High genetic similarity of *Streptococcus agalactiae* and *Streptococcus diffcilis*: *S. diffcilis* Eldar *et al.* 1995 is a later synonym of *S. agalactiae* Lehmann and Neumann 1896 (Approved Lists 1980)

Yoshiaki Kawamura,¹ Yoko Itoh,¹ Noriko Mishima,¹ Kiyufumi Ohkusu,¹ Hiroaki Kasai² and Takayuki Ezaki¹

Correspondence

Yoshiaki Kawamura
kawamura@cc.gifu-u.ac.jp

¹Department of Microbiology, Regeneration and Advanced Medical Science, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan

²Marine Biotechnology Institute, 3-75-1 Heita, Kamaishi, Iwate, Japan

The genetic relationship between *Streptococcus agalactiae* and *Streptococcus diffcilis* was studied. *S. diffcilis* was originally described as serologically non-typable but was later reported to be a group B, type Ib streptococcus. Upon comparative analysis of five gene sequences, it was found that *S. agalactiae* and *S. diffcilis* are closely related. Sequence similarity values between these two species were 100.0% for 16S rRNA, 99.6% for *gyrB*, 98.6% for *sodA*, 99.5% for *gyrA* and 99.8% for *parC* genes. These data strongly suggest that *S. agalactiae* and *S. diffcilis* are synonyms. The biochemical characteristics of *S. diffcilis*, which differ slightly from those of typical *S. agalactiae*, are similar to those of other group B, type Ib streptococci isolated from fish and frogs. Whole genome DNA–DNA hybridization values between the type strains of both species were greater than 78.6%. On the basis of these data, it is proposed that *S. diffcilis* is a later synonym of *S. agalactiae*.

INTRODUCTION

Group B streptococci are synonymous with *Streptococcus agalactiae*. Strains of this species have been recovered predominantly from bovine and human sources but have been isolated occasionally from several homeothermic animals (cats, dogs, hamsters) and also from some poikilothermic animals, including frogs and fish (Dow *et al.*, 1987; Kornblatt *et al.*, 1983; Kummeneje *et al.*, 1975). Most strains of the species show β -haemolysis on blood agar, although a number of non-haemolytic, type Ib variants have been isolated from humans, cows and fish (Amborski *et al.*, 1983; Wilkinson *et al.*, 1973). Wilkinson *et al.* (1973) and Elliott *et al.* (1990) characterized individual, non-haemolytic, serotype Ib strains of group B streptococci by biochemical analysis and whole-cell protein analysis, respectively. Wilkinson *et al.* (1973) reported several biochemical

differences between fish isolates and those from other sources (humans and cows), whereas Elliott *et al.* (1990) showed no differences in whole-cell protein patterns in strains isolated from fish, mice and humans.

Eldar *et al.* (1994) proposed two novel streptococcal species, *Streptococcus shiloi* and *Streptococcus diffcilis* [*Streptococcus diffcile* (sic), the species epithet was corrected by Euzéby (1998)] isolated from meningoencephalitis in fish. However, the next year these authors reported that *S. shiloi* was a later synonym of *Streptococcus iniae*, isolated from a dolphin (Eldar *et al.*, 1995). Vandamme *et al.* (1997) reported that *S. diffcilis* is a group B, serotype Ib streptococcus with whole-cell protein characteristics indistinguishable from those of *S. agalactiae*. Furthermore, Berridge *et al.* (2001) reported genetic similarity between *S. diffcilis* and *S. agalactiae* by analysis of the 16S–23S intergenic rRNA gene sequence.

In a study of the fluoroquinolone resistance of *S. agalactiae* strains, we found that the gene sequences of quinolone resistance determining regions (QRDRs) of both *gyrA* and *parC* between *S. agalactiae* and *S. diffcilis* were almost identical (Kawamura *et al.*, 2003). In the initial phase of the study, we hypothesized that this was due to horizontal gene transfer. However, we first needed to clarify the taxonomic relationship between these two species.

Published online ahead of print on 12 November 2004 as DOI 10.1099/ijs.0.63403-0.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyrB*, *sodA*, *gyrA* and *parC* gene sequences discussed in this study can be found in Fig. 1.

Tables giving details of the biochemical characteristics of *Streptococcus diffcile* and *Streptococcus agalactiae*, and the sequence similarity data for various *Streptococcus* species are available as supplementary material in IJSEM Online.

The aim of the present study was to clarify the relationship between *S. agalactiae* and *S. diffcilis*. For this, we collected sequence data on the genetic similarity of three house-keeping genes (16S rRNA, *sodA* and *gyrB*) and two quinolone resistance-related genes (*gyrA* and *parC*) (Kasai *et al.*, 2000; Kawamura *et al.*, 1999, 2003). We also confirmed the whole genome DNA similarity of the type strains of *S. agalactiae* and *S. diffcilis*, because Eldar *et al.* (1994) did not use the type strain of *S. agalactiae* in their study. On the basis of our results, we propose that *S. diffcilis* is a later synonym of *S. agalactiae*.

METHODS

Bacterial strains. We used the type strain and a reference strain of *S. diffcilis* [GTC 730^T (=ATCC 51487^T) and GTC 2049 (=CIP 103853)]. We also used the type strains of *S. agalactiae* (GTC 1234^T=NCTC 8181^T), *Streptococcus pyogenes* (GTC 262^T=ATCC 12344^T), *Streptococcus canis* (GTC 423^T=ATCC 43496^T), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (GTC 431^T=NCFB 2023^T), *Streptococcus dysgalactiae* subsp. *equisimilis* (GTC 842^T=NCFB 1356^T), *Streptococcus equi* subsp. *equi* (GTC 269^T=NCTC 9682^T), *Streptococcus equi* subsp. *zooeidemicus* (GTC 542^T=ATCC 43079^T), *Streptococcus iniae* (GTC 244^T=ATCC 29178^T), *Streptococcus porcinus* (GTC 543^T=ATCC 43138^T), *Streptococcus uberis* (GTC 271^T=NCTC 3858^T) and *Streptococcus parauberis* (GTC 539^T=NCDO 2020^T).

All strains were grown on Columbia blood (5% defibrinated sheep blood) agar plates at 30 or 37 °C under aerobic conditions unless indicated otherwise.

Biochemical profiles. Biochemical traits were determined by using the Rapid ID32 Strep (bioMérieux) and STREPTOGRAM (Wako Pure Chemical) systems according to manufacturers' recommendations, i.e. suggested incubation of test plates at 37 °C under aerobic conditions (Kawamura *et al.*, 1995). We also collected data on biological activity at 30 °C under aerobic conditions, at 37 °C in 5% CO₂ and at 37 °C under anaerobic conditions.

Gene sequences and phylogenetic analysis. Sequences from 16S rRNA, superoxide dismutase (*sodA*), DNA gyrase subunit B (*gyrB*), DNA gyrase subunit A (*gyrA*) and DNA topoisomerase subunit C (*parC*) genes were used to investigate the genetic relationship between *S. diffcilis* and *S. agalactiae*. PCR primers used for amplification of the 16S rRNA, *sodA*, *gyrA* and *parC* genes were as described previously (Kawamura *et al.*, 1999, 2003). The primers for amplification of *gyrB* were GY105G-f (5'-GAA GTC ATC ATG ACC GTT CTG CAY GSN GGN GGN AAR TTY GG-3') and GY404-r (5'-TGT AAA ACG ACG GCC AGT TCN GCN NAR YTT NCC NGG-3'). After confirming single amplification products for each gene on 1% agarose gels, sequences were determined by using an automated sequencer (model 3100; Applied Biosystems) using a dye-terminator reaction kit (Applied Biosystems).

The CLUSTAL W software originally described by Thompson *et al.* (1994) was used to align sequences, and phylogenetic distances were calculated by using the neighbour-joining method. Phylogenetic trees were drawn using TreeView software (Page, 1996).

Microplate DNA–DNA hybridization. DNA from each strain was carefully prepared by the standard procedure of Marmur (1961). We also used the silica–guanidinium thiocyanate DNA purification method described previously (Boom *et al.*, 1990).

Quantitative microplate DNA–DNA hybridization was carried out as

described previously (Ezaki *et al.*, 1989). Hybridization experiments were carried out at 31 °C (optimal condition) and 41 °C (stringent condition) using 2 × SSC and 50% formamide. The optimal temperature was 55 °C below the thermal denaturation temperature, because formamide lowered the hybridization temperature (Meinkoth & Wahl, 1984).

RESULTS AND DISCUSSION

Physiological and biochemical characteristics

The type strain and reference strain of *S. diffcilis* grew well at 30 °C under aerobic, 5% CO₂ and anaerobic conditions. The first generation of these strains from stock cultures (stored at –80 °C in 10% skimmed milk) did not grow well at 37 °C in any atmosphere (air, 5% CO₂ or anaerobic). However, from the second generation, both strains grew under each of those conditions.

With a Slidex Strepto-kit (bioMérieux), we confirmed that these two strains possessed the Lancefield group B antigen. They were non-haemolytic when cultured on sheep blood agar (under both aerobic and anaerobic conditions).

Biochemical traits determined with the two commercial kits are summarized in Supplementary Table A in IJSEM Online. The biochemical profiles did not differ, even under different incubation conditions (37 °C aerobic, 37 °C 5% CO₂, 37 °C anaerobic and 30 °C aerobic). Almost all traits were similar to those described by Eldar *et al.* (1994), with the exception of the hydrolysis activity of hippurate, for which they reported no activity, but we observed a clear positive reaction. However, acid production from ribose, which has been reported to be a positive characteristic, was negative in this study. Some of the biochemical characteristics of the *S. diffcilis* strains did not fit the typical pattern of *S. agalactiae* (see Supplementary Table A).

The biochemical patterns of the *S. diffcilis* strains were similar to those of other group B, type Ib streptococci isolated from poikilothermic host animals, such as frogs and fish. Elliott *et al.* (1990) reported that group B, type Ib streptococci isolated from frogs were negative for reactivity in fermentation tests of lactose, salicin and trehalose, whereas isolates from cows, mice and humans showed positive reactivity in these tests. With respect to group B streptococci isolated from fish, Elliott *et al.* (1990) reported positive reactivity in these three fermentation tests. However, Wilkinson *et al.* (1973) reported no reactivity in these. Wanger & Dunny (1985) described the association of transposon infection and mutation in some biochemical reactions. They showed that insertion of the Tn916 transposon in *S. agalactiae* strains causes slow lactose fermentation and also defective fermentation of trehalose. Other researchers explained that the biochemical divergence of frog or fish isolates from cow or human isolates may lead to phenotypic adaptations in the host (Elliott *et al.*, 1990). We did not investigate whether the strains of *S. diffcilis* carried transposons. These elements may be a reason for the phenotypic diversity.

Fig. 1. Comparative phylogenetic trees constructed from *gyrB*, *sodA*, *gyrA* and *parC* gene sequences. Distances were calculated by using the neighbour-joining method. The scales of the phylogenetic trees are identical. Bar, 10% sequence divergence. *S. agalactiae* and *S. diffcilis* were neighbours, with very little sequence divergence, in all of the phylogenetic trees.

Table 1. DNA–DNA hybridization values

Each value represents the mean $\pm$ SD of three experiments.

Strains	DNA hybridization (%) with biotin-labelled DNA from:					
	<i>S. agalactiae</i> GTC 1234 ^T		<i>S. diffcilis</i> GTC 730 ^T		<i>S. diffcilis</i> GTC 2049	
	Optimal	Stringent	Optimal	Stringent	Optimal	Stringent
<i>S. agalactiae</i> GTC 1234 ^T	100·0	100·0	78·6 $\pm$ 1·6	75·7 $\pm$ 0·6	77·8 $\pm$ 0·7	75·4 $\pm$ 0·1
<i>S. diffcilis</i> GTC 730 ^T	84·2 $\pm$ 1·5	79·7 $\pm$ 1·0	100·0	100·0	100·0 $\pm$ 1·3	93·9 $\pm$ 0·4
<i>S. diffcilis</i> GTC 2049	85·3 $\pm$ 1·2	80·3 $\pm$ 1·5	100·0 $\pm$ 1·9	91·8 $\pm$ 0·5	100·0	100·0
Salmon DNA	0·0	0·0	0·0	0·0	0·0	0·0

Sequence similarity

Phylogenetic analysis of the 16S rRNA gene sequence clearly revealed that *S. diffcilis* is a member of the pyogenic group of the genus *Streptococcus* (data not shown). The closest relative was *S. agalactiae*, with which it shared identical sequence.

It is well known that comparative analysis of the 16S rRNA gene sequence is not suitable for the investigation of genetically closely related strains, because the rate of evolution of the 16S rRNA gene is relatively slow (Kawamura *et al.*, 1999). Therefore, we used four other genes (*gyrB*, *sodA*, *gyrA* and *parC*) to investigate the genetic relationship between *S. diffcilis* and other members of the pyogenic group, particularly *S. agalactiae*. Sequence similarity values for selected members of the pyogenic group of the genus *Streptococcus* are available as Supplementary Table B in IJSEM Online, and the phylogenetic trees are shown in Fig. 1. *S. diffcilis* and *S. agalactiae* constituted a cluster that was separate from other *Streptococcus* species in all four gene-based phylogenetic trees (Fig. 1). Two different strains of *S. diffcilis* (the type strain and a reference strain) shared identical sequences in all five of the genes studied. *S. agalactiae* and *S. diffcilis* showed very high sequence similarity in the five gene sequences: 100·0 % for 16S rRNA (1320 bp compared), 99·6 % for *gyrB* (900 bp), 98·6 % for *sodA* (366 bp), 99·5 % for *gyrA* (420 bp) and 99·8 % for *parC* (528 bp).

Two pairs of subspecies (*S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis*; *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*) constituted single clusters in all four trees, with the exception of *S. dysgalactiae* subspecies in the *sodA* tree (Fig. 1). However, the genetic distances of the *S. diffcilis*/*S. agalactiae* clusters were less than those of the two subspecies pairs. The sequence similarity values also showed the relatedness of these two species (see Supplementary Table B). Both sets of data clearly show that the relatedness of *S. diffcilis* and *S. agalactiae* is greater than that of subspecies level. Along with the report of Berridge *et al.* (2001) on the genetic similarity of *S. diffcilis* and *S. agalactiae* by analysis of 16S–23S intergenic rRNA gene sequences, our data strongly suggest that these two species are genetically very similar.

Whole genome DNA hybridization analysis

The DNA–DNA hybridization values obtained under optimal (31 °C) and stringent (41 °C) conditions are shown in Table 1. The two strains of *S. diffcilis* were almost identical, with a hybridization value greater than 91·8 % obtained even under stringent conditions.

Minimum hybridization values of 77·8 and 75·4 % were obtained between the type and reference strains of *S. agalactiae* and *S. diffcilis*, under optimal and stringent conditions, respectively. These values proved that the two species are synonyms.

Eldar *et al.* (1994) reported a DNA–DNA hybridization value of only 30·8 % between *S. diffcilis* and *S. agalactiae*, but they did not use the type strain of *S. agalactiae*. However, we observed values greater than 77·8 % between the type strain of *S. agalactiae* (GTC 1234^T=NCTC 8181^T) and two different strains of *S. diffcilis* (GTC 730^T=ATCC 51487^T and GTC 2049=CIP 103853). Even though the strains were different, these data are quite different. We cannot explain this discrepancy with certainty. One possible reason is the difference in the DNA–DNA hybridization methods used; Eldar *et al.* (1994) used the hydroxyapatite method, whereas we used microplate hybridization method. Another possibility is that a contaminating component of the DNA preparation interfered with the re-association of the DNA heteroduplex. The strains of *S. diffcilis* (we used two reference strains) were very mucoidal, and some capsule components (polysaccharides or glycopeptides) were difficult to remove during DNA extraction and easily contaminated the samples. In our experience, DNA extracted from the strains of *S. diffcilis* by the Marmur method was very sticky, probably due to such contamination. With these samples, the DNA–DNA hybridization data were inconsistent (data not shown), and we could not obtain stable and reliable data. Consequently, we used the silica–guanidinium thiocyanate DNA purification method, which resulted in relatively pure DNA. Thus, we collected stable and reliable data with this purification method.

Wanger & Dunny (1985) reported that human and bovine strains of *S. agalactiae* of various serotypes (serotypes Ia, Ib, Ic, II and III) show significantly high DNA–DNA

hybridization values, indicating no correlation between DNA–DNA hybridization values and serotypes within group B streptococci. Vandamme *et al.* (1997) also reported that strains isolated from various hosts, such as humans, cows, dogs, cats and pigs, belonged to a single cluster based on the numerical analysis of whole-cell protein profiles. Furthermore, Elliott *et al.* (1990) showed that strains isolated from humans, mice and fish shared identical whole-cell protein profiles. These reports strongly suggest that the majority of strains of group B streptococci isolated from different hosts belong to a single cluster.

Considering the data presented here and data from other researchers, we conclude that *S. difficilis* isolated from meningoencephalitis in fish is indistinguishable from *S. agalactiae*, particularly the non-haemolytic, type Ib variant strains, physiologically, biochemically and genetically. Therefore, we propose that *S. difficilis* is a later synonym of *S. agalactiae*.

REFERENCES

- Amborski, R. L., Snider, T. G., 3rd, Thune, R. L. & Culley, D. D., Jr (1983). A non-hemolytic, group B *Streptococcus* infection of cultured bullfrogs, *Rana catesbeiana*, in Brazil. *J Wildl Dis* **19**, 180–184.
- Berridge, B. R., Bercovier, H. & Frelief, P. F. (2001). *Streptococcus agalactiae* and *Streptococcus difficile* 16S-23S intergenic rDNA: genetic homogeneity and species-specific PCR. *Vet Microbiol* **78**, 165–173.
- Boom, R., Sol, C. J., Salimans, M. M., Jansen, C. L., Wertheim-van Dillen, P. M. & van der Noordaa, J. (1990). Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* **28**, 495–503.
- Dow, S. W., Jones, R. L., Thomas, T. N., Linn, K. A. & Hamilton, H. B. (1987). Group B streptococcal infection in two cats. *J Am Vet Med Assoc* **190**, 71–72.
- Eldar, A., Bejerano, Y. & Bercovier, H. (1994). *Streptococcus shiloi* and *Streptococcus difficile*: two new streptococcal species causing a meningoencephalitis in fish. *Curr Microbiol* **28**, 139–143.
- Eldar, A., Frelief, P. F., Assenta, L., Varner, P. W., Lawhon, S. & Bercovier, H. (1995). *Streptococcus shiloi*, the name for an agent causing septicemic infection in fish, is a junior synonym of *Streptococcus iniae*. *Int J Syst Bacteriol* **45**, 840–842.
- Elliott, J. A., Facklam, R. R. & Richter, C. B. (1990). Whole-cell protein patterns of nonhemolytic group B, type Ib, streptococci isolated from humans, mice, cattle, frogs, and fish. *J Clin Microbiol* **28**, 628–630.
- Euzéby, J. P. (1998). Taxonomic note: necessary correction of specific and subspecific epithets according to Rules 12c and 13b of the International Code of Nomenclature of Bacteria (1990 Revision). *Int J Syst Bacteriol* **48**, 1073–1075.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Kasai, H., Ezaki, T. & Harayama, S. (2000). Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. *J Clin Microbiol* **38**, 301–308.
- Kawamura, Y., Sultana, F., Hou, X. G. & Ezaki, T. (1995). Evaluation of a new streptococcal identification kit “STREPTOGRAM”. *J Jpn Soc Clin Microbiol* **5**, 4–12 (in Japanese).
- Kawamura, Y., Whitley, R. A., Shu, S. E., Ezaki, T. & Hardie, J. M. (1999). Genetic approaches to the identification of the mitis group within the genus *Streptococcus*. *Microbiology* **145**, 2605–2613.
- Kawamura, Y., Fujiwara, H., Mishima, N., Tanaka, Y., Tanimoto, A., Ikawa, S., Itoh, Y. & Ezaki, T. (2003). First *Streptococcus agalactiae* isolates highly resistant to quinolones, with point mutations in *gyrA* and *parC*. *Antimicrob Agents Chemother* **47**, 3605–3609.
- Kornblatt, A. N., Adams, R. L., Barthold, S. W. & Cameron, G. A. (1983). Canine neonatal deaths associated with group B streptococcal septicemia. *J Am Vet Med Assoc* **183**, 700–701.
- Kummeneje, K., Nesbakken, T. & Mikkelsen, T. (1975). *Streptococcus agalactiae* infection in a hamster. *Acta Vet Scand* **16**, 554–556.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *Mol Biol* **3**, 208–218.
- Meinkoth, J. & Wahl, G. (1984). Hybridization of nucleic acids immobilized on solid supports. *Anal Biochem* **138**, 267–284.
- Page, R. D. M. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Vandamme, P., Devriese, L. A., Pot, B., Kersters, K. & Melin, P. (1997). *Streptococcus difficile* is a nonhemolytic group B, type Ib *Streptococcus*. *Int J Syst Bacteriol* **47**, 81–85; erratum **48**, 331.
- Wanger, A. R. & Dunne, G. M. (1985). Development of a system for genetic and molecular analysis of *Streptococcus agalactiae*. *Res Vet Sci* **38**, 202–208.
- Wilkinson, H. W., Thacker, L. G. & Facklam, R. R. (1973). Nonhemolytic group B streptococci of human, bovine, and ichthyic origin. *Infect Immun* **7**, 496–498.