Taxonomic Position of Lecithinase-negative Strains of *Clostridium sordellii*

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(Received 21 December 1984; revised 26 February 1985)

Eleven out of 43 strains of *Clostridium sordellii* from clinical sources did not produce lecithinase activity and were not toxic to mice. However, these strains did belong to the *C. sordellii* group and could readily be differentiated from *C. bifermentans* and *C. difficile* on the basis of DNA–DNA homologies, carbohydrate fermentation patterns, enzyme activities, GLC analysis of fatty acid fermentation products and the electrophoretic analysis of whole cell protein extracts.

INTRODUCTION

Clostridium sordellii is commonly encountered in soil and sewage, and in the large bowel of mammals (Willis, 1977). *C. sordellii* is closely related to *C. bifermentans*, and these two species are usually clustered in the so-called 'lecithinase-producing *Clostridium* group' (Holdeman *et al.*, 1977). However, pathogenic and non-pathogenic *C. sordellii* strains can be differentiated from *C. bifermentans* strains by several phenotypic characters (Brooks & Epps, 1958; Nishida *et al.*, 1964; Novotny, 1969; Tataki & Huet, 1953) and by DNA–DNA homology (Nakamura *et al.*, 1975, 1976). On the other hand, *C. sordellii* and *C. bifermentans* strains exhibit marked serological cross-reactions with *C. difficile*, a well-known lecithinase-negative species (Poxton & Byrne, 1981, 1984; Rifkin *et al.*, 1977).

Recently we have isolated from clinical sources 11 lecithinase-negative strains of *Clostridium* which display the main phenotypic traits of *C. sordellii*. Because lecithinase activity is an important diagnostic test for *C. sordellii*, the unclear taxonomic position of the isolates led us to attempt the present study.

METHODS

Bacterial strains. The origins of the 50 strains included in this study are presented in Table 1. Four strains (*C. sordellii* NCIB 10717 and IP82; *C. bifermentans* ATCC 638; *C. difficile* ATCC 9689) are type or reference strains from international collections (Skerman *et al.*, 1980).

Media and growth conditions. Strains were normally grown in TYG broth, containing (g l⁻¹): trypticase (BBL), 30; yeast extract (Difco), 20; glucose 0.5; cysteine, 1; pH 7.2. In some experiments, TY broth (TYG broth without glucose) or peptone broth (meat pancreatic peptone (Osi, Paris, France), 20 g l⁻¹; NaCl, 5 g l⁻¹; pH 7.2) and complex meat/yeast extract agar medium (Beerens, 1954) were used.

All cultures were incubated at 35 °C in anaerobic jars (Baird and Tatlock) containing a hydrogen atmosphere and a palladium catalyst.

Biochemical studies. Strains received in our laboratory were assigned either to *C. sordellii* (41 strains) or to *C. bifermentans* (5 strains) as recommended by Sebald & Brefort (1977). Sugar fermentation studies were performed in TY broth supplemented with the appropriate carbohydrate (1%, w/v) inoculated with a 2% (v/v) dilution in TY broth of an overnight culture in TYG broth. After overnight incubation at 35 °C, the reaction was scored positive if the pH was below 5.5, and weak if the pH was between 5.5 and 5.7.

Enzyme activities. Lecithinase activity was determined on Columbia agar (Oxoid) supplemented with 1% (v/v) egg yolk. Strains were scored lecithinase negative if no opacity was seen after 4 d incubation, after removal of the colonies. Weak reactions were considered lecithinase positive. Urease activity was studied in 0.5 ml 'urée-indole'

Table 1. *Designation and source of the 50 strains of Clostridium*

Strain	Source	Strain	Source
<i>C. sordellii</i>		<i>C. sordellii</i>	
Group I		Group III	
IP82	Unknown; A. R. Prevot collection	CS5682	Human (1982)
CS174 4	Newborn faeces; R. Raibaud, CNRZ, Jouy-en-Josas, France (1981)	CS42	Sheep (1981)
CS175 1			
CS181 1			
CS183 1			
CS186 2			
Group II		CS9780	
NCIB 10717	Unknown; National Collection of Industrial Bacteria (Skerman <i>et al.</i> , 1980)	CS611 4	Cattle (1981)
CS190220	Newborn faeces; P. Raibaud (1981)	CS1124	Chamois (1982)
		CS4384	
		CS3000B	Duck (1981)
		<i>C. bifermentans</i>	
CSA1	Sheep (1981)	ATCC 638	Unknown; American Type Culture Collection (Skerman <i>et al.</i> , 1980)
CSA2		CB1791	Newborn faeces; P. Raibaud (1981)
CSA3		CB190221	
CSA4		CBR1	Sheep (1981)
CS12		CBR5	
CS15		CB21684	Goat (1981)
CS21			
CS29			
CS32			
CS35			
CS46			
CSG23			
CS12631			
CS2089B			
CS3393			
CS5801			
CS8192A			
CS1228			
CS1575B			
CS2352		Cattle (1982)	
CS5911			
CS1227A	Rabbit (1981)		
CS1227B			
CS6569	Horse (1981)		

medium (Institut Pasteur Production, Paris). Gelatinase activity was determined in gelatine film-TY broth (Institut Pasteur Production). These three tests and the indole production test were performed as previously described (Sebald & Brefort, 1977).

Other enzyme activities, reported in Table 3, were detected using API-ZYM galleries (API System). An overnight culture in 5 ml TYG broth was washed once in distilled water and suspended in 1.7 ml 0.15 M-NaCl. This suspension was used to inoculate each well of the strips. Incubation conditions and reading of results followed the manufacturer's instructions.

Toxicity tests. Supernatant (0.5 ml) from an overnight culture in TYG broth was injected intraperitoneally into 16–20 g male white mice (Swiss) obtained from CESAL (Montmedy, France). Strains were considered toxigenic if the animals died within 3 d of inoculation.

DNA-DNA homology. Cells from a 500 ml culture in TYG broth incubated anaerobically at 35 °C for 3 h (OD_{600} 0.9–1.0) were washed with distilled water and suspended in 20 ml 0.05 M-Tris pH 8.0 containing 25% (w/v) sucrose and 0.12% (w/v) lysozyme (Sigma). After incubation at 35 °C for 1 h, 2 ml predigest protease XIV (Sigma; 5 mg ml⁻¹) and 16 ml Sarkosyl (Ciba-Geigy; 2%, w/v) in 0.05 M-Tris/0.05 M-EDTA pH 8 were added. The mixture was incubated at 35 °C for 1 h and 8 ml 0.25 M-EDTA was added. DNA was purified and sheared following established procedures (Brenner *et al.*, 1972).

The DNA from strains NCIB 10717 and ATCC 638, the type strains of *C. sordellii* and *C. bifermentans* respectively (Skerman *et al.*, 1980), and from IP82, the laboratory reference strain of pathogenic *C. sordellii*, were labelled *in vitro* with ³H-labelled nucleotides (Grimont *et al.*, 1980).

For DNA base pairing experiments, the S1 nuclease method (Crosa *et al.*, 1973) was used. The DNA duplexes remaining after S1 nuclease treatment for 30 min at 45 °C were separated from free nucleotides by using DEAE-

cellulose filters (DE81 filters; Whatman) (Popoff & Coynault, 1980). These DNA-homology studies were performed at optimum renaturation temperature (58 °C) (Nakamura *et al.*, 1975) for 18 h. The degree of polynucleotide sequence homology was calculated by determining the ratio between the average counts in the S1-treated and untreated samples (Brenner *et al.*, 1976).

SDS-PAGE of cell proteins. Total cell proteins were extracted by a modification of the procedure described by Poxton & Byrne (1981). Bacteria from 5 ml of an overnight culture in TYG broth were harvested by centrifugation (5000g, 10 min) and washed in phosphate-buffered saline (50 mM-sodium phosphate buffer, pH 7.2, containing 0.15 M-NaCl). The pellet was suspended in 0.2 ml 5 mM-CHES pH 9.8 containing 100 mM-EDTA and incubated for 30 min at 70 °C. After centrifugation (5000g, 10 min) the supernatant was retained. The samples were analysed on 12% polyacrylamide gels containing 0.1% (w/v) SDS and stained with Coomassie blue according to the method of Laemmli (1970).

GLC. This was done as described by Carlier (1985). The bacteria were grown in TYG broth for 48 h at 35 °C. A Tracor 560 gas chromatograph (Tracor Instruments) equipped with a flame ionization detector and connected to a model ICRIB integrator (Intersmat France, Suresnes, France) was used. A glass column (2 m × 4 mm) packed with Resoflex LAC1R296 acid-washed on Chromosorb G DMcs (70–80 mesh) was used for volatile fatty acids. The instrument was operated at 125 °C for 6 min and then programmed at 20 °C min⁻¹ to 145 °C. The methyl esters were assayed on a glass column (2 m × 4 mm) packed with 10% (w/w) SP1000 (Supelco) containing 1% (w/w) H₃PO₄ acid-washed on Chromosorb W (100–120 mesh). The instrument was operated isothermally at 145 °C. For both assays, the operating conditions were: injector temperature, 200 °C; detector temperature, 250 °C; carrier gas (nitrogen) flow rate, 30 ml min⁻¹. Each peak of the GLC patterns was identified by the retention time compared with those obtained with standards (Merck). The amounts of fatty acids were calculated by comparison with internal standards.

Presentation of data and statistical methods. The data are presented as the mean ± standard error of the mean and were evaluated statistically by Student's *t*-test.

RESULTS

Phenotypic characterization

Forty-three motile, sub-terminal spore-forming, strictly anaerobic strains were identified to *C. sordellii* on the basis of the following criteria: cell width; motility; Gram-positive character; gelatinase and urease activity; production of indole; fermentation of glucose, fructose, maltose and ribose with the production of gas; lack of hydrolysis of aesculin; production of acetic, propionic, isobutyric, isovaleric, isocaproic, pyruvic, lactic, phenylacetic and 3-phenylpropionic acids as fermentation end products in TYG broth. None of these strains produced acid from arabinose, dextrin, cellobiose, galactose, inositol, lactose, melezitose, raffinose, rhamnose, saccharose, trehalose or xylose. All the strains showed a similar enzyme activity pattern with API-ZYM tests (Table 2). Three groups were differentiated according to lecithinase reaction and toxigenicity: Group I, 6 lecithinase-positive and toxigenic strains including the reference strain IP82; Group II, 26 lecithinase-positive and non-toxigenic strains including the type strain NCIB 10717; Group III, 11 lecithinase-negative and non-toxigenic strains (Table 3).

The six strains assigned to *C. bifermentans*, including the type strain ATCC 638, showed morphological characters similar to those of the *C. sordellii* strains. However, the sporulation rate in TYG broth was higher than for *C. sordellii*. The *C. bifermentans* strains showed lecithinase and gelatinase activities, were urease negative and non-toxigenic, produced indole, hydrolysed aesculin and fermented glucose, laevulose, maltose, mannose, sorbitol, salicin and aesculin. The type strain *C. bifermentans* ATCC 638 produced only weak acidification from salicin, sorbitol and aesculin when compared to the other *C. bifermentans* strains. All the *C. bifermentans* strains produced enzyme activity patterns with API-ZYM tests, and GLC patterns, similar to those obtained with the *C. sordellii* strains. However, the *C. bifermentans* strains produced more propionic, isocaproic and 3-phenylpropionic acids than the *C. sordellii* strains (Table 4). Two *C. bifermentans* strains (CB1791 and CB190221) produced low amounts of propionic acid and did not produce lactic acid. Similar amounts of acetic acid were produced by the *C. sordellii* and *C. bifermentans* strains. Isobutyric, isovaleric, pyruvic, lactic and phenylacetic acids were produced in variable amounts by both these *Clostridium* species. The characters differentiating *C. bifermentans* strains from *C. sordellii* strains are shown in Tables 3 and 4.

Table 2. *Enzyme activities of C. sordellii and C. bifermentans strains in API-ZYM tests*

Species/group	No. of strains tested	No. of strains positive in each enzyme test*																		
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
<i>C. sordellii</i>																				
Group I	26	26	26	26	0	25	0	7	1	1	26	25	0	4	0	1	0	1	0	1
Group II	6	6	6	6	0	5	0	1	0	0	6	6	0	0	0	1	1	1	0	0
Group III	11	11	11	11	0	10	0	0	0	0	11	11	1	1	0	1	0	0	0	0
<i>C. bifermentans</i>	6	6	6	6	0	6	1	3	0	0	6	6	0	0	0	0	1	0	0	0
<i>C. difficile</i>	1	1	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0

* A, alkaline phosphatase; B, esterase (C4); C, esterase lipase (C8); D, lipase; E, leucine arylamidase; F, valine arylamidase; G, cystine arylamidase; H, trypsin; I, α -chymotrypsin; J, acid phosphatase; K, phosphoamidase; L, α -galactosidase; M, β -galactosidase; N, β -glucuronidase; O, α -glucosidase; P, β -glucosidase; Q, *N*-acetyl- β -glucosaminidase; R, α -mannosidase; S, α -fucosidase.

Table 3. *Phenotypic differentiation between C. sordellii, C. bifermentans and C. difficile strains*

Test	<i>C. sordellii</i>			<i>C. bifermentans</i>	<i>C. difficile</i>
	Group I	Group II	Group III		
Lecithinase	+	+	—	+	—
Mouse toxicity	+	—	—	—	+
Urease		+		—	—
Indole		+		+	—
Aesculin hydrolysis		—		+	+
Fermentation of:					
Aesculin		—		+ / w*	—
Maltose		+		+	—
Mannose		—		+	—
Ribose		+		+	—
Salicin		—		+ / w*	—
Sorbitol		—		+ / w*	—

* Strain ATCC 638 gave a weak reaction for these three tests.

Table 4. *Quantitative gas liquid chromatography of fermentation products from TYG differentiating C. sordellii from C. bifermentans*

The results are mean concentrations (mM) in the culture supernatant, $\pm$ SEM.

Species/group (no. of strains)	Propionic acid	Isocaproic acid	3-Phenylpropionic acid
<i>C. sordellii</i>			
Group I (4)	3.20 $\pm$ 0.35	7.80 $\pm$ 0.39 <i>P</i> = 0.001*	1.55 $\pm$ 0.36 <i>P</i> = 0.05*
Group II (24)	2.47 $\pm$ 0.74 <i>P</i> = 0.001*	7.50 $\pm$ 2.38 <i>P</i> = 0.001*	0.64 $\pm$ 0.63 <i>P</i> = 0.001*
Group III (10)	2.01 $\pm$ 0.63 <i>P</i> = 0.001*	6.00 $\pm$ 2.00 <i>P</i> = 0.001*	0.78 $\pm$ 0.74 <i>P</i> = 0.01*
<i>C. bifermentans</i> (7)	8.08 $\pm$ 4.17	12.64 $\pm$ 1.67	3.45 $\pm$ 1.33

* Values significantly different from *C. bifermentans*.

Table 5. DNA homologies of *C. sordellii* and *C. bifermentans* strains with *C. sordellii* IP82 and NCIB 10717, and *C. bifermentans* ATCC 638

The results are mean percentage homologies $\pm$ SEM, with the range in parentheses.

Unlabelled DNA	³ H-labelled DNA . . .	<i>C. sordellii</i> IP82	<i>C. sordellii</i> NCIB 10717	<i>C. bifermentans</i> ATCC 638
<i>C. sordellii</i>				
All 43 strains		70 $\pm$ 14 (49–101)	80 $\pm$ 15 (52–105)	31 $\pm$ 6 (17–45)
Group I (6)		86 $\pm$ 26 (56–101)	89 $\pm$ 14 (61–103)	31 $\pm$ 5 (26–37)
Group II (26)		72 $\pm$ 10 (62–93)	80 $\pm$ 16 (52–105)	33 $\pm$ 6 (17–45)
Group III (11)		63 $\pm$ 11 (49–81)	76 $\pm$ 13 (56–93)	25 $\pm$ 3 (20–29)
<i>C. bifermentans</i> (6)		26 $\pm$ 2 (24–28)	30 $\pm$ 5 (25–34)	81 $\pm$ 11 (71–87)
<i>C. difficile</i> ATCC 9689		15	13	15

The *C. difficile* type strain ATCC 9689 was clearly different from the *C. sordellii* and *C. bifermentans* strains. This strain did not sporulate in TGY broth, did not exhibit lecithinase or urease activity; did not produce indole but did hydrolyse aesculin, was gelatinase positive and fermented glucose and fructose. The fermentation end products in TYG broth were acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, pyruvic, lactic, phenylacetic and 3-phenylpropionic acids. The enzyme activity pattern with API-ZYM tests was slightly different from those obtained with the *C. sordellii* and *C. bifermentans* strains (Table 2).

All the *C. sordellii* and *C. bifermentans* strains, and the one *C. difficile* strain, produced an unknown component in GLC with a retention time between those for caproic and heptanoic acids. Some *C. sordellii* and *C. bifermentans* strains, and the *C. difficile* strain, produced a low amount (0.10–0.20 mm) of heptanoic acid.

DNA–DNA homologies

DNAs from all the putative *C. sordellii* strains tested, including lecithinase-negative strains, showed high homologies with DNA from the *C. sordellii* type and reference strains (lowest value 49%), whereas DNAs from the *C. difficile* and *C. bifermentans* strains tested had low homologies (<30%) with DNA from the *C. sordellii* type and reference strains. DNAs from the *C. bifermentans* isolates showed high homologies (>70%) with DNA of the *C. bifermentans* type strain, whereas *C. difficile* DNA showed very low homology (<15%) with all the type strains (Table 5).

Polyacrylamide gel electrophoresis patterns

EDTA-extracted proteins of all the *C. sordellii* strains showed similar electrophoretic patterns, different from those obtained with the *C. bifermentans* or *C. difficile* strains. Our *C. bifermentans* isolates had slightly different electrophoretic patterns compared to the *C. bifermentans* type strain ATCC 638 (Fig. 1).

DISCUSSION

Our results are in agreement with those of Tataki & Huet (1953), Brooks & Epps (1958), Nishida *et al.* (1964), Novotny (1969) and Nakamura *et al.* (1975), who showed that *C. sordellii* and *C. bifermentans* are two distinct but related species and that urease activity is a useful criterion for their differentiation. However, Nakamura *et al.* (1976) did find urease-negative *C. sordellii* strains. Acid production from aesculin, maltose, mannose, ribose, salicin and sorbitol enabled us to distinguish *C. sordellii*, *C. bifermentans* and *C. difficile* strains, but some strains showed weak reactions that were difficult to interpret (Table 3). All the *C. bifermentans* strains we tested hydrolysed aesculin, and all the *C. sordellii* strains did not hydrolyse this substrate. The interpretation of this reaction was easy in all cases. In our study, quantitative GLC and SDS-PAGE patterns were useful additional characters for differentiating *C. sordellii* from *C.*

Fig. 1. Patterns of EDTA-extracted proteins from *C. sordellii*, *C. bifermentans* and *C. difficile* strains in SDS-PAGE on a 12% acrylamide gel stained with Coomassie blue. Lane 1, *C. difficile* ATCC 9689; lanes 2 and 3, non-pathogenic *C. sordellii* NCIB 10717 and CSA1 respectively; lanes 4 and 5, pathogenic *C. sordellii* 1P82 and CS1744 respectively; lanes 6 and 7, lecithinase-negative *C. sordellii* CS4447 and CS2089C respectively; lanes 8 to 11, *C. bifermentans* (ATCC 638, CBR1, CBR5 and CB1791 respectively). Molecular weight markers are shown at the sides of the photograph.

bifermentans. Cato *et al.* (1982) also showed different electrophoretic patterns for these two *Clostridium* species for total protein extracts run in the absence of SDS.

The lecithinase-negative non-pathogenic *C. sordellii* strains we studied shared all the other phenotypic characteristics defining classical pathogenic and non-pathogenic *C. sordellii* strains, including GLC patterns of fermentation products (Holdeman *et al.*, 1977; Sebald & Bifort, 1977). The production of phenylacetic acid by *Clostridium* spp. was previously reported by Mayrand & Bourgeau (1982). Their enzyme activity profiles using API-ZYM tests were closely related to those of lecithinase-positive *C. sordellii* strains, and were similar to those obtained by Mead *et al.* (1981). No significant differences in protein PAGE patterns were found between the *C. sordellii* strains tested. Moreover, DNA-DNA homologies confirmed that these lecithinase-negative, urease-positive and proteolytic *Clostridium* strains belonged to the *C. sordellii* homology group. Thus the only significant difference between these and the other *C. sordellii* strains was the lack of lecithinase activity.

C. sordellii is occasionally associated with gas gangrene in man (Willis, 1977) and animals (Smith *et al.*, 1962), and it has recently been isolated from lambs (Richards & Hunt, 1982), sheep (Al Mashat & Taylor, 1983a; Popoff, 1984) and cattle (Al Mashat & Taylor, 1983b) with enteric pathology. It is also often encountered in cases of sudden death in animals in France (unpublished). The majority of these isolates did not produce lethal toxin for mice. In our study, the lecithinase-negative *C. sordellii* strains were isolated mainly from sheep, but also from cattle, horse, goat, duck, wild animals and man. The clinical and epidemiological significance of these biovars of *C. sordellii* has yet to be established.

The authors are very grateful to M. Y. Popoff (Service des Enterobactéries, Institute Pasteur) for his assistance in performing the DNA hybridization and preparing the manuscript, and to Professor M. Sebald for her support during the course of this work.

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