a small (1.5 to 2.5 by 0.5 μm), gram-negative, nonmotile, straight rod with rounded ends and occurred singly, in pairs, or occasionally in short chains. Strain S_2 required strictly anaerobic conditions and the presence of CO_2 and vitamin K in the culture medium. In addition, no visible growth (OD ≤ 0.01) developed in any of the media tested, unless when supplemented with 0.1% taurine (OD = 0.35 on CB^+ medium) or 0.1% sodium sulfite (OD = 0.13). Higher concentrations of taurine did not cause supplementary growth and more than 0.1% sulfite was toxic. Sulfur sources such as 0.02 to 0.5% sodium sulfate and thiosulfate, as well as 0.1% cysteine and methionine, failed to stimulate growth. Addition of 0.1% sodium pyruvate to media supplemented with 0.1% sodium sulfite clearly enhanced growth (OD = 0.32), but was without effect in media supplemented with sodium sulfate or taurine. No growth enhancement was obtained by additives such as 0.5 to 1.0% of different carbohydrates, the mixtures of volatile and nonvolatile fatty acids described by Holdeman et al. (12), 0.02% Tween 80, or vitamin-rich preparations such as 0.5% yeast extract, 1% PolyVitex, and 1% Wensinck vitamin solution (30). Growth was inhibited by 0.2% oxgall powder.

As growth of strain S_2 was very poor on peptone-yeast extract or peptone-yeast extract-glucose medium, even when supplemented with 0.1% taurine, the identification procedure was carried out on CB^+ medium on which good growth occurred. Strain S_2 did not ferment carbohydrates and did not produce gelatinase, indole, urease, acetoine, lecithinase, catalase, or esculin hydrolase. Nitrates were not reduced. Strain S_2 produced ammonia, hydrogen sulfide, and acetic acid, but no other volatile or nonvolatile fatty acids. Desulfoviridin was not detected. The guanine-plus-cytosine content of DNA of strain S_2 was 40.8 versus 42.8 mol% in *Clostridium* sp. strain S_1. The strain grew well at 43°C on CB^+ medium, but growth was weak at 45°C.

Heat tests (10 min at 70 or 80°C) on 4- to 7-day-old cultures and on suspensions of cultures grown for 4 to 18 days on CB^+ agar slants supplemented with 1% cooked-meat medium usually were negative. However, spore stains on 4- to 7-day-old cultures occasionally revealed small, spherical, terminal or subterminal bodies, slightly bulging from the cells. In vivo spore formation by strain S_2 might be suspected, as this strain was isolated from heated fecal suspensions. Confirmation of in vivo sporulation was obtained by survival of strain S_2 in heat tests (10 min at 80°C) on fecal suspensions of gnotobiotic rats associated with strain S_2 plus *Clostridium perfringens* ATCC 19574.

Surface colonies of strain S_2 on CB^+ agar plates after 3 days of incubation were <0.2 mm in diameter, convex, circular, entire, shiny, colorless, and transparent. However, it was observed that colonies increased in diameter to 1 mm and became slightly irregular and opaque in the neighborhood of colonies of certain other bacteria (e.g., *Eubacterium lentum*, *Propionibacterium* sp.). Culture filtrates of these strains, added to the agar plates, also enhanced colony growth of strain S_2. When vitamin K_1 or K_3 was added in ascending concentrations of 0.0001 to 0.0025%, colonies increased in size from 0.2 to 0.7 mm and from 0.2 to 0.9 mm, respectively, and attained the same morphology as those in the neighborhood of colonies of growth-stimulating strains. Concentrations of vitamin K_1 or K_3 of >0.0001% did not cause supplementary growth in liquid CB^+ medium.

Strain S_2 resembled the previously described bile salt-desulfating *Clostridium* sp. strain S_1. Nevertheless, apparent differences were observed when the two strains were cultured simultaneously under the same conditions. Cultures of strain S_1 developed higher cell densities than strain S_2 in medium CB^+ (OD = 0.45 versus 0.35) and taurine-supplemented peptone-yeast extract-glucose medium (OD = 0.30 versus 0.10). Moreover, on all media tested colony diameters of strain S_1 were about twice those of strain S_2.

Bile salt transformations. (i) Unsulfated bile salts. *Clostridium* sp. strain S_2 showed 12α-hydroxysteroid dehydrogenase (HSDH) activity (Table 1). The 12α-hydroxyl group of DCA was more easily dehydrogenated (>30%) than that of CA (<5%). The 6β-hydroxyl group of muroCA, α-MCA, and β-MCA was dehydrogenated to a 6-oxo group or isomerized to a 6α-hydroxyl group. By these reactions muroCA was 33% converted into HDCA, whereas β-MCA and α-MCA were almost completely isomerized into ω-MCA and HCA, respectively. The 6α-hydroxyl group of HDCA was more than 70% dehydrogenated into a 6-oxo group. Cultures incubated with ω-MCA or HCA also contained small amounts of β-MCA (traces) and α-MCA (10%), respectively. The 3α-, 3β-, 7α-, and 7β-hydroxyl groups were not affected by strain S_2. The previously isolated strain S_1 examined simultaneously and under the same experimental conditions also showed 12α-HSDH activity on DCA and CA. On the other hand, strain S_1 differed from strain S_2 by the absence of epimerization of the 6β-hydroxyl groups of muroCA, α-MCA, and β-MCA. Hence, strain S_1 did not convert β-MCA into ω-MCA or α-MCA into HCA.

TABLE 1. Transformations of unconjugated bile salts by *Clostridium* sp. strain S_2

Bile salt[a]	Configuration of hydroxyl groups	5β-cholanoates formed (% transformed)[b]
LCA	3α	—[c]
isoLCA	3β	—
CDCA	3α,7α	—
isoCDCA	3β,7α	—
UDCA	3α,7β	—
HDCA	3α,6α	3α-ol,6-oxo (78)
muroCA	3α,6β	3α-ol,6-oxo (67) + 3α,6α-diol (33)
HCA	3α,6α,7α	3α,6β,7α-triol (10)
α-MCA	3α,6β,7α	3α,6α,7α-triol (92)
ω-MCA	3α,6α,7β	3α,6β,7β-triol (tr)[d]
β-MCA	3α,6β,7β	3α,6α,7β-triol (87) + 3α,7α-diol,6-oxo (12)
DCA	3α,12α	3α-ol,12-oxo (33)
CA	3α,7α,12α	3α,7α-diol,12-oxo (tr)

[a] The culture medium was medium CB^+, containing 100 μg of monohydroxy bile salts or 200 μg of di- or trihydroxy bile salts per 6 ml.
[b] Percent transformed (mean of four determinations) after 4 days of incubation.
[c] —, Unchanged.
[d] tr, trace amounts; <5% of total.

Tauro- and glyco-conjugates of LCA, CDCA, DCA, CA, and β-MCA were nearly 100% deconjugated by strain S_2 and were further transformed as described for the unconjugated unsulfated bile salts.

(ii) Bile salt sulfates. Under optimal growth conditions (CB^+ medium) the 3α-sulfates of the unconjugated 5β-bile salts LCA, CDCA, DCA, and CA and the 3α-sulfates of the unconjugated 5α-bile salts alloLCA and alloCDCA were more than 60% desulfated (Table 2). Neither the 3β-sulfates of isoLCA, isoCDCA, isoalloLCA, and isoalloCDCA nor the 7α- or 12α-sulfate ester of DCA and CA was affected. Comparison of bile salt sulfatase activities of strains S_1 and S_2 revealed different substrate specificities. The 5β-bile salt-3α-sulfates were desulfated by both strains. The 5α-bile salt-3α-sulfates were hydrolyzed only by strain S_2, whereas the 5β- and 5α-bile salt-3β-sulfates were desulfated only by strain S_1.

Tauro- and glyco-conjugated 3α-sulfates of LCA, CA, DCA, and CDCA were extensively deconjugated and desulfated by strain S_2 (Table 3). Glyco-CDCA-7-sulfate was 90% deconjugated. Tauro-DCA-12-sulfate was 50% deconjugated, while the glycine conjugate was deconjugated by only 4%. The 7α- and 12α-sulfate groups were not hydrolyzed. This indicated that the deconjugating enyzme was active, even when the bile salts were sulfated. Whether or not deconjugation must precede the desulfation of the conjugated bile salt-3-sulfates could not be derived from these data because of the pronounced deconjugating activity of strain S_2.

Sulfatase activity in relation to cell growth was studied by adding 0.05 mM CDCA-3-sulfate or alloCDCA-3-sulfate to cultures at different phases of growth and additional incubation for 3 to 6 h. Sulfatase activity started 4 to 6 h after inoculation, was maximal in the exponential growth phase, and decreased strongly in the late exponential and stationary growth phases 12 to 15 h after inoculation. When substrate was added to growing cultures, no sulfatase activity was detected in the first 2 to 3 h after addition of the substrate, suggesting that the sulfatase had to be induced. A similar pattern of sulfatase activity was observed with both substrates. No sulfatase activity was found in the supernatant from cultures centrifuged in the exponential phase of growth. The cell pellet resuspended in 0.1 M phosphate buffer (pH 7) was only sulfatase active in anaerobic conditions and when the cultures were grown in the presence of bile salt sulfates.

(iii) In vivo bile salt transformation. Strain S_2 could not be established as a monoassociate in the digestive tract of gremfree rats, probably because of the strictly anaerobic character of the microorganism. Association was, however, successful in rats monoassociated with *C. perfringens* ATCC 19574 or with *Clostridium* sp. strain Cl_8 previously isolated from conventional rat cecum (14). These accompanying strains did not produce bile salt sulfatase. In gnotobiotic rats associated with *C. perfringens* and strain S_2 the fecal bile salts were nearly completely desulfated, except for isoalloCDCA (12% of total bile salts) which was more than 90% sulfated. This bile salt did not occur in germfree rats. Presumably, it was formed by *C. perfringens* from unsulfated alloCDCA by isomerization of the 3α-hydroxyl group into a 3β-hydroxyl group since this transformation also occurred in cultures in vitro.

The data in Table 4 show that in gnotobiotic rats associated with *Clostridium* sp. strains Cl_8 plus S_2 the fecal bile salts were nearly completely desulfated. In addition, the alloCDCA fraction of total bile salts decreased to less than 20% of the amounts found in germfree rats or rats monoassociated with strain Cl_8; no isoalloCDCA was formed. Furthermore, β-MCA was more than 90% converted into two bile salts, each with a double bond in the side chain between C-22 and C-23. The first substance was the unsaturated derivative of β-MCA and was also present in rats monoassociated with strain Cl_8 or *C. perfringens*. The second substance was the corresponding unsaturated derivative of

TABLE 2. Desulfation of monosulfated bile salts by *Clostridium* sp. strain S_2

Bile salt sulfate[a]	Configuration of:		% Desulfation[b]
	SO_4 group	C-5 hydrogen	
LCA-3-sulfate	3α	5β	70
CDCA-3-sulfate	3α	5β	>95
DCA-3-sulfate	3α	5β	63 (73% 3α-ol,12-oxo)[c]
CA-3-sulfate	3α	5β	64 (<5% 3α,7α-diol,12-oxo)[c]
isoLCA-3-sulfate	3β	5β	0
isoCDCA-3-sulfate	3β	5β	0
alloLCA-3-sulfate	3α	5α	79
alloCDCA-3-sulfate	3α	5α	>95
isoalloLCA-3-sulfate	3β	5α	0
isoalloCDCA-3-sulfate	3β	5α	0
CDCA-7-sulfate	7α	5β	0
CA-7-sulfate	7α	5β	0
DCA-12-sulfate	12α	5β	0
CA-12-sulfate	12α	5β	0

[a] The culture medium CB^+ contained 100 μg of monohydroxy bile salts or 200 μg of di- or trihydroxy bile salts per 6 ml. Cultures were incubated for 4 days.
[b] Means of four determinations.
[c] Percentages of the 12-oxo derivative found in the desulfated fraction of DCA and CA.

TABLE 3. Transformation of tauro- and glyco-conjugated 5β-bile salt sulfates by *Clostridium* sp. strain S_2

Bile salt[a]	Location and configuration of SO_4 group	Transformation products[b] of:	
		Glycine conjugates	Taurine conjugates
LCA	3α	LCA (78)	LCA (77)
CA	3α	CA (85) + 3α,7α-diol,12-oxo (12)	CA (87) + 3α,7α-diol,12-oxo (10)
DCA	3α	DCA (80) + 3α-ol,12-oxo (16)	DCA (69) + 3α-ol,12-oxo (26)
DCA	12α	DCA-12-sulfate (4)	DCA-12-sulfate (50)
CDCA	3α	CDCA (98)	CDCA (>95)
CDCA	7α	CDCA-7-sulfate (90)	NT[c]

[a] The culture medium CB^+ contained 100 μg of conjugated monohydroxy or 200 μg of conjugated di- or tri-hydroxy bile salt sulfates per 6 ml. Cultures were incubated for 4 days.
[b] Values in parentheses are the percentages of transformation products including deconjugated bile salts, deconjugated plus desulfated bile salts, and the 12-oxo derivatives of deconjugated plus desulfated CA and DCA (means of two determinations).
[c] NT, Not tested.

ω-MCA and was only found in rats associated with *Clostridium* sp. strain S_2. Formation of a double bond in the side chain of β-MCA, tauro-β-MCA, or ω-MCA was not observed in vitro either in pure cultures or in mixed cultures of *C. perfringens*, *Clostridium* sp. strain Cl_8, or strain S_2. In gnotobiotic rats associated with strains Cl_8 plus S_2, about 84% of total fecal bile salts were deconjugated versus 58% in rats monoassociated with strain Cl_8.

DISCUSSION

LCA-3-sulfate is extensively hydrolyzed in the rat intestine prior to fecal excretion (23) and this transformation is thought to be of microbial origin. Microbial bile salt sulfatase activity in the intestine was also proposed by Eyssen et al. (9) as a possible explanation for the difference in amounts of sulfated bile salts in the large intestine and the feces of germfree and conventional rats. Direct evidence of this was provided by the isolation from rat intestinal microflora of *Clostridium* sp. strain S_1 by Huijghebaert et al. (13, 14). The isolate hydrolyzed 5β-bile salt-3α-sulfates and 5α-bile salt-3β-sulfates. The 3β-sulfates of 5α-bile salts, including alloCDCA-3-sulfate, the predominant naturally occurring bile salt sulfate in the rat, were not affected by strain S_1. However, alloCDCA-3-sulfate was desulfated by mixed cultures from human, rat, and mouse intestinal bacteria (15).

In the present investigations we isolated from rat intestinal microflora an unnamed bacterium, termed strain S_2, performing sulfatase activity on 5α- and 5β-bile salt 3α-sulfates. Because of its strictly anaerobic character, its rod shape, and its sporeforming capacity, strain S_2 belongs to the genus *Clostridium* or to the genus *Desulfotomaculum*, but it did not correspond to any of the species described in *Bergey's Manual* (2) or in the *Virginia Polytechnic Institute Anaerobic Laboratory Manual* (12). Since growth of strain S_2 was enhanced by sulfite-pyruvate supplements in the culture medium, the strain might be related to the dissimilatory sulfate-reducing, gram-negative, flagellated, sporeforming genus *Desulfotomaculum*. Dissimilatory reduction of sulfate, which distinguishes the genus *Desulfotomaculum* from the genus *Clostridium* (2), was, however, not observed in strain S_2 in media containing sulfate-pyruvate supplements. In addition, strain S_2 was not flagellated. The guanine-plus-cytosine content of DNA of strain S_2 was 40.8 versus 23 to 54 mol% in *Clostridium* and 41.7 to 45.5 mol% in *Desulfotomaculum* (2, 12). Taking these data into consideration, strain S_2 was tentatively classified in the genus *Clostridium*. Strain S_2 required CO_2 and vitamin K, a common feature in anaerobes. In addition, growth was strongly stimulated by taurine, a sulfur-containing substance, while the sulfur-containing amino acids cysteine and methionine were without effect. The metabolic role of taurine is at present unknown, but one could speculate that both the sulfonic acid group of taurine and inorganic sulfite might be used as terminal electron acceptors. As far as we know, strain S_2 and the previously isolated strain S_1 are the only

TABLE 4. Composition of fecal bile salts of germfree and gnotobiotic female rats

Bile salt	Germfree		Strain Cl_8[a]		Strains Cl_8 plus S_2[b]	
	% of total	% sulfated	% of total	% sulfated	% of total	% sulfated
CDCA	3.0	53	2.4	78	0.8	3
alloCDCA	8.3	90	9.8	95	1.4	14
3α-ol,7-oxo-cholanoate	2.8	91	3.7	95	0.7	69
CA	35.0	22	33.4	22	29.9	5
alloCA	5.6	53	5.2	71	4.6	
α-MCA	5.6	32	5.6	53	1.2	
HCA					2.3	
β-MCA	36.7	8	14.3	9	1.3	
ω-MCA					2.5	
Unsaturated β-MCA[c]			19.6	11	12.2	tr
Unsaturated ω-MCA[c]					43.0	tr
Unidentified	3.0	>95	6.0	>95	0.1	
Total % sulfated		30		37		2

[a] Gnotobiotic rats associated with *Clostridium* sp. Cl_8.
[b] Gnotobiotic rats associated with *Clostridium* sp. Cl_8 plus *Clostridium* sp. strain S_2.
[c] Derivatives of β-MCA and ω-MCA presenting with a double bond in the side chain between C-22 and C-23.

microorganisms in which taurine is reported to act as a growth factor.

Among the bile salt degradative activities of the intestinal microflora, the most common types are deconjugation and dehydrogenation of the 3α-, 7α-, or 12α-hydroxyl group. Strain S_2 produced 6-HSDH activity and incubation with muroCA or HDCA resulted in a similar mixture of reaction products: HDCA and 3α-hydroxy-6-oxo-5β-cholanoate. These observations can be explained by a reversible 6-epimerization reaction via a 6-oxo intermediate involving a 6α- and 6β-HSDH system and are in analogy with previous investigations on bacterial 3- and 7-epimerization of bile salts (21). It should be noted that 6-epimerization was not observed in cultures of *Clostridium* sp. strain S_1. Sacquet et al. (26) were first to report 6-epimerization of β-MCA into ω-MCA by a *Clostridium* sp. termed strain R6X76 which was isolated from the rat intestine. Conversion of β-MCA into ω-MCA was also reported to occur by the concerted activity of a *Eubacterium lentum* strain, producing 6β-HSDH, and an atypical *Fusobacterium* sp. reducing the 6-oxo group formed into a 6α-hydroxyl group (6). *Clostridium* sp. strain S_2 differs from Sacquet's *Clostridium* sp. strain R6X76 by the inability to ferment carbohydrates, the possession of bile salt deconjugating activity, and the production of hydrogen sulfide. Hence, strain S_2 is the second strain known to epimerize β-MCA into ω-MCA in pure cultures. In addition, strain S_2 converted α-MCA into HCA.

Bile salt sulfatase activity of *Clostridium* sp. strains S_1 and S_2 was limited to sulfate esters of the 3-hydroxyl group and depended upon the configuration at carbon atom C-5. The 3α-sulfate esters of 5β-bile salts were hydrolyzed by both strains and the 3β-sulfates of 5β-bile salts were hydrolyzed only by strain S_1. The 3α-oriented sulfate groups of 5α-bile salts were hydrolyzed only by strain S_2 and 3β-oriented sulfates were hydrolyzed only by strains S_1. The 7α- and 12α-monosulfated bile salts were not desulfated by either strain. The existence of microbial bile salt-7-sulfatase activity in the intestine might be questioned as bile salt sulfates, predominantly sulfoconjugated at the 7-hydroxyl group, are found in large amounts in the feces of both germfree and conventional mice (8). In vitro experiments on desulfation of 3-, 7-, and 12-monosulfated bile salts by mixed cultures of the intestinal flora of humans, rats, and mice also failed to demonstrate 7α- or 12α-sulfatase activity (15). It should also be noted that strains S_1 and S_2 produced bile salt sulfatase in media in which good growth occurred, whereas the previously described *Pseudomonas aeruginosa* produced sulfatase only under aerobic conditions on a minimal support medium with LCA-3-sulfate as the only sulfur source (16).

When female gnotobiotic rats were associated with *Clostridium* sp. Cl_8 plus strain S_2, the fecal 5α- and 5β-bile salt-3-sulfates were almost completely desulfated. Hence, the sulfatase activity of strain S_2 differed from that of strain S_1 which selectively left the 5α-bile salt sulfates unchanged (14). Moreover, in rats associated with *Clostridium* sp. Cl_8 plus strain S_2, the percentage of total alloCDCA decreased to less than one-fifth of the amounts found in germfree rats, in rats monoassociated with *Clostridium* sp. Cl_8, or rats associated with strains Cl_8 plus S_1. Fecal bile salts of gnotobiotic rats associated with strain S_2 and *C. perfringens* ATCC 19574 contained 12% of isoalloCDCA, which was more than 90% sulfated, and <1% of alloCDCA. This was not unexpected since *C. perfringens* also isomerized unsulfated alloCDCA into isoalloCDCA in vitro. Obviously, isoalloCDCA was reabsorbed and sulfated in the liver; subsequently, the compound accumulated in the large intestine because it was not desulfated by strain S_2. The results of these experiments indicate that microbially formed unsulfated alloCDCA and isoalloCDCA are efficiently reabsorbed from the cecum and the large intestine. These observations are in consonance with the results of previous studies on gnotobiotic rats associated with *Clostridium* sp. strain S_1. In these animals, microbially desulfated CA was efficiently reabsorbed from the intestine, resulting in a fivefold increase of the time required for excretion of 50% of the injected 24-^{14}C-labeled cholate-3-sulfate in the feces (10).

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