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Isolation of a Bile Salt Sulfatase-Producing *Clostridium* Strain from Rat Intestinal Microflora

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Bile acid sulfates, formed in human and rat livers, are desulfated by the intestinal microflora. In our study we first isolated from conventional rat feces an unnamed bacterium, termed strain S_1, which desulfated the 5β-bile salt 3α-sulfates in vitro and in vivo after association with gnotobiotic rats. Strain S_1 also possessed 12α-hydroxysteroid dehydrogenase and bile salt-deconjugating activities. The strain was a strict anaerobic, CO_2-requiring, gram-negative, sporeforming rod and was designated as belonging to the genus *Clostridium*. Growth was scarce in culture media, unless in the presence of 0.1% taurine, a sulfur-containing amino acid. Addition of this substance raised the number of bacteria in thioglycolate and peptone yeast media from 10^4 per ml to 10^6 to 10^7 per ml and increased the colony diameter on agar medium from 0.2 mm to 0.5 to 0.9 mm. Sulfatase activity was specific for the 5β-bile salt sulfates, leaving the 5α-bile salt sulfates unchanged. In addition, the sulfatase activity was cell bound, and its production was dependent on the composition of the culture medium, although no minimal sulfur medium was required for sulfatase activity.

Sulfation is an important step in bile acid metabolism and seems to be more pronounced in hepatobiliary diseases in humans (1, 3, 6, 18, 22, 25, 26). Sulfation of bile acids also occurs in normal rat liver (7, 20). After excretion of the bile acid sulfates via the bile, reabsorption of these more polar metabolites from the small intestine is limited (16); hence, sulfation of bile acids should tend to promote excretion via the fecal route. In the large intestine, however, the bile acid sulfates are desulfated due to bile acid sulfatase activity of the intestinal microflora. Significant amounts of bile acid sulfates could be demonstrated in the large intestine of germfree rats, whereas only trace amounts were detected in conventional rats (7, 8). Other authors (4, 14, 20) found that mixed populations of fecal bacteria hydrolyzed lithocholic acid sulfate in vitro, whereas they were unable to isolate individual strains carrying out this transformation. Imperato et al. (13) found that a strain of *Pseudomonas aeruginosa*, isolated from human feces, desulfated LCA sulfate when grown in a minimal medium under aerobic conditions.

Isolation and purification of a bacterial enzyme capable of hydrolyzing the bile salt sulfate ester bond would significantly improve the methodology of bile salt analysis, because the 18 h of acid solvolysis (23) could be replaced by a shorter enzymatic process. In this study we report on the isolation of a strictly anaerobic bile salt sulfatase-producing microorganism from the fecal flora of a conventional rat.

MATERIALS AND METHODS

Bacteriology. (i) Media and incubation techniques. The following media were used for isolation and identification of strain S_1: medium T, thioglycolate medium without added dextrose (BBL Microbiology Systems; no. 11718), supplemented with 0.5% yeast extract (Difco Laboratories); medium BB, containing brucella broth (GIBCO Diagnostics) and 0.05% 1,4-dithiothreitol; medium CMP, consisting of 1% tryptone yeast (Difco; no. 0769-01), 0.5% $Na_2HPO_4 \cdot 2H_2O$, 0.05% 1,4-dithiothreitol, and 1% powdered cooked-meat Phytone (BBL); and medium PYt, the peptone yeast medium of Holdeman et al. (12), supplemented with 0.1% taurine. Agar plates were prepared by adding 2% agar to medium T or BB. Other media used for growth and activity studies were Columbia broth (GIBCO), Schaedler broth (GIBCO), Todd-Hewitt broth (Oxoid Ltd.), tryptic soy broth (BD-Mérieux), reinforced clostridial medium (Oxoid), and brain heart infusion (BBL), all supplemented with 0.05% 1,4-dithiothreitol.

Inoculations and incubations were carried out at 37°C under 90% nitrogen–10% hydrogen in an anaerobic glove box, similar to that described by Aranki et al. (2). All media were prereduced in the glove box at least 48 h before inoculation. Tubes contained a maximum of 6 ml of liquid medium; agar plates were poured in the isolator. Large inocula (0.5 to 1 ml) were used for subculturing. Inoculated media were incubated in an anaerobic jar in the glove box; when required, 4%

CO_2 was added by placing a GasPak envelope (BBL) in the jar.

To study the sulfatase activity and growth in aerobic conditions, blood agar plates or 100-ml flasks with 10 ml of liquid culture broth were placed at 37°C in an aerobic incubator; liquid media were shaken to obtain adequate aeration.

(ii) Isolation techniques. Feces from conventional rats were immediately transferred into the anaerobic isolator. The sample was serially diluted in medium T; in addition, each dilution was streaked twice on blood agar and on medium T or BB agar plates for aerobic and anaerobic incubation. Isolates from the plates corresponding to the highest sulfatase-positive dilutions were subcultured in medium T or BB and checked for sulfatase activity. The same procedure was used to obtain simplified cultures from serial dilutions of fecal suspensions heated for 20 min at 70°C.

(iii) Identification procedures. Strain S_1 was identified according to the methods described by Holdeman et al. (12), with the following modifications: (a) the basal peptone yeast broth was supplemented with 0.1% taurine; (b) reduction of nitrates was investigated in the basal medium to which 0.1% sterile potassium nitrate solution was added after autoclaving; (c) hydrogen sulfide was detected in medium T plus 0.03% ferric ammonium citrate and 0.1% taurine; (d) colonial appearance and Gram staining were studied on medium T agar plates with and without 0.1% taurine; (e) motility was determined in semisolid basal medium; (f) gelatinase activity was examined by the method of Willis (28) on agar plates, consisting of medium T, 1.5% agar, 0.4% gelatin, and 0.2% taurine; (g) medium T agar plates with 0.1% taurine were prepared with 1% egg yolk (Oxoid) for study of catalase, lipase, or lecithinase production or with 10% sterile horse blood for detection of hemolysin production; (h) a heat test (10 min, 80°C) was performed on bacterial suspensions obtained from PYt agar slants with 1% cooked-meat Phytone.

(iv) Bacterial counts. Viable counts in pure cultures of strain S_1 were determined by making 10-fold dilutions in medium PYt and subsequently inoculating 0.1 ml of these dilutions on medium T agar plates with 0.1% taurine. Growth curves of strain S_1 in medium PYt were established by measurement of the optical density at 460 nm in a Fluoropoint Spectro-fluorimeter (Baird-Atomic Ltd.). At least two samples were taken every 2 h during the first day of incubation and every 6 h during the next 48 h.

Study of the in vitro bile acid transformations. (i) Bile acids used. Bile acids are referred to in the text by the following abbreviations for their trivial names: DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid); CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid); alloCDCA, allochenodeoxycholic acid (3α,7α-dihydroxy-5α-cholan-24-oic acid); CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid); alloCA, allocholic acid (3α,7α,12α-trihydroxy-5α-cholan-24-oic acid); 12-ketoLCA, 12-ketolithocholic acid (3α-hydroxy-12-oxo-5β-cholan-24-oic acid); 12-ketoCDCA, 12-ketochenodeoxycholic acid (3α,7α-dihydroxy-12-oxo-5β-cholan-24-oic acid); LCA, lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid).

DCA, CDCA, CA, 12-ketoLCA, 12-ketoCDCA, and 23-nordeoxycholic acid were obtained from Steraloids. The glycine and taurine conjugates of CDCA and CA were obtained from Maybridge. Tauro-β-muricholate was prepared from freeze-dried intestinal contents of germfree male rats (8).

LCA-3-sulfate, CDCA-3-sulfate, and the taurine and glycine conjugates were synthesized according to previously described methods (23). To prepare alloCDCA-3-sulfate, bile salts were extracted with aqueous 80% ethanol from freeze-dried feces of cholesterol-fed germfree female rats. After the extract was desalted over Amberlite XAD-2, bile acid sulfates were separated from their free acids (8) on Sephadex LH-20; the bile acid sulfate fraction was desalted, enzymatically deconjugated by cholylglycine hydrolase (19), and again desalted. This crude preparation mainly contained alloCDCA-3-sulfate and CDCA-3-sulfate in a ratio of 9:1. Conjugated alloCDCA-3-sulfate was prepared by the same method except that the enzymatic deconjugation was omitted.

(ii) Analysis of bile acid transformations in cultures. The culture media contained 50 μg of bile acid sulfate or bile acid conjugate per ml. To determine the amount of bacterially desulfated or deconjugated bile acids, 3 ml of culture medium was mixed with 0.5 ml of internal standard solution, containing 50 μg of 23-nordeoxycholic acid, and saponified for 1 h at 60°C with 1 ml of 40% KOH in ethanol. After extraction of sterols with light petroleum (bp, 40 to 60°C), the aqueous layer was acidified to pH 4.5, and the free bile acids were extracted twice with 6 ml of diethyl ether. The extract was evaporated, and bile acids were derivatized to their methyl ester acetates for gas-liquid chromatography on a column of 3% OV-1 at 265°C or 3% QF-1 at 240°C. Bile acid sulfates and conjugates were not affected by the saponification or by the acidification to pH 4.5 and remained in the aqueous layer at the diethyl ether extraction. Samples from sulfatase-negative cultures yielded minor amounts of free bile acids (3 to 7%); hence, cultures containing more than 15% unsulfated bile acids were considered to be sulfatase positive.

The amount of bile acid sulfates in the culture medium was calculated by subtracting the amount of unsulfated bile acids from the total bile acids. To determine the total bile acids, 3 ml of culture medium plus 0.5 ml of internal standard solution (50 μg of 23-nordeoxycholic acid) were diluted with 7 ml of water and mixed with 2 g of Amberlite XAD-2 for 15 min; after centrifugation at 3,000 × *g* for 10 min, the XAD-2 was washed with 10 ml of water and recentrifuged. Total bile acids were eluted from XAD-2 with 2× 10 ml of methanol–25% ammonium hydroxyde (100:1); the eluate was filtered, 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. After evaporation, 5 ml of 20% NaOH was added, and the samples were further processed for gas-liquid chromatography as described above. By this procedure, more than 80% of the unsulfated bile acids were measured; recovery of total bile acids was 75 to 85%.

Studies in gnotobiotic and conventional rats. (i) Animals and diet. Germfree and gnotobiotic female Fischer rats were kept in Trexler plastic isolators. Conventional rats were housed in normal animal quarters. All animals were 3 months old at the start of the experi-

ment and received a steam-sterilized commercial diet (SMRA-1210, Hope Farms). Implantation of the strains in the digestive tract of gnotobiotic rats occurred via the anal route with a soft plastic cannula fitted to a syringe.

(ii) Analysis of fecal bile acids. Samples of feces were homogenized with an equal volume of water and freeze dried. Fecal bile acid composition was determined by methods described elsewhere in detail (8). Briefly, bile salts were extracted from 0.25 to 0.5 g of freeze-dried feces with aqueous 80% ethanol for 4 h at 65°C; after evaporation of the solvent and desalting over a column of Amberlite XAD-2, the methanol eluate was divided into two equal portions (Fig. 1). One aliquot was evaporated, solvolyzed, and again divided into two equal samples, A and B. To determine the total bile acids, sample A was submitted to alkaline deconjugation, followed by removal of sterols with light petroleum, acidification of the aqueous layer to pH 1, extraction of the bile acids with diethyl ether, and gas chromatography of their methyl ester acetates. To determine the unconjugated bile acids, sample B was acidified and the unconjugated bile acids were extracted and converted into the methyl ester acetates, which were purified from sterols by chromatography over a column of silica gel before analysis by gas-liquid chromatography. The second aliquot was chromatographed on a column of Sephadex LH-20 to separate sulfated and unsulfated bile acids. The unsulfated fraction was divided into samples C and D. To determine the total unsulfated bile acids, sample C was submitted to alkaline deconjugation and further treated as for sample A. In sample D, sterols were removed with light petroleum, the water phase was acidified, and the unconjugated unsulfated bile acids were extracted and derivatized for gas-liquid chromatography. The sulfated fraction was desalted, solvolyzed, and divided into samples E and F; sample E was treated as for sample A to determine the conjugated bile acid sulfates; sample F was treated as for sample B to determine the unconjugated bile acid sulfates.

RESULTS

Isolation of strain S_1. A 1% suspension of female rat feces in medium T developed sulfatase activity only in anaerobic conditions. When feces was serially diluted in medium T or BB, three of five of the 10^{-7} dilutions were sulfatase positive after 5 days of incubation; total counts showed 5×10^9 to 10×10^9 anaerobes per g of feces. However, none of 200 isolates from plates inoculated with unheated fecal dilutions were sulfatase positive. Isolate S_1 was obtained through progressive purification of cultures from a fecal suspension that had been heated for 20 min at 70°C. During this isolation procedure, the most simplified cultures consisted of strain S_1 and three sulfatase-negative *Clostridium* strains. Colonies of strain S_1 were smaller than 0.2 mm and failed to develop when transferred to medium T or BB. However, isolate S_1 could be subcultured in medium CMP or PYt when incubated with 4% CO_2. Later, the isolation procedure was improved after it was found that growth of strain S_1 was stimulated by taurine.

Characterization of strain S_1. Strain S_1 appeared as nonmotile straight rods, 2 to 5 by 0.5 to 0.6 μm, sometimes exhibiting central swellings and occurring singly, in pairs, or occasionally in short chains in young cultures (24 to 36 h) or surface colonies. Cells from older cultures (4 to 5 days) or cells grown in less reduced conditions were pleomorphic, often swollen, and arranged in long filaments of 40 to 75 μm. The microorganism stained gram negative; on the other hand, it was inhibited on agar plates by disks with 5 μg of vancomycin, whereas it was resistant to disks with 10 μg of colimycin.

Strain S_1 required strict anaerobic conditions

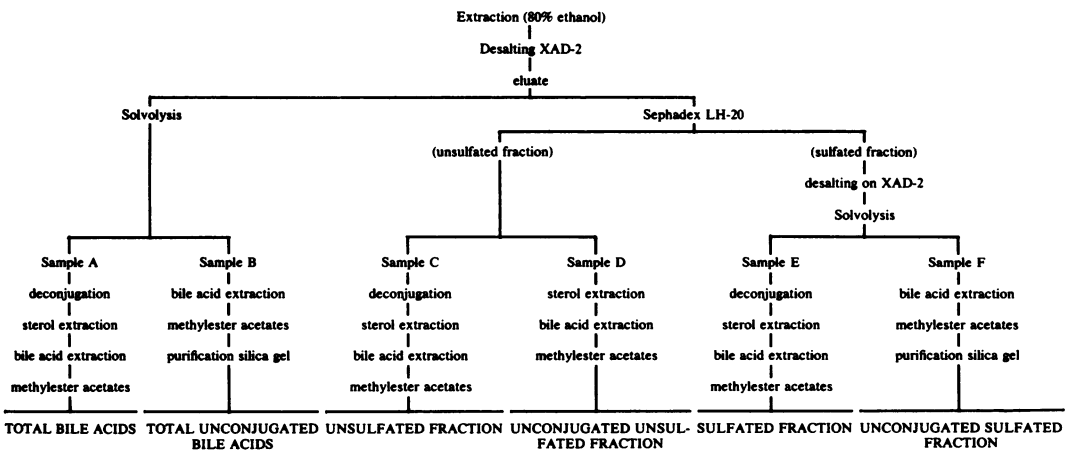

FIG. 1. Analytical procedure to determine (un)conjugated and (un)sulfated fecal bile acids; total bile acids (sample A) = unsulfated fraction (sample C) + sulfated fraction (sample E); total unconjugated bile acids (sample B) = unconjugated unsulfated fraction (sample D) + unconjugated sulfated fraction (sample F).

and the presence of CO_2. Even in the anaerobic isolator, the strain did not grow in media without a reducing agent, e.g., 0.06% thioglycolate, 0.05% dithiothreitol, or 0.05% cysteine. Respecting these requirements, growth still remained poor, without development of turbidity or sediment on 10 nutritive media tested (Table 1). Viable counts showed that cultures in these media contained, at maximum, 10^4 bacteria per ml. Incubation with more CO_2 (8 to 12%) did not enhance growth. No better growth was obtained by addition of the following supplements to medium T: 10% serum, 0.5% Biosate peptone (BBL), 0.5% proteose peptone (BBL), 2% Phytone (BBL), 1% cooked-meat Phytone, 2% yeast extract, 0.5% liver digest (Oxoid), 0.5% beef extract (Difco), 1% glucose, 1 to 20 μg of hemin or menadione per ml, organic (27) and inorganic (12) salt mixtures, 0.01 to 0.01% potassium nitrate, or 0.1 to 0.01% sodium sulfate, sulfite, bisulfite, or sulfide. Concentrations of 0.1% of the following amino acids also failed to stimulate growth: glycine, L-alanine, L-serine, L-threonine, DL-methionine, DL-lysine, L-cystine, D-glutamine, L-arginine, and L-cysteine. Growth was inhibited by 0.1% Tween 80 and 2% oxgall (Oxoid). On increasing the amount of CDCA-3-SO_4 10-fold, growth was preserved, but not stimulated.

In contrast, addition of 0.1% taurine to medium T induced moderate turbidity: viable counts showed 10^6 to 10^7 bacteria per ml. Higher concentrations of taurine did not cause additional growth stimulation, and the growth-stimulating effect of taurine did not occur in all media (Table 1): it was present in media T, CMP, and PY, Columbia broth, and Schaedler broth, but absent in medium BB, reinforced clostridial medium, Todd-Hewitt medium, tryptic soy broth, and brain heart infusion broth. In medium PY and in Schaedler broth plus taurine, a smooth, black or greyish sediment was formed, which disappeared after 3 to 5 days of incubation due to lysis of the cells. This lysis was reduced by adding 0.6% agar, 0.5% Casamino Acids, or 0.5% beef extract. In medium CMP, the cooked-meat particles turned dark during incubation with 0.1% taurine.

The presence of taurine also increased the size of the surface colonies of strain S_1 on agar plates. After 7 days of incubation on medium T agar plates without supplements of taurine, colonies were <0.2 mm in diameter, entire, convex, smooth, colorless, shiny, and transparent (Fig. 2A); when grown on the same agar medium admixed with 0.1% taurine, the colony diameter increased to 0.9 mm (Fig. 2B), and they became dull and opaque. Surface colonies were not observed on agar media without reducing agent or on blood agar plates, regardless of the presence of taurine and CO_2.

The heat test (10 min, 80°C) was positive on suspensions of cultures grown for 8 to 12 days on PYt agar slants supplemented with 1%

TABLE 1. Growth, sulfatase activity, and deconjugating activity of strain S_1 in different culture media with or without 0.1% taurine

Medium[a]	Growth[b]		% Desulfated[c]		% Deconjugated[d]	
	− Taurine	+ Taurine	− Taurine	+ Taurine	− Taurine	+ Taurine
PY	+	+++ sL	94	90	90	90
Schaedler broth	+	+++ sL	86	93	94	89
T	+	+++	93	17	90	92
Columbia broth	+	++ L	90	28	92	86
CMP	+	++ e	80	26	84	81
Reinforced clostridial medium	+	+	88	13	89	88
Tryptic soy broth	+	+	94	12	93	88
Brucella broth	+	+	14	11	90	85
Todd-Hewitt broth	+	+	9	5	97	93
Brain heart infusion	+	+	4	5	85	85
T + ferric ammonium citrate (0.03%)	+	++ f	85	88	90	91

[a] All media contained 0.05% dithiothreitol, except medium PYt (containing 0.05% cysteine) and medium T (containing 0.06% thioglycolate), and were inoculated with 0.5 ml of fresh suspensions in prereduced NaCl (0.9%) of strain S_1 grown for 5 days on medium T agar slants with 0.1% taurine.

[b] +, No turbidity, only microscopically visible growth; ++, turbidity, weak growth; +++, turbidity, moderate growth; s, small sediment; L, lysis after 4 days of incubation; e, darkening of the cooked-meat Phytone sediment; f, precipitation of ferric sulfide.

[c] Presence of sulfatase activity is expressed by the percentage of free CDCA after incubation with 50 μg of CDCA-3-sulfate per ml; each value represents the mean of the results of three cultures and their respective subcultures.

[d] Presence of deconjugating activity is expressed by the percentage of free CA plus its 12-keto derivative, after incubation with 50 μg of tauro-CA per ml.

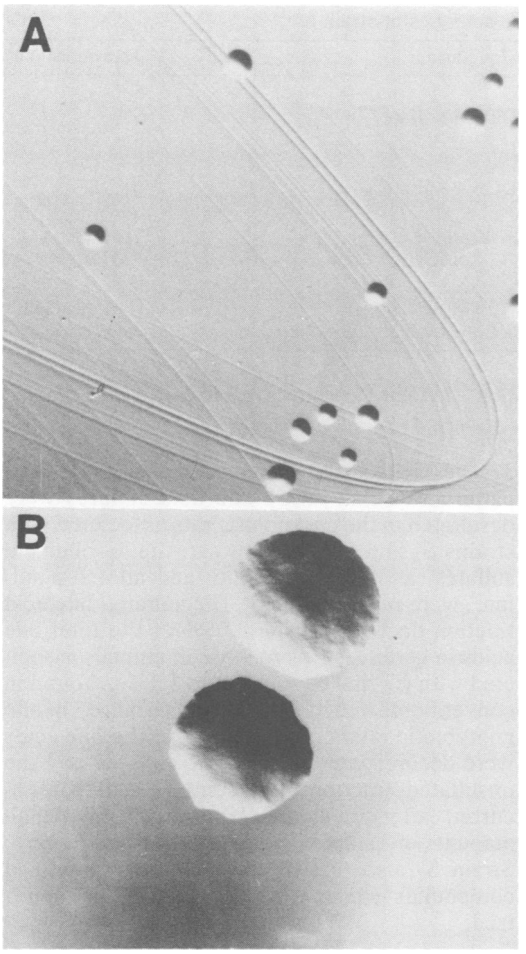

FIG. 2. Colony morphology of strain S_1 on medium T agar plates (×20). (A) Without taurine supplement: round, shiny colonies <0.2 mm in diameter; (B) with 0.1% taurine: round or irregular, dull colonies ≃0.9 mm in diameter.

cooked-meat Phytone; spores were small, spherical, and mostly subterminal. Sporulation in culture broth was rarely observed.

Strain S_1 did not ferment glucose, fructose, maltose, D-mannose, saccharose, xylose, ribose, mannitol, or starch. Volatile fatty acids, produced in 5-day-old cultures on medium PYt with or without glucose, were acetic acid (<1 meq/100 ml) and traces of propionic, butyric, isobutyric, and isovaleric acids. Nonvolatile fatty acids were not produced. The strain did not produce indole, catalase, gelatinase, lecithinase, lipase, or urease, did not hydrolyze esculin, and did not digest meat or milk. Neither threonine, lactate, nor pyruvate was used. The strain produced hydrogen sulfide and small amounts of ammonia. Nitrates were reduced; nitrites, however, were absent, even in the presence of excess nitrates.

Strain S_1 belongs to the genus *Clostridium* because of its sporeforming capacity. It should be classified as a member of group I (subterminal spores, no gelatin liquefactions) or group V (species with special growth requirements), as defined in *Bergey's Manual* (5); it does not correspond to any of the species described.

In vitro enzyme activities of strain S_1. Strain S_1 developed bile salt sulfatase, bile salt deconjugating, and 12α-hydroxysteroid dehydrogenase activities (Table 2).

In medium PYt, strain S_1 desulfated LCA-3-sulfate and CDCA-3-sulfate, but not alloCDCA-3-sulfate. Conjugated alloCDCA-3-sulfate was readily deconjugated but not desulfated, whereas the glycine and taurine conjugates of CDCA-3-sulfate were deconjugated and desulfated.

The bile salt sulfatase activity was not produced in any of the culture media tested and was influenced by the presence of taurine (Table 1). In medium PY and in Schaedler broth, CDCA-3-sulfate was desulfated, irrespective of taurine supplementation. In T, CMP, Columbia broth, tryptic soy broth, and reinforced clostridial medium, addition of 0.1% taurine depressed the sulfatase activity, regardless of the growth-stimulating effect: at maximum, 28% CDCA-3-sulfate was hydrolyzed after 5 or more days of incubation. In medium BB, Todd-Hewitt broth, and brain heart infusion broth, no sulfatase activity was found, irrespective of the presence of taurine. In medium T, the sulfatase activity reappeared when 0.03% ferric ammonium citrate was added before incubation.

Strain S_1 deconjugated the glycine and taurine conjugates of CDCA and CA and also tauro-β-muricholate. This deconjugating activity was present in all of the media tested and was not influenced by the addition of taurine (Table 1).

Strain S_1 developed 12α-hydroxysteroid dehydrogenase activity on DCA and CA, with the production of the respective 12-keto derivatives. These 12-keto compounds were only present during the logarithmic stage of growth and were quickly reconverted into DCA and CA, respectively, at the stationary stage. 3α-Dehydrogenase and 7α-dehydrogenase activities were absent.

Studies on the correlation between cell growth and enzyme activity were carried out in medium PYt supplemented with CDCA-3-SO_4 and tauro-CA. The results of these experiments demonstrated that desulfation was associated with the phase of active growth and started 6 to 8 h after inoculation (Fig. 3). Desulfation was complete in 12 h. In contrast, deconjugation was already completed before active growth started, probably due to extracellular deconjugating enzyme

TABLE 2. In vitro enzyme activities of strain S_1

Substrate[a]	End product(s)	Transformation[b]
CDCA-3-sulfate	CDCA	S+
AlloCDCA-3-sulfate	AlloCDCA-3-sulfate	S−
Tauro-CDCA-3-sulfate	CDCA	S+, D+
Glyco-CDCA-3-sulfate	CDCA	S+, D+
Tauro-alloCDCA-3-sulfate	AlloCDCA-3-sulfate	S−, D+
LCA-3-sulfate	LCA	S+
DCA	DCA + 12-ketoLCA	12αDeh
CA	CA + 12-ketoDCA	12αDeh
Tauro-CDCA	CDCA	D+
Tauro-CA	CA + 12-ketoDCA	D+, 12αDeh
Tauro-β-muricholic acid	β-Muricholic acid	D+

[a] Substrates were added to medium PYt.
[b] S+, Desulfation; S−, no desulfation; D+, deconjugation; 12αDeh, 12α-dehydrogenation.

transferred with the large inoculum (0.5 to 1 ml). The deconjugating activity was also preserved in the filtrate of a 3-day-old culture, filtered through a membrane filter (0.22 μm; Millipore Corp.) and in the supernatant of 1-day-old cultures centrifuged for 30 min to 1 h at 6,250 × *g*. In contrast, both of the other enzyme activities were absent from the filtrate and the supernatant.

Bile acid transformation in gnotobiotic rats. Stain S_1 did not become established as a monoassociate in the digestive tract of germfree female rats, even after eight successive inoculations via the oral and anal routes. Implantation of the strain was successful after previous lowering of the oxidation-reduction potential of the cecum by administration of another *Clostridium* strain, Cl_8, previously isolated from the cecal content of a conventional rat. Strain Cl_8 did not produce sulfatase activity or other bile salt transformations in gnotobiotic rats.

The composition of fecal bile acids of the germfree and gnotobiotic rats is shown in Table 3. The most striking change in fecal bile acid pattern was that all 5β-bile acid sulfates were desulfated in the gnotobiotic rats associated with strains S_1 and Cl_8. In contrast, the 5α-bile acid sulfates, alloCDCA-3-sulfate and alloCA-3-sulfate, were not desulfated. The sulfated bile acid fraction decreased from 22.5% of the total bile acids in germfree rats to 9.8% in animals associated with Cl_8 and S_1, but not to 1.1% as found in conventional female rats. Furthermore, in the gnotobiotic rats more than 90% of the bile acids were deconjugated in both the sulfated and the unsulfated fractions. In contrast to what occurred after incubation in vitro, only small amounts of 12-ketoCDCA were formed in vivo. Strain S_1 also produced 16.7% of unidentified compounds which were not further characterized.

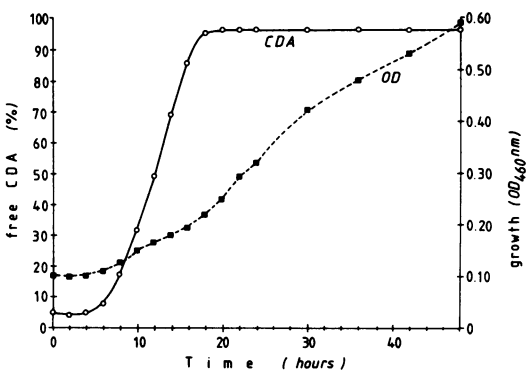

FIG. 3. Desulfation of CDCA-3-SO_4 by strain S_1 versus its cell growth: percent free CDCA (○) and optical density (OD) at 460 nm (■) in medium PYt containing 50 μg of CDCA-3-SO_4 per ml.

DISCUSSION

Since toxic bile acids such as LCA (15) may be converted in the liver into less toxic (9) and less efficiently reabsorbable (16) sulfate esters, the questions arise as to whether these products are desulfated by the intestinal microflora and whether this transformation occurs in intestinal segments from where absorption of the free bile acid still takes place. In the present investigations, we isolated from feces of a conventional rat an unnamed *Clostridium* strain, termed S_1, which desulfates 5β-bile salt 3α-sulfates, the predominant bile salt sulfates formed by the human liver (1). This strain, which belongs to the indigenous microflora of rats, was characterized by an extreme sensitivity to oxygen, poor growth in nutritive broth, stimulation of growth by taurine, and the presence of three bile salt-transforming enzyme activities: desulfation, deconjugation, and 12α-dehydrogenation.

Among the bile salt degradative activities of the intestinal microflora, the most common types are deconjugation and dehydrogenation of the 3α-, 7α-, and 12α-hydroxyl groups. Strain S_1

TABLE 3. Composition of fecal bile acids of germfree and gnotobiotic female rats

Bile acid	Germfree[a]		Gnotobiotic[b]		
	% of total	% Sulfated	% of total	% Sulfated	% Deconjugated
3β-Hydroxy-5-cholenic acid	tr[c]	>90	tr	—[d]	?
CDCA	3.3	54.1	3.0	—	>95
AlloCDCA	9.5	84.6	9.2	90.3	>95
CA	43.2	10.6	41.4	—	90.3
AlloCA	3.0	51.3	2.9	50.2	92.5
α-Muricholic acid	4.7	35.2	4.1	—	90.0
β-Muricholic acid	34.9	5.7	17.5	—	80.6
12-KetoCDCA	—	—	5.2	—	>95
Unidentified compounds	1.4	33	16.7	—	?

[a] Bile acids of germfree rats were more than 95% conjugated. Total sulfated, 22.5%.
[b] Gnotobiotic rats associated with *Clostridium* strains Cl_8 and strain S_1. Total sulfated, 9.8%.
[c] tr, Less than 1%.
[d] —, Not detected.

deconjugated glycine and taurine conjugated bile acids in vitro and in vivo. The bile salt hydrolases were secreted extracellularly, as revealed by the results of studies on bacteria-free culture filtrates and by comparison of the enzyme activity versus cell growth. Concerning the bile salt dehydrogenase activity, strain S_1 only dehydrogenated the 12α-hydroxyl group of DCA and CA during active growth; 3α- and 7α-dehydrogenase activities were not found. The presence of 12α-dehydrogenases seems to be less common among *Clostridium* strains, which more frequently possess 3α- or 7α-dehydrogenase or both (17).

Available information concerning bile salt-desulfating microorganisms is scarce and mostly limited to results from mixed fecal cultures (4, 14), except for the studies of Imperato et al. (13), who described a desulfating strain of *P. aeruginosa*. Since *Pseudomonas* requires aerobic growth conditions, its significance as a desulfating microorganism in the colonic ecosystem is questionable. Hence, desulfating activity is supposed not to be widely distributed among the intestinal microorganisms. However, our study also indicated that it may be difficult to demonstrate bile salt sulfatase activity in pure cultures since the sulfatase activity of strain S_1 was influenced by the composition of the culture medium.

Growth of strain S_1 was stimulated by the sulfur-containing amino acid taurine, but not by inorganic sulfur compounds (e.g., sodium sulfate, sodium sulfite, sodium bisulfite) or by amino acids with a sulfydryl group (e.g., methionine and cysteine). However, the growth-stimulating effect of taurine did not occur in all media. Whether this was due to the absence of other, as yet unknown growth factors remains to be investigated.

It also remains to be established why sulfatase activity, first, did not occur in all media tested and, second, was depressed by the presence of taurine in some media. Somehow we can refer to the complex mechanisms of inhibition, repression, and derepression, observed in studies on arylsulfatases and short-chain alkylsulfatases of aerobic soil microorganisms; the formation and activity of these enzymes are influenced by the presence of other sulfur sources (10), the carbon content of the medium (10), and the energy availability, such as ATP levels (11). The bile salt sulfatase produced by *P. aeruginosa* (13) is also controlled by the sulfur and carbon content of the medium, and the sulfatase is only produced in a minimal support medium with a bile salt sulfate as the sole sulfur source (13). Strain S_1, in contrast, displayed bile salt sulfatase activity in vitro in several types of nutritive broth and in vivo in the intestinal tract after association with gnotobiotic rats. The bile salt sulfatase, however, was depressed by the growth-stimulating factor taurine, except in medium PY and in Schaedler broth, which might be due to the presence of a derepressing substance in these two media. Methionine, for instance, serves as a derepressor for synthesis of the arylsulfatase of *Aerobacter aerogenes* (24). To obtain insight into the regulation mechanisms of sulfatase production, studies on a minimal medium should be carried out. Such a medium, however, has not yet been developed for strain S_1.

The bile salt sulfatase activity of strain S_1 was shown to be cell bound and specific in respect to the stereoconfiguration of carbon atom C-5 on the bile acid molecule; in vitro, as well as in vivo, strain S_1 desulfated the 5β-bile acid sulfates, but not the 5α-bile acid sulfates. Hence, alloCDCA-3-sulfate, the predominant bile acid sulfate in female rats (8), was not desulfated. Since 5α-bile acid sulfates are desulfated in the

intestinal tract of conventional rats (8), other desulfating microorganisms should be present in the intestinal microflora.

Whether desulfation of the 5β-bile acid-3α-sulfates in the human intestinal tract is due to the same or a related microorganism remains an open question. In our studies, strain S_1 produced LCA as the sole metabolite from LCA-3-sulfate. In contrast, Kelsey et al. (14) found four major metabolites after incubation of LCA-3-sulfate with human feces: 3β-palmitoyl-isoLCA, chol-3-en-24-oic acid, LCA, and isoLCA. It is not known, however, whether these products were formed by a single microorganism or through combined action of several microorganisms in the mixed cultures.

To what extent microbial desulfation influences the reabsorption and excretion rates of bile acids cannot be deduced from our in vivo experiments with gnotobiotic rats associated with strain S_1 since the microorganism only desulfated the 5β-bile acids. In addition, strain S_1 also deconjugated the bile acids, and this activity could affect bile acid excretion. Further, it remains to be established whether deconjugation must precede the desulfation. Isolation of the sulfatase would answer this question and would provide a means for application of the enzyme to facilitate the determination of bile acids in human serum and urine.

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