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Specificity of Bile Salt Sulfatase Activity from *Clostridium* sp. Strain S_1

SUZANNE M. HUIJGHEBAERT* AND HENDRIK J. EYSSEN

The Rega Institute for Medical Research, University of Leuven, B-3000 Leuven, Belgium

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Clostridium sp. strain S_1, an unnamed bile acid-desulfating strain from rat intestinal microflora (S. M. Huijghebaert, J. A. Mertens, and H. J. Eyssen, Appl. Environ. Microbiol. **43**:185–192, 1982), was examined for its ability to desulfate different bile acid sulfates and steroid sulfates in growing cultures. *Clostridium* sp. strain S_1 desulfated the 3α-monosulfates of chenodeoxycholic, deoxycholic, and cholic acid, but not their 7α- or 12α-monosulfates. Among the 3-sulfates of the 5α- and 5β-bile acids, only bile acid-3-sulfates with an equatorial sulfate group were desulfated. Hence, *Clostridium* sp. strain S_1 desulfated the 3-sulfates of bile acids with a 3α,5β-, a 3β,5α- or a 3β,Δ^5-structure. In contrast, the bile acid-3-sulfates with a 3β,5β- or a 3α,5α-structure were not desulfated. In addition, *Clostridium* sp. strain S_1 did not hydrolyze the equatorial 3-sulfate esters of C_{19} and C_{21} steroids and cholesterol or the phenolic 3-sulfate esters of estrone and estradiol. 23-Nordeoxycholic acid with a C-23 carboxyl group was also not desulfated, in contrast to the 5β-bile acid 3α-sulfates with a C-24 or C-26 carboxyl group. Therefore, the specificity of the sulfatase of *Clostridium* sp. strain S_1 is related to the location of the sulfate group on the bile acid molecule, the equatorial orientation of the sulfate group, and the structure of the C-17 side chain, its carboxyl group, and chain length.

Mammalian sulfatases (11, 13, 20), as well as sulfatases from aerobic soil bacteria (12), fungi (21), and the snail *Helix pomatia* (17) have been studied extensively for the hydrolysis of phenolic sulfate esters, 3β-sulfate esters of Δ^5-steroids, or short-chain alkylsulfates. The desulfation of lithocholic acid (LCA)-3-sulfate by cultures of *Pseudomonas aeruginosa* has been described by Imperato et al. (16). Recently, we isolated a strictly anaerobic, bile salt sulfatase-producing bacterium, termed *Clostridium* sp. strain S_1, from indigenous rat intestinal microflora (15). Little is known about the specificity of these bacterial sulfatases or their physiological significance in the metabolism of bile acid and steroid sulfoconjugates. Since sulfated bile acids and steroids are reabsorbed from the small intestine less efficiently than are their unsulfated form (2, 5, 27), sulfoconjugation should promote fecal excretion during enterohepatic circulation. Likewise, bacterial sulfatase activity might interfere with the excretion and promote reabsorption.

In our present study, the substrate specificity of the sulfatase of *Clostridium* sp. strain S_1 was further characterized, using growing cultures. In vivo, the location of sulfate esterification may vary from species to species (1, 3, 8, 10, 22, 23) and, presumably, from organ system to organ system (4, 7, 8). Also di- and trisulfated bile acids may be formed in cases of cholestasis (26). For these reasons, *Clostridium* sp. strain S_1 was incubated with bile acid substrates having their sulfate group in the 3α-, 7α-, or 12α-position. Further, *Clostridium* sp. strain S_1 was tested for its ability to desulfate bile acid substrates with a different configuration in carbon atoms C-3 and C-5. We noted previously a specificity of *Clostridium* sp. strain S_1, in vitro in culture and in vivo after association with gnotobiotic rats, for the 5β-bile acid 3α-sulfates of the rat, whereas the 5α-bile acid 3α-sulfates were unchanged (15).

In addition, since C_{19} and C_{21} steroid sulfates are desulfated in the gut through microbial action (2, 6), these steroid sulfates and bile acid 3-sulfates of varying chain lengths were used to further examine the substrate specificity of the sulfatase activity.

Since it was difficult to prepare buffered cell suspensions under strictly anaerobic conditions and sulfatase activity was lost during aerobic cell washing, growing cultures of *Clostridium* sp. strain S_1 were used for all experiments in this study.

MATERIALS AND METHODS

Strains and cultural procedures. *Clostridium* sp. strain S_1 was previously isolated in our laboratory from the feces of female conventional rats and was

characterized as a strict anaerobic sporeforming species (15). The strain was cultured at 37°C in an anaerobic glove box, under 10% hydrogen and 90% nitrogen.

Stock cultures were maintained in a medium corresponding to the peptone-yeast extract medium of Holdeman et al. (14); the 0.5% peptone, however, was replaced by 0.5% Trypticase (BBL Microbiology Systems, Cockeysville, Md.) to avoid the interference with taurocholic acid present in peptone (Difco Laboratories, Detroit, Mich.), and 0.1% taurine was added to promote growth (15). After autoclaving, the media (5 ml) were immediately introduced into the isolator and prereduced for at least 48 h. Cultures were incubated in the isolator in an anaerobic jar with a carbon dioxide-generating GasPak (BBL) envelope. Inocula were about 10% (vol/vol) from 2-day-old cultures.

Transformation of bile acid sulfates or steroid sulfates by growing cultures. *Clostridium* sp. strain S_1 was cultured in the anaerobic glove box as described for the stock cultures, but was incubated for 5 days before analysis. The assay medium contained 0.005% chenodeoxycholic acid 3-sulfate (internal control) or 0.005% of the sulfated substrate under investigation or both. The substrates were added to the culture media in the anaerobic isolator before inoculation: 0.25 ml of aqueous bile salt sulfate solution (1 mg/ml, sterilized by filtration) or 0.1 ml of ethanolic steroid sulfate suspension (2.5 mg/ml, aseptically prepared). The bile salt sulfates were soluble in water as disodium salts. The steroid sulfates dissolved in the culture medium, and the small amount of ethanol did not interfere with the growth of strain S_1 or the desulfation of chenodeoxycholic acid 3-sulfate.

Analysis of the desulfated bile acids and steroids. The amount of unsulfated bile acids was determined as described previously (15), but with the omission of the sterol extraction. Briefly, after the addition of an internal standard (23-nordeoxycholic acid), 3 ml of culture broth was saponified for 1 h at 60°C with 1 ml of 40% aqueous KOH and 3 ml of ethanol. The free bile acids were extracted from the acidified aqueous layer (pH 4.5) with diethylether. After evaporation, bile acids were estimated by gas-liquid chromatography of their methylester acetates on a column of 3% OV_1 at 265°C.

To measure sulfatase activity on steroid 3-sulfates other than bile acid sulfates, 3 ml of the grown culture was mixed with 0.2 ml of a 500-μg/ml 5α-cholestane solution in methanol (the internal standard) and 5 ml of 20% NaCl in water; free steroids were extracted with light petroleum (bp, 40 to 60°C) and quantified by gas-liquid chromatography on a column of 3% OV_1 at 230°C.

Bile acids and steroids. Bile acids are referred to in the text by the following abbreviations or trivial names for their systematic names: LCA, (3α-hydroxy-5β-cholan-24-oic acid); isoLCA, isolithocholic acid (3β-hydroxy-5β-cholan-24-oic acid); isoalloLCA, isoallolithocholic acid (3β-hydroxy-5α-cholan-24-oic acid); 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); 3β-OH-cholenoic acid (3β-hydroxy-5-cholen-24-oic acid); 23-norDCA, 23-nordeoxycholic acid (3α,12α-dihydroxy-24-nor-5β-cholan-23-oic acid); trihydroxycoprostanic acid (3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid).

The following steroids are mentioned with their trivial names: androsterone (3α-hydroxy-5α-androstan-17-one); epiandrosterone (3β-hydroxy-5α-androstan-17-one); etiocholanolone (3α-hydroxy-5β-androstan-17-one); dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one); cholesterol (3β-hydroxy-5-cholestene); pregnenolone (3β-hydroxy-5-pregnen-20-one); 5α-pregnanolone (3β-hydroxy-5α-pregnan-20-one); 17β-estradiol (3,17β-dihydroxy-1,3,5-estratriene); estrone (3-hydroxy-1,3,5-estratriene-17-one). These free steroids and their 3-sulfates were purchased from Steraloids (Wilton, N.H.).

LCA, isoLCA, isoalloLCA, DCA, CDCA, CA, and 3β-OH-cholenoic acid were obtained from Steraloids. The taurine and glycine conjugates of LCA, DCA, CDCA, and CA were supplied by Maybridge (Trevilett, Tintagel, U.K.). Trihydroxycoprostanic acid was isolated from the bile of *Alligator mississippiensis* (9).

The following bile acid sulfates were synthesized in our laboratory by previously described methods (24, 25): LCA-3-sulfate, isoLCA-3-sulfate, isoalloLCA-3-sulfate, DCA-3-sulfate, DCA-12-sulfate, CDCA-3-sulfate, CDCA-7-sulfate, CA-3-sulfate, CA-7-sulfate, CA-12-sulfate, 5-cholenoic acid 3β-sulfate, and trihydroxycoprostanic acid 3-sulfate. The monosulfates in position C-3, C-7, or C-12 of the taurine and glycine conjugates of LCA, DCA, CDCA, and CA were prepared from their unsulfated conjugated bile acids by the same methods (25). AlloCDCA-3-sulfate and its conjugated form were obtained from the feces of cholesterol-fed germfree female rats as described elsewhere (15).

The purity of the bile salt sulfates and steroid sulfates was assured by chromatographing these conjugates on silica gel-coated plates (thin layer) with different solvent systems as described elsewhere (24, 25).

RESULTS AND DISCUSSION

Influence of the location of the sulfate group in the bile acid molecule. As shown in Table 1, *Clostridium* sp. strain S_1 desulfated the 3α-monosulfates of LCA, DCA, CDCA, and CA. In contrast, it did not desulfate the 7α-monosulfates of CDCA and CA or the 12α-monosulfates of DCA and CA. In humans and rats, the hydroxyl group at C-3 is the most important place of sulfation (1, 7, 8, 26). However, the feces of humans and conventional rats contain only trace amounts of sulfated bile acids, due to the bacterial sulfatase activity in the intestine (8, 19). Conventional mice excrete quantitative amounts of cholic acid 7-sulfate, suggesting that bacterial sulfatase activity for bile acid 7α-sulfates is absent (10).

Clostridium sp. strain S_1 also deconjugated and desulfated the glycine and taurine conjugates of the bile acid 3α-sulfates. Glycine- or taurine-conjugated bile acid 7α- or 12α-monosul-

TABLE 1. Influence of the position of the sulfate group and effect of taurine and glycine conjugation on the sulfatase activity of *Clostridium* sp. strain S_1

Position of sulfate	Bile acid sulfate of:	% Desulfated[a]		
		Unconjugated	Taurine conjugated	Glycine conjugated
3α-Sulfate	LCA	88	87	100
	DCA	85	90	87
	CDCA	96	100	85
	CA	90	85	85
7α-Sulfate	CDCA	7	7	9
	CA	3	2	NT[b]
12α-Sulfate	DCA	2	1	3
	CA	1	NT	NT

[a] The presence of sulfatase activity is expressed by the percent free bile acid formed by *Clostridium* sp. strain S_1 after 5 days of incubation in culture medium with 50 μg of unconjugated, taurine-conjugated, or glycine-conjugated bile acid sulfate per ml. More than 80% = sulfatase activity present; less than 9% = sulfatase activity absent.

[b] NT, Not tested.

fates were deconjugated, but not desulfated. Hence, sulfation of bile acids probably does not prevent bacterial deconjugation, but we could not determine whether deconjugation precedes the microbial desulfation of these substrates. In this respect, one could refer to the microbial dehydroxylation of bile acid conjugates by an intestinal *Eubacterium* species, where the induction of the 7α-dehydroxylase requires a free C-24 carboxyl group (28).

Influence of the configuration of C-3 and C-5 of the bile acid molecule. The isomeric forms of LCA were utilized as substrates (Table 2). For the 3α-sulfate, 5α-hydrogen A/B-trans configuration, only alloCDCA-3-sulfate was available. Among the 5α-bile acid sulfates tested, *Clostridium* sp. strain S_1 only desulfated LCA-3-sulfate and CDCA-3-sulfate, but not isoLCA-3-sulfate. For the 5α-bile acids tested, isoalloLCA-3-sulfate was desulfated by strain S_1, but not alloCDCA-3-sulfate. Strain S_1 also desulfated 5-cholenoic acid 3β-sulfate. The trace amounts of free bile acids found for isoLCA-3-sulfate and alloCDCA-3-sulfate were probably due to some hydrolysis during the analysis (15). These results and our previous investigations confirm that gnotobiotic female rats associated with strain S_1 do not hydrolyze alloCDCA-3-sulfate, the predominant bile acid sulfate of the female rats, and smaller amounts of alloCA-3-sulfate. The smaller quantities of the 3α-sulfates of the 5β-bile acids, CDCA, CA, α-muricholic acid, and β-muricholic acid, and trace amounts of 3β-OH-cholenoic acid were completely desulfated (15).

The results also demonstrate the influence of substrate stereoconfiguration on strain S_1 sulfatase activity. Desulfation of the bile acid-3-sulfates occurred only in substrates with an equatorial orientation of the C-3 sulfate group. As seen in Table 2, bile acids with 3α-sulfate, 5β-A/B-*cis* configuration, or 3β-sulfate, 5α-A/B-*trans* configuration or 3β-sulfate, Δ^5-configuration were desulfated by strain S_1. The sulfate groups of the 5α-bile acid 3α-sulfates and the 5β-bile acid 3β-sulfates, which were not desulfated by *Clostridium* sp. strain S_1, are oriented in an axial position. We conclude that the substrate stereospecificity of *Clostridium* sp. strain S_1 sulfatase is determined by the equatorial position of the sulfate group, which in turn is determined by the configuration of carbon atom C-5. Jarrige et al. (17) established a similar importance of the equatorial sulfate group for the hydrolysis of steroid 3-sulfates by the arylsulfatase of *Helix pomatia*. These investigators found that the steroid sulfates with a 3β,5α or a 3β,Δ^5 structure, as well as the sulfates of estrogens and corticosteroids, were hydrolyzed efficiently; using the steroid substrates with an axial sulfate

TABLE 2. Influence of the configuration of carbon atoms C-3 and C-5 of the bile acid molecule on the sulfatase activity of *Clostridium* sp. strain S_1

Bile acid 3-sulphate of:	Configuration at:		Structure A/B-rings[a]	% Desulfated[b]
	C-3	C-5[a]		
LCA	3α-SO_4 (equat.)	5β-H	A/B-*cis*	88
CDCA	3α-SO_4 (equat.)	5β-H	A/B-*cis*	90
IsoLCA	3β-SO_4 (axial)	5β-H	A/B-*cis*	3
AlloCDCA	3α-SO_4 (axial)	5α-H	A/B-*trans*	2
IsoalloLCA	3β-SO_4 (equat.)	5α-H	A/B-*trans*	82
3β-OH-cholenoic acid	3β-SO_4 (equat.)	Δ^5	Δ^5	80

[a] The α- or β-orientation of the hydrogen (H) at C-5 determines the equatorial (equat.) or the axial position of an α- or β-oriented sulfate group (SO_4) at C-3 and the *cis*- or *trans*-configuration of the A- and B-rings of the bile acid nucleus; a Δ^5-configuration has a structure comparable to a A/B-*trans* configuration.

[b] The presence of sulfatase activity is expressed by the percent free bile acid, formed by *Clostridium* sp. strain S_1 after 5 days of incubation in culture medium with 50 μg of bile salt 3-sulfate per ml.

TABLE 3. Influence of the substitution at C-17 of the steroid nucleus on the sulfatase activity of *Clostridium* sp. strain S_1

C_x steroid	Substrate (3-sulfate of:)	Configuration at C-3/C-5[a]	Substitution at C-17	Carboxyl group	% Desulfated[b]
C_{27}	Trihydroxycoprostanic acid	3α-SO_4, 5β-H	-$CH(CH_3)CH_2CH_2CH_2CH(CH_3)COOH$	C-26	85
	Cholesterol	3β-SO_4, Δ^5	-$CH(CH_3)CH_2CH_2CH_2CH(CH_3)_2$	None	Tr
C_{24}	LCA	3α-SO_4, 5β-H	-$CH(CH_3)CH_2CH_2COOH$	C-24	85
	DCA	3α-SO_4, 5β-H	-$CH(CH_3)CH_2CH_2COOH$	C-24	90
	AlloCDCA	3α-SO_4, 5α-H	-$CH(CH_3)CH_2CH_2COOH$	C-24	Tr
	IsoalloLCA	3β-SO_4, 5α-H	-$CH(CH_3)CH_2CH_2COOH$	C-24	82
	3β-OH-cholenoic acid	3β-SO_4, Δ^5	-$CH(CH_3)CH_2CH_2COOH$	C-24	75
C_{23}	23-NorDCA	3α-SO_4, 5β-H	-$CH(CH_3)CH_2COOH$	C-23	Tr
C_{21}	5α-Pregnanolone	3β-SO_4, 5α-H	-$CH(CH_3)O$	None	Tr
	Pregnenolone	3β-SO_4, Δ^5	-$CH(CH_3)O$	None	Tr
C_{19}	Ethiocholanolone	3α-SO_4, 5β-H	=O	None	Tr
	Androsterone	3α-SO_4, 5α-H	=O	None	Tr
	Epiandrosterone	3β-SO_4, 5α-H	=O	None	Tr
	Dehydroepiandrosterone	3β-SO_4, Δ^5	=O	None	Tr
	17β-Estradiol	Phenolic 3-SO_4	-OH	None	Tr
	Estrone	Phenolic 3-SO_4	=O	None	Tr

[a] α- or β-orientation of the sulfate group (SO_4) at C-3 and the hydrogen at C-5.

[b] The presence of sulfatase activity is expressed by the percent free bile acid or steroid set free by *Clostridium* sp. strain S_1 after 5 days of incubation in culture medium with 50 μg of bile acid sulfate or steroidsulfate per ml.

group (3β,5β-structures and 3α,5α-structures), they found that the velocity of hydrolysis was very low for those with a 3β,5β-structure and zero for those with a 3α,5α-structure.

In conventional rats, alloCDCA-3-sulfate and alloCA-3-sulfate are desulfated by intestinal microflora (8). The desulfation of these sulfates probably occurs through yet unknown desulfating microorganisms other than *Clostridium* sp. strain S_1. On the other hand, one could speculate that a single isomerization of the hydrogen at carbon atom C-5 would allow the desulfation of the original allobile acid sulfate by *Clostridium* sp. strain S_1. Microbial isomerization of the 5α-hydrogen of unsulfated allobile acids has been observed by Kallner (18) after intracecal administration of tritium-labeled alloDCA. Hence, one cannot exclude the possibility that the desulfation of the 5α-bile acid 3α-sulfates in the rat might occur through two enzymes produced by one or more microorganisms: a 5α-isomerase and an equatorial 3-sulfatase. However, bacteria which develop 5α-isomerase activity have not as yet been isolated.

Influence of the substitution at C-17 of the steroid molecule. As shown in Table 3, *Clostridium* sp. strain S_1 did not desulfate the 3-sulfates of the C_{19} steroids ethiocholanolone, androsterone, and (dehydro)epiandrosterone, or the C_{21} steroids 5α-pregnanolone and pregnenolone. These substrates possess the same nuclear configuration and sulfate group location as the sulfatase-sensitive bile acids, whereas they differ only by substitution at carbon atom C-17. Likewise, the phenolic 3-sulfates of estrone and estradiol, both C_{19} steroids, were not desulfated. The trace amounts detected by gas-liquid chromatography were comparable to those resulting from the analysis of blank samples not incubated with *Clostridium* sp. strain S_1. It cannot be excluded that this specificity may be the result of a permeability barrier (cell wall or cell membrane) between the substrates and the enzyme. Sulfatase activity in broken cells of *Clostridium* sp. strain S_1 has not been established, probably because of the oxygen sensitivity of the desulfating enzyme. Since the intestinal microflora of humans and rats is able to hydrolyze C_{19} and C_{21} steroid sulfates (2, 6), sulfatases of other microorganisms must be responsible for these biotransformations.

Furthermore, the carboxyl group in the side chain is of functional import for the development of sulfatase activity. *Clostridium* sp. strain S_1 did not desulfate the C_{27} steroid 3-sulfate, cholesteryl-3-sulfate, in contrast to the C_{27} bile acid 3-sulfate, trihydroxycoprostanic acid 3-sulfate. Notably, both C_{27} substrates possess a nuclear configuration sensitive to sulfatase activity, but their side chains differ by the absence of the C-26 carboxyl group in cholesterol.

In addition, *Clostridium* sp. strain S_1 did not desulfate the 3α-sulfate of the C_{23} bile acid 23-norDCA (C-23 carboxyl), in contrast to the 3α-sulfate of the C_{24} bile acid DCA (C-24 carboxyl). Hence, the distance from the carboxyl group to the five-member ring, determined by the number of carbon atoms in the side chain, may be of importance. However, the significance of the length of the side chain is not yet clear, since the

3α-sulfate of trihydroxycoprostanic acid with a C-26 carboxyl was also desulfated by strain S_1.

In conclusion, the specificity of the sulfatase activity of cultured *Clostridium* sp. strain S_1 seems to be determined by three factors: (i) the location of the sulfate group on the bile acid molecule at carbon atom C-3 (*Clostridium* sp. strain S_1 did not desulfate bile acid 7- or 12-sulfates); (ii) the equatorial position of the sulfate group which depends on the configuration of carbon atoms C-3 and C-5 of the bile acid molecule (*Clostridium* sp. strain S_1 only desulfated the 5β-bile acid 3α-sulfates, the 5α-bile acid 3β-sulfates, and the Δ^5-bile acid 3β-sulfates); (iii) the substitution at C-17 of the steroid molecule and the presence of a carboxyl group at a minimum distance from this carbon atom (a C-24 or C-26 carboxyl group) (*Clostridium* sp. strain S_1 hydrolyzed the 3-sulfate esters of the C_{24} and C_{27} bile acids [C-24 and C-26 carboxyl group], in contrast to the C_{23} bile acid [C-23 carboxyl group] and the C_{19}, C_{21}, and C_{27} steroids). Therefore, other sulfatases responsible for the desulfation of allobile salt sulfates and steroid sulfates must be produced by as yet unidentified intestinal microorganisms other than *Clostridium* sp. strain S_1.

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