Streptococcus difficile Is a Nonhemolytic Group B, Type Ib Streptococcus

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Whole-cell protein electrophoretic analysis of the type strain of *Streptococcus difficile* (LMG 15799) revealed that this organism was indistinguishable from *Streptococcus agalactiae* strains. Although LMG 15799^T (T = type strain) was originally described as serologically untypeable, we found that this strain was a group B streptococcus belonging to the capsular polysaccharide antigen type Ib group. The biochemical reactivity of *S. difficile*, which differed from the biochemical reactivity of typical *S. agalactiae* strains mainly by being less versatile, is similar to the biochemical reactivity of other group B, type Ib streptococci isolated from poikilothermic animals, such as fish and frogs.

Group B streptococci are isolated mainly from human and bovine sources; however, occasional infections in dogs, cats, camels, frogs, and fish have been reported (10, 23). The Lancefield serotyping method based on carbohydrate antigens is widely used as an aid for the identification of streptococci (18), yet, to date, only serogroup B corresponds to an individual species, *Streptococcus agalactiae* (7, 11). Other streptococcal species comprise strains belonging to multiple serogroups or untypeable strains, and members of several serogroups occur in different species (7, 11, 23).

Recently, Eldar et al. (8) described two new streptococcal species, *Streptococcus shiloi* and *Streptococcus difficile*, which were isolated from fish with meningoencephalitis. The strains were assigned to the genus *Streptococcus* based on their growth characteristics. A micro-scale DNA-DNA hybridization assay in which 5 ng of labeled reference DNA and DNAs from five other streptococcal and enterococcal species were used and a biochemical analysis were used to demonstrate that the fish isolates did not correspond to known *Streptococcus* species. After validation of the new species names, Eldar et al. (9) performed another study "... to elucidate the taxonomic position of *S. shiloi*." This study revealed that *Streptococcus iniae* (20) and *S. shiloi* are synonyms.

In an ongoing study on the taxonomy and epidemiology of streptococcal and enterococcal species, we found additional discrepancies between our data and the conclusions of Eldar et al. (8). Below, we demonstrate that *S. difficile* is a group B streptococcus belonging to the capsular serotype Ib group with a whole-cell protein pattern and phenotypic characteristics similar to those of other type Ib variants of *S. agalactiae* (10).

MATERIALS AND METHODS

Bacterial strains. Type and other well-characterized reference strains of all present streptococcal species were obtained from international culture collections. The other strains examined were field isolates obtained by two of us (L.A.D. and P.M.). Strains belonging to *S. difficile*, *Streptococcus phocae*, and reference species of the pyogenic species group (1, 23) and their sources are listed in Table 1. Bacteriological purity was checked by plating and examining living and Gram-stained cells.

Whole-cell protein analysis. All strains were grown for 24 h on brain heart infusion agar (catalog no. 0037-17-8; Difco Laboratories, Detroit, Mich.) and

were incubated at 36 to 37°C in a microaerobic atmosphere containing approximately 5% O₂, 10% CO₂, and 85% N₂. Preparation of cellular protein extracts, polyacrylamide gel electrophoresis, densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed as described by Pot et al. (22), using the GelCompar 4.0 software package (Applied Maths, Kortrijk, Belgium). The profiles were recorded and stored on a personal computer. The similarity between pairs of traces was expressed by the Pearson product moment correlation coefficient converted for convenience to a percentage.

Physiological tests. Strains were grown on Columbia agar (Lab M, Paisley, United Kingdom) supplemented with 5% sheep or cattle blood at 37°C in air enriched with 5% CO₂.

The Lancefield group antigen was determined with an Oxoid Streptococcal Grouping kit (Unipath, Basingstoke, United Kingdom) and a Slidex Strepto-kit latex B kit (bioMérieux, Marcy l'Etoile, France). Capsular typing was performed by a coagglutination assay (DARO group B *Streptococcus* serotyping test; DAKO A/S, Glostrup, Denmark).

Phenotypic characteristics as determined by the API 20 Strep and API 50 CH (bioMérieux, La Balme-les-Grottes, Montalieu-Vercieu, France) and VITEK GPI (bioMérieux Vitek, Inc., Hazelwood, Mo.) microtest systems were analyzed at 30 and at 37°C according to the recommendations of the manufacturer; reagents were added after 24 h. The medium used to inoculate the API 50 CH gallery was a laboratory-prepared API 50 CHS medium without added cystine.

RESULTS

Polyacrylamide gel electrophoresis of whole-cell proteins.

Duplicate protein extracts of several strains were prepared to check the reproducibility of the growth conditions and the preparation of the extracts. The correlation level between duplicate protein patterns was at least 94% (data not shown).

Figure 1 is a dendrogram obtained after numerical comparison and clustering of some of the species examined. All *S. agalactiae* strains and the *S. difficile* type strain (LMG 15797) form a single homogeneous cluster above a similarity level of 86%. *S. phocae* and the reference taxa of the pyogenic hemolytic species group each constitute a distinct protein electrophoretic cluster. The type strains of the other streptococcal species occupied separate positions when they were included in the numerical comparison (data not shown).

Physiological tests. *S. difficile* LMG 15797^T (T = type strain) had an optimal growth temperature below 30°C. It did not grow at 37°C in air; growth was observed when the organism was incubated anaerobically, and good growth (at 30 and 37°C) was obtained in a microaerobic atmosphere containing about 5% O₂, 10% CO₂, and 85% N₂. Strain LMG 15797^T was nonmotile and nonhemolytic on bovine blood agar and did not grow on Slanetz-Bartley enterococcal selective medium. The CAMP test (3) was negative.

Coagglutination tests with the Oxoid Streptococcal Group-

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TABLE 1. Strains used

Species or subspecies	LMG no. ^a	Other designation(s) ^b	Depositor ^{b,c}	Source (type)
<i>Streptococcus agalactiae</i>	14608	STR 43		Bovine
<i>Streptococcus agalactiae</i>	14609	STR 631		Bovine
<i>Streptococcus agalactiae</i>	14694 ^T	CCUG 4208 ^T	CCUG	Bovine milk
<i>Streptococcus agalactiae</i>	14747	STR 765		Bovine mastitis
<i>Streptococcus agalactiae</i>	14836	STR 432		Canine vagina
<i>Streptococcus agalactiae</i>	14838	1247/77 ^T		Bovine mastitis
<i>Streptococcus agalactiae</i>	14839	STR 631		Bovine mastitis
<i>Streptococcus agalactiae</i>	14840	STR 623		Feline oral cavity
<i>Streptococcus agalactiae</i>	14847	SHV 357	Hommeez	Porcine vagina
<i>Streptococcus agalactiae</i>	15081	ATCC 12400, 090	ATCC	Human (type Ia)
<i>Streptococcus agalactiae</i>	15083	ATCC 27591, A909	ATCC	Human (type Ic)
<i>Streptococcus agalactiae</i>	15084	CDC SS619, 18RS21	CDC	Human throat (type II)
<i>Streptococcus agalactiae</i>	15085	M732	ATCC	Human (type III)
<i>Streptococcus agalactiae</i>	15086	CNCTC 1/82	CNCTC	Human (type IV)
<i>Streptococcus agalactiae</i>	15087	CNCTC 10/84	CNCTC	Human (type V)
<i>Streptococcus agalactiae</i>	15088	ATCC 12386, 090R	ATCC	Mutant strain of LMG 15081 (no type)
<i>Streptococcus agalactiae</i>	15089	419290		Human urinary infection (type III)
<i>Streptococcus agalactiae</i>	15090	419944		Human urinary infection (type Ib)
<i>Streptococcus agalactiae</i>	15091	421390		Human urinary infection (type IV)
<i>Streptococcus agalactiae</i>	15092	426260		Human vagina (type II)
<i>Streptococcus agalactiae</i>	15093	454117		Human vagina (type V)
<i>Streptococcus agalactiae</i>	15094	455511		Human urinary infection (type III)
<i>Streptococcus agalactiae</i>	15095	455889		Human respiratory infection (type III)
<i>Streptococcus canis</i>	14833	CCUG 27660, STR 290		Canine
<i>Streptococcus canis</i>	15890 ^T	STR-T1 ^T		Bovine mastitis
<i>Streptococcus canis</i>	15894	23668/2140		Mink
<i>Streptococcus difficile</i>	15977 ^T	CIP 103768 ^T	CIP	Fish brain
<i>Streptococcus dysgalactiae</i> subsp. <i>dysgalactiae</i>	16023 ^T	NCFB 2023 ^T	NCFB	Bovine
<i>Streptococcus dysgalactiae</i> subsp. <i>dysgalactiae</i>	16024	NCFB 2024	NCFB	Bovine mastitis
<i>Streptococcus dysgalactiae</i> subsp. <i>dysgalactiae</i>	16029	NCFB 2652	NCFB	Canine
<i>Streptococcus dysgalactiae</i> subsp. <i>equisimilis</i>	15844	CCUG 27479	CCUG	Human
<i>Streptococcus dysgalactiae</i> subsp. <i>equisimilis</i>	15846	CCUG 502	CCUG	Human
<i>Streptococcus dysgalactiae</i> subsp. <i>equisimilis</i>	16026 ^T	NCFB 1356 ^T	NCFB	Human
<i>Streptococcus equi</i> subsp. <i>equi</i>	15764	STR 674		Equine
<i>Streptococcus equi</i> subsp. <i>equi</i>	15886 ^T	CCUG 23255 ^T	CCUG	Equine
<i>Streptococcus equi</i> subsp. <i>zooepidemicus</i>	15887 ^T	NCFB 1358 ^T	NCFB	Bovine mastitis
<i>Streptococcus hyointestinalis</i>	14579 ^T	S93 ^T		Porcine
<i>Streptococcus hyointestinalis</i>	14581	S85		Porcine
<i>Streptococcus hyointestinalis</i>	14582	EV 28b		Porcine
<i>Streptococcus iniae</i>	14520 ^T	CCUG 27303 ^T	CCUG	Dolphin
<i>Streptococcus iniae</i>	14521	CCUG 27623	CCUG	Dolphin
<i>Streptococcus parauberis</i>	12173	NCFB 2018	NCFB	Bovine mastitis
<i>Streptococcus parauberis</i>	12174 ^T	NCFB 2020 ^T	NCFB	Bovine mastitis
<i>Streptococcus phocae</i>	16735 ^T	CCUG 35103 ^T	CCUG	Seal liver
<i>Streptococcus phocae</i>	16736	CCUG 35104	CCUG	Seal spleen
<i>Streptococcus phocae</i>	16737	CCUG 35105	CCUG	Seal kidney
<i>Streptococcus porcinus</i>	14615	STR 827		Human
<i>Streptococcus porcinus</i>	15980 ^T	CCUG 27628 ^T	CCUG	Porcine
<i>Streptococcus porcinus</i>	15981	CCUG 27629	CCUG	Porcine
<i>Streptococcus pyogenes</i>	14237	CIP 56.58	CIP	Patient with rheumatic fever, throat
<i>Streptococcus pyogenes</i>	14238	CIP 70.3	CIP	Patient with bronchopneumonia, pleural fluid
<i>Streptococcus pyogenes</i>	14700 ^T	CCUG 4207 ^T	CCUG	Patient with scarlet fever
<i>Streptococcus shiloi</i>	15978 ^T	CIP 103769 ^T	CIP	Fish brain
<i>Streptococcus uberis</i>	14395	STR 192		Duck lung
<i>Streptococcus uberis</i>	14610	266/STR 203		Bovine
<i>Streptococcus uberis</i>	14686	ATCC 13386	ATCC	

^a LMG, Laboratorium voor Microbiologie Gent Culture Collection, Universiteit Gent, Ghent, Belgium.

^b ATCC, American Type Culture Collection, Rockville, Md.; CCUG, Culture Collection of the University of Göteborg, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; CIP, Collection bactérienne de l'Institut Pasteur, Paris, France; CNCTC, Czechoslovak National Collection of Type Cultures, Institute of Hygiene and Epidemiology, Prague, Czech Republic; Hommeez, J. Hommeez, Regional Veterinary Investigation Centre, Torhout, Belgium; NCFB, The National Collection of Food Bacteria, Agricultural and Food Research Council, Institute of Food Research, Reading Laboratory, Reading, Berkshire, United Kingdom.

^c Strains for which no depositor is indicated are our isolates.

FIG. 1. Dendrogram derived from unweighed pair group average linkage of correlation coefficients (expressed for convenience as percentages) between whole-cell protein patterns of streptococci belonging to the pyogenic species group. Strain LMG 15978 is the type strain of *S. shiloi*.

ing kit and the latex agglutination test with the Slidex Streptokit both revealed that the strain possessed the Lancefield group B carbohydrate antigen. Capsular typing revealed the presence of the type Ib antigen.

Most results obtained with the API 20 Strep and API 50 CH microtest systems confirmed the results of Eldar et al. (8). In contrast, hydrolysis of hippurate and beta-glucuronidase activity were detected when the gallery was incubated at 30°C, but not when the gallery was incubated at 37°C.

When the VITEK GPI microtest system was used, resistance to optochin and novobiocin, susceptibility to bacitracin, and acid production from D-glucose and sucrose were recorded. Acid was weakly produced from ribose. No growth occurred in the presence of 6% NaCl, 10% bile, or 40% bile. Esculin and urea hydrolysis, arginine dihydrolase activity, reduction of tetrazolium chloride, and acid production from hemicellulase, lactose, mannitol, raffinose, salicin, sorbitol, trehalose, arabinose, pyruvate, pullulan, inulin, melibiose, melezitose, cellobiose, and xylose were all absent.

DISCUSSION

Comparative whole-organism protein electrophoresis has been used by numerous investigators to examine relationships between bacteria (for reviews see references 6, 16, and 22). The results of numerical analyses of one-dimensional whole-organism protein fingerprints are often equivalent to the results obtained by DNA-DNA hybridization, as strains with very similar whole-cell protein patterns for the most part exhibit high DNA-DNA binding values. This equivalence has been widely documented in gram-negative bacteria, including the genera *Alcaligenes*, *Arcobacter*, *Bordetella*, *Campylobacter*, and *Pseudomonas* (19, 27, 28, 30). In gram-positive bacteria, this relationship is less well documented because of the difficulty of obtaining sufficient amounts of DNA for the hybridization experiments, yet it has been demonstrated in several genera, including the genera *Clostridium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Paenibacillus* (4, 6, 13–15, 17, 21, 26).

The same reference strains of several *Streptococcus* species (including *Streptococcus dysgalactiae*, *Streptococcus equi*, *S.*

inae, *Streptococcus parasanguis*, *Streptococcus parauberis*, and *S. phocae*) have been included in DNA-DNA hybridization and protein electrophoretic studies (Fig. 1) (5, 9, 12, 25, 29, 33). Comparison of the results obtained with the two techniques indicates that the congruence between whole-cell protein pattern similarity and percentage of DNA-DNA binding is also present in the genus *Streptococcus*.

Type and other well-characterized strains of all known *Streptococcus* species have been examined by whole-cell protein electrophoresis in order to construct a database for identification. When the type strains of *S. shiloi* and *S. difficile* were examined, it was obvious that these strains did not represent new streptococcal species as described by Eldar et al. (8). Numerical comparison of the whole-cell protein patterns confirmed that *S. shiloi* is identical to *S. iniae* (29) (Fig. 1), as stated in a subsequent paper by Eldar et al. (9). However, we also found that *S. difficile* did not occupy a distinct position in the dendrogram. *S. difficile* LMG 15977^T is situated among the *S. agalactiae* strains which form a homogeneous cluster (Fig. 1). Numerical analysis of whole-cell protein patterns of *S. agalactiae* strains representing types Ia, Ib, Ic, II, III, IV, and V did not reveal differences (Fig. 1), confirming the DNA-DNA hybridization data of Wanger and Dunny (31). The latter authors reported that *S. agalactiae* strains with different serotypes (serotypes Ia, Ib, Ic, II, and III) that were isolated from human and bovine sources all exhibit high DNA-DNA hybridization values. Elliot et al. (10) reported that group B type Ib streptococci isolated from humans, cows, mice, fish, and frogs all have identical protein patterns despite salient phenotypic differences. These phenotypic variants of the normal beta-hemolytic group B *S. agalactiae* strains are mainly characterized by being less versatile (i.e., by being nonhemolytic, by being CAMP negative, or by not producing acid from various sources) (10, 34). This was confirmed in the present study, which included different group B type Ib strains, strains from unusual sources, such as cat and dog isolates (Table 1), and a phenotypically aberrant strain from bovine mastitis (strain LMG 14838, which is negative in the CAMP assay). All of these isolates formed a single homogeneous protein electrophoretic cluster with the other *S. agalactiae* and *S. difficile* strains (Fig. 1).

Eldar et al. (8) reported that *S. difficile* was serologically untypeable. However, in two independent assays, we found that the *S. difficile* type strain clearly reacted with the group B antigen. Moreover, we found that capsular polysaccharide antigen Ib, which is specific for *S. agalactiae* strains (23), was present. Virtually all of the phenotypic characteristics present in the API 50 CH and API 20 Strep galleries as reported by Eldar et al. (8) were confirmed in our analyses. Acid production from ribose was weakly positive after prolonged incubation in the API galleries and after 15 h of incubation in the VITEK GPI system. This phenotypic profile of *S. difficile* corresponds to that of other fish-pathogenic, group B, type Ib streptococci described by Wilkinson et al. (34). We found that the *S. difficile* type strain grows well at 37°C, provided that it is incubated under microaerobic conditions. *S. difficile* LMG 15977^T cells adhered strongly to the agar layer and were difficult to suspend, which may influence the outcome of test results. Characteristics such as beta-glucuronidase activity and hippurate hydrolysis were detected when the organisms were incubated at 30°C but not when they were incubated at 37°C.

The serological classification of strain LMG 15977^T as a group B type Ib streptococcus and the protein electrophoretic similarity to *S. agalactiae* strains representing different serotypes, hosts, and phenotypic variants identify this strain as an *S. agalactiae* strain. Eldar et al. (8) obtained a DNA-DNA hy-

bridization value of 30.8% between strain LMG 15977^T and *S. agalactiae* ATCC 27956. The latter strain is not the type strain of *S. agalactiae*, but there is no evidence to question its identity. Eldar et al. used a microscale version of the classical hydroxyapatite method (2) and 5 ng of labeled reference DNA (8) in their hybridization assays. They considered the 17% homology between *S. difficile* LMG 15977^T and *S. shiloi* LMG 15978^T significant, although in the same study a DNA-DNA binding value of 37.7% was calculated between *S. difficile* LMG 15977^T and an *Enterococcus faecalis* strain, which belongs to a distinct phylogenetic lineage (24). It is difficult to interpret these DNA-DNA binding values. Perhaps the conditions used for DNA-DNA hybridization were too stringent to reveal the level of DNA-DNA hybridization expected from the standard hydroxyapatite method and from the evidence supplied by the high similarity in whole-cell protein content, the common group B antigen, and the identification of capsular type Ib. It is not contradictory in this context that the fish isolates classified as *S. difficile* all exhibited high levels of DNA binding (8). These strains could represent a distinct marine variant of *S. agalactiae* which originated from a common ancestor and which is phenotypically adapted to its host, as suggested by Elliot et al. (10). This hypothesis is supported by the DNA-DNA hybridization data of Wanger and Dunny (31), who found that under highly stringent conditions, DNA-DNA hybridization values were higher between strains isolated from the same host, irrespective of the capsular serotype, than between strains isolated from different hosts even if they were the same capsular serotype. Obviously, a methodological comparison between the DNA binding values obtained with the standard and microscale hydroxyapatite methods is warranted before the latter technique is validly used for species delineation as described by Wayne et al. (32).

In the present study, we also included three reference strains of *S. phocae*, a beta-hemolytic species isolated from diseased seals (25). *S. phocae* strains are serologically heterogeneous (group antigens F and C, and untypeable strains are present) and exhibited no significant DNA hybridization with other *Streptococcus* species. Our protein electrophoretic analysis confirmed the separate status of these organisms within the group of beta-hemolytic streptococci (Fig. 1).

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