***Clostridium hiranonis* sp. nov., a human intestinal bacterium with bile acid 7 α -dehydroxylating activity**

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The *Clostridium*-like organisms TO-931^T and HD-17, isolated from human faeces, have high levels of bile acid 7 α -dehydroxylating activity. Sequencing of their 16S rDNA demonstrated that they belong to cluster XI of the genus *Clostridium* and that they represent a new and distinct line of descent. *Clostridium bifermentans* and *Clostridium sordellii* in cluster XI also possess bile acid 7 α -dehydroxylating activity. DNA–DNA hybridization experiments with the isolates, TO-931^T and HD-17, and *C. bifermentans* and *C. sordellii* revealed that the isolates are a single species distinct from *C. bifermentans* and *C. sordellii*. On the basis of phylogenetic analysis, using 16S rDNA sequences, and DNA–DNA hybridization analysis, it is concluded that strains TO-931^T and HD-17 are members of a new species of the genus *Clostridium*, for which the name *Clostridium hiranonis* is proposed. The type strain is strain TO-931^T (= JCM 10541^T = DSM 13275^T).

Keywords: *Clostridium hiranonis*, bile acid, 7 α -dehydroxylation

INTRODUCTION

Some intestinal species of the genus *Clostridium* exhibit bile acid 7 α -dehydroxylating activity (Archer *et al.*, 1982; Hayakawa & Hattori, 1970; Hirano *et al.*, 1981; Stellwag & Hylemon, 1979; Wells *et al.*, 2000). The presence of this activity in the intestinal microflora results in the 7 α -dehydroxylation of cholic acid and chenodeoxycholic acid, yielding deoxycholic acid and lithocholic acid, respectively. Secondary bile acids have been strongly implicated in colorectal cancer as co-carcinogens (Cheah & Bernstein, 1990; Hill, 1975; Mastromarino *et al.*, 1976; Reddy *et al.*, 1977, 1996), indicating that bile acid 7 α -dehydroxylation is an important physiological reaction in the intestinal ecosystem.

Strains TO-931^T and HD-17, isolated from human faeces, were reported to exhibit high levels of bile acid 7 α -dehydroxylating activity (Doerner *et al.*, 1997; Hirano *et al.*, 1981). Most of the strains that have high levels of bile acid 7 α -dehydroxylating activity were identified as *Clostridium scindens* (Kitahara *et al.*,

2000). Strains TO-931^T and HD-17 and *C. scindens* have activity levels at least 10 times higher than those of *Clostridium bifermentans*, *Clostridium hylemonae*, *Clostridium leptum* and *Clostridium sordellii* (Doerner *et al.*, 1997; Kitahara *et al.*, 2000), so strains TO-931^T and HD-17 and *C. scindens* are noted as being highly bile acid 7 α -dehydroxylating bacteria. On the phylogenetic tree, *C. leptum* is positioned in cluster I of the genus *Clostridium* (Collins *et al.*, 1994), *C. sordellii* and *C. bifermentans* are in cluster XI, and *C. hylemonae* and *C. scindens* are in cluster XIVa. Although the bile acid 7 α -dehydroxylating activity of strains TO-931^T and HD-17 was studied in several papers (Masuda & Oda, 1983; Narushima *et al.*, 1999; Wells & Hylemon, 2000), the strains have remained unidentified at the species level.

The purpose of this study was to clarify the taxonomic positions of strains TO-931^T and HD-17, which possess high levels of bile acid 7 α -dehydroxylating activity, by phylogenetic analysis of 16S rDNA sequences and DNA–DNA hybridization experiments.

METHODS

Bacterial strains and cultivation. Two strains known to have bile acid 7 α -dehydroxylating activity, and other reference strains, were used in this study (Table 1). Strain TO-931^T was

The DDBJ accession numbers for the 16S rDNA sequences of *Clostridium hiranonis* TO-931^T and *Clostridium hiranonis* HD-17 are AB023970 and AB023971, respectively.

Table 1. *Clostridium* strains used in this study

Strain*	Source	Bile acid 7 α -dehydroxylating activity	Reference
<i>C. hiranonis</i> TO-931 ^T JCM 10541 ^T	Faeces of a healthy adult	+	Doerner <i>et al.</i> (1997)
<i>C. hiranonis</i> HD-17 JCM 10542	Faeces of a healthy adult	+	Hirano <i>et al.</i> (1981)
<i>C. bifermentans</i> JCM 1386 ^T	Soil	+	Archer <i>et al.</i> (1982)
<i>C. difficile</i> JCM 1296 ^T	Faeces of a newborn infant	NR	
<i>C. ghonii</i> JCM 1400 ^T	Post-operative human peritonitis	NR	
<i>C. glycolicum</i> JCM 1401 ^T	Mud	NR	
<i>C. irregulare</i> JCM 1425 ^T	—	NR	
<i>C. lituseburens</i> JCM 1404 ^T	Mud	NR	
<i>C. mangenotii</i> JCM 1428 ^T	African soil	NR	
<i>C. sordellii</i> JCM 3814 ^T	Gangrene in humans	+	Hayakawa & Hattori (1970)

NR, Not reported.

* JCM, Japan Collection of Microorganisms, RIKEN, Wako, Japan.

isolated from the faeces of a healthy human by F. Takamine, using previously described procedures (Doerner *et al.*, 1997), and strain HD-17 was isolated from the faeces of a healthy human by S. Hirano (Hirano *et al.*, 1981).

Two strains were cultured on Eggerth–Gagnon (EG) agar (Eiken) plates with 5% horse blood for 2 d at 37 °C in anaerobic jars (Hirayama Manufacturing Corporation) filled with 100% CO₂.

Growth experiments. The basal medium for growth experiments was PYF medium containing 0.5% glucose. The PYF medium was composed of the following (in 1000 ml): 10 g trypticase (BBL; Becton Dickinson), 10 g yeast extract (Difco), 40 ml salts solution, 40 ml Fildes solution and 0.5 g L-cysteine.HCl.CH₂O. The salts solution contained 0.2 g CaCl₂, 0.2 g MgSO₄, 1 g K₂HPO₄, 1 g KH₂PO₄, 10 g NaHCO₃, 2 g NaCl and 1000 ml distilled water. The Fildes solution contained 150 ml physiological saline, 6 ml concentrated HCl, 50 ml horse blood and 1 g pepsin (1:10000; Difco); the pH was adjusted to 7.6 after digestion.

Cultures were incubated at 15, 20, 25, 30, 37, 40, 45 and 50 °C to determine the optimum temperature at the initial pH of 7.6. The optimum pH was determined by incubating cultures at 37 °C at initial pH values of 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. The pH was adjusted by adding HCl or Na₂CO₃.

16S rDNA sequence analysis. Fragments (approx. 1500 bases) of the 16S rDNA genes of strains used in this study were amplified by a PCR with primers 5'-dAGAGTTTGATCC-TGGCTCAG-3' (designated primer 27F) and 5'-dGGTTA-CCTTGTTACGACTT-3' (designated primer 1492R), using a Takara PCR thermal cycler MP (Takara Shuzo). The PCR products were purified using a GENE Mate PCR pure SPIN purification kit (Intermountain Scientific Corporation) and were sequenced using an AutoCycle sequencing kit and an ALF express DNA sequencer (Pharmacia Biotech). A homology search, using FASTA of DDBJ, was used to determine the taxonomic neighbours of strains TO-931^T and HD-17. The new sequences were compared with the sequences of reference organisms from the GenBank database. Phylogenetic analysis was performed with CLUSTAL W software (Thompson *et al.*, 1994), and a phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987). The topology of the tree was evaluated by bootstrap analysis using CLUSTAL W.

Nucleotide sequence accession numbers. The 16S rDNA

sequences of strains TO-931^T and HD-17 have been deposited in the DDBJ database. The following 16S rRNA/rDNA sequences were used for the phylogenetic analysis: '*Clostridium aminobutyricum*' DSM 2634 (X76161), *Clostridium bifermentans* ATCC 638^T (X75906), *Clostridium butyricum* NCIMB 8082 (X68178), *Clostridium difficile* DSM 11209 (X73450), *Clostridium felsineum* DSM 794^T (X77851), *Clostridium formicaceticum* DSM 92^T (X77836), *Clostridium ghonii* DSM 10636 (X73451), *Clostridium glycolicum* DSM 1288^T (X76750), *Clostridium halophilum* DSM 5387^T (X77837), *Clostridium irregulare* DSM 2635^T (X73447), *Clostridium litorale* DSM 5388^T (X77845), *Clostridium lituseburens* ATCC 25759^T (M59107), *Clostridium mangenotii* ATCC 25761^T (M59098), *Clostridium paradoxum* DSM 7308^T (Z69939), *C. sordellii* ATCC 9714^T (M59105), *Clostridium sticklandii* VPI 14603 (L04167), *Clostridium thermoalcaliphilum* ATCC 51508^T (L11304), *Eubacterium tenue* ATCC 25553^T (M59118), *Filifactor villosus* DSM 1645^T (X73452) and *Peptostreptococcus anaerobius* ATCC 27337^T (D14150). *C. butyricum* NCIMB 8082 was used as an outgroup organism.

DNA base composition. DNA was extracted from the cells harvested from EGF broth [2.4 g lab-lemco powder (Oxoid), 10 g proteose pepton No. 3 (Difco), 5 g yeast extract, 4 g Na₂HPO₄, 5 g glucose, 0.5 g soluble starch, 0.5 g L-cystein.HCl.H₂O and 1000 ml distilled water; pH was adjusted to 7.6] after 12 h at 37 °C and purified by the methods of Saito & Miura (1963). DNA base compositions were determined using HPLC (Tamaoka & Komagata, 1984) after enzyme digestion of DNA to the deoxyribonucleosides. An equimolar mixture of four deoxyribonucleotides from the Yamasa GC kit (Yamasa Shoyu) was used as the quantitative standard.

DNA–DNA hybridization. Levels of DNA–DNA hybridization were determined by the method of Ezaki *et al.* (1989), using photobiotin and microplates.

Physiological and biochemical tests. For physiological and biochemical tests, PYF medium was used in the same way as for growth experiments. The initial pH values of media were adjusted to 7.6 and the incubation temperature was 37 °C. H₂S production, indole production, nitrate reduction, motility, aesculin hydrolysis, starch hydrolysis and gelatin liquefaction were detected by using the methods of Holdeman *et al.* (1977). Acid production from 30 sugars was determined by using PYF medium containing 0.5% (w/v) sugar, except for amygdalin and aesculin (0.25%). The pH

was measured directly in the culture tubes after incubation for 7 d.

The metabolic end products were analysed by means of gas-liquid chromatography (GC-14A; Shimadzu) using a 2.1 m glass column (i.d. 2.8 mm, FAL-M 25%, Chromosorb W, AW-DMCS H₂PO₄, 80/100-mesh). The short fatty acids were analysed by means of acidified ether-extraction.

Enzymic activity test. The enzymic activity tests were performed using AN-IDENT (API bioMérieux) according to the instructions of the manufacturer.

RESULTS AND DISCUSSION

Biological characterization

Phase-contrast microscopy showed that the cells of strain TO-931^T were straight or slightly curved rods that occurred in pairs or as single cells (Fig. 1). Spores were observed in strains TO-931^T and HD-17. Strain TO-931^T grew at initial pH values between 6.5 and 9.0, and the optimum pH was between 7.5 and 8.0. The temperature range for growth was 30–50 °C, and the optimum temperature was approximately 37 °C.

16S rDNA sequence analysis

The 16S rDNA sequences of strains TO-931^T and HD-17 were determined for approximately 1500 bases. The sequences clearly indicated that these isolates were related to the strains in cluster XI of the genus *Clostridium* (Collins *et al.*, 1994), as shown in Fig. 2. Strains TO-931^T and HD-17 formed a single cluster and a new and distinct line of descent. Strain TO-931^T was more closely related to *C. sordellii* than to any of the other species, according to sequence similarity

(94.8%), and all of the levels of sequence similarity between strain TO-931^T and strains in cluster XI were below 94.8%. These low levels of sequence similarity are consistent with the phylogenetic tree.

C. bifermentans and *C. sordellii* (cluster XI) were reported as the species that had bile acid 7 α -dehydroxylating activity (Archer *et al.*, 1982; Hayakawa & Hattori, 1970), but their activities were very low compared with those of strains TO-931^T and HD-17 (Doerner *et al.*, 1997). It is interesting that strains TO-931^T and HD-17 were found to be closely related to *C. bifermentans* and *C. sordellii* on the phylogenetic tree. *C. leptum*, *C. hylemonae* and *C. scindens* were also reported as the other bile acid 7 α -dehydroxylating bacteria. *C. leptum* is positioned in cluster I, whereas *C. hylemonae* and *C. scindens* are positioned in cluster XIVa; these phylogenetic positions indicate that bile acid 7 α -dehydroxylating bacteria are phylogenetically heterogeneous.

DNA base compositions and DNA–DNA hybridization

The DNA base compositions and levels of DNA–DNA hybridization are shown in Table 2. *C. difficile* JCM 1296^T, *C. bifermentans* JCM 1386^T, *C. sordellii* JCM 3814^T and *C. glycolicum* JCM 1401^T were used as the reference strains because they were closely related to strains TO-931^T and HD-17 on the phylogenetic tree and because *C. bifermentans* and *C. sordellii* showed bile acid 7 α -dehydroxylating activity. The G+C contents of strains TO-931^T and HD-17 were 31.1 and 31.9 mol%, respectively. The levels of DNA–DNA hybridization between strains TO-931^T and HD-17 were greater than 82%, whereas the levels between strain TO-931^T and the reference strains were less than 39%. These data clearly revealed that strains TO-931^T and HD-17 were closely related genetically and distinct from *C. difficile*, *C. bifermentans*, *C. sordellii* and *C. glycolicum*.

On the basis of phylogenetic analysis of the 16S rDNA sequences and the results of DNA–DNA hybridizations, strains TO-931^T and HD-17 should belong to a new species of the genus *Clostridium*. We propose the name *Clostridium hiranonis* for strains TO-931^T and HD-17.

Now, the bile acid 7 α -dehydroxylating bacteria are divided into two groups: one group, with relatively high activity, contains *C. hiranonis* and *C. scindens*; the other group, with low activity, contains *C. bifermentans*, *C. hylemonae*, *C. leptum* and *C. sordellii*.

Physiological and biochemical properties

Strains TO-931^T and HD-17 produce acid from fructose, glucose, mannose and sucrose, but not from adonitol, arabinose, amygdalin, cellobiose, dulcitol, aesculin, erythritol, aesculin, galactose, glycerol, glycogen, inositol, inulin, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose,

Fig. 1. Phase-contrast micrograph of strain TO-931^T. Cells were cultured on Eggerth–Gagnon blood agar. Bar, 10 μ m.

Fig. 2. Phylogenetic relationships within *Clostridium* cluster XI and *Clostridium butyricum*. The tree was created by the neighbour-joining method and using K_{nuc} values. The numbers on the tree indicate bootstrap values greater than 50%. Bar, evolutionary distance (K_{nuc}) of 0.02.

Table 2. DNA base composition and levels of DNA–DNA hybridization among *Clostridium hiranonis* and related strains

Strain	G + C content (mol %)	DNA–DNA complementarity (%) with labelled DNA from:				
		TO-931 ^T	HD-17	JCM 1386 ^T	JCM 3814 ^T	JCM 1401 ^T
<i>C. hiranonis</i> TO-931 ^T JCM 10541 ^T	31.1	100	84	30	28	24
<i>C. hiranonis</i> HD-17 JCM 10542	31.9	82	100	32	30	24
<i>C. bifermentans</i> JCM 1386 ^T	33.1	21	28	100	39	26
<i>C. sordellii</i> JCM 3814 ^T	32.2	19	29	41	100	25
<i>C. glycolicum</i> JCM 1401 ^T	29*	18	22	23	23	100
<i>C. difficile</i> JCM 1296 ^T	28*	16	17	19	26	20

* According to *Bergey's Manual of Systematic Bacteriology* (Cato *et al.*, 1986).

salicin, sorbitol, sorbose, starch, trehalose or xylose. Strains TO-931^T and HD-17 exhibit gas formation, do not produce indole or H₂S, do not reduce nitrate and do not hydrolyse aesculin, gelatin or starch.

Enzymic activity

The enzymic activity tests, using AN-IDENT, of strains TO-931^T and HD-17 are shown in Table 3.

Strains phylogenetically related to *C. hiranonis* in cluster XI were tested, as reference strains, at the same time. Strains TO-931^T and HD-17 gave positive reactions on *N*-acetyl- β -D-glucosamidase and proline aminopeptidase, but the reference strains used in this test gave negative reactions on *N*-acetyl- β -D-glucosamidase. The enzymic activity of *N*-acetyl- β -D-glucosamidase is useful as a distinguishing characteristic of *C. hiranonis*.

Table 3. Enzymic activity of *Clostridium* strains

For the two strains of *C. hiranonis* and closely related strains, the following activities were not detected: α -arabinofuranosidase, β -glucosidase, α -L-fucosidase, α -galactosidase, β -galactosidase, indoxyl-acetate, arginine, leucine aminopeptidase, pyroglutamic acid arylamidase, tyrosine aminopeptidase, arginine aminopeptidase, alanine aminopeptidase, histidine aminopeptidase, phenylalanine aminopeptidase, glycine aminopeptidase and catalase. For *C. lituseburens* JCM 1404^T, the test below were all negative.

Test	<i>C. hiranonis</i> TO-931 ^T	<i>C. hiranonis</i> HD-17	<i>C. bifermentans</i> JCM 1386 ^T	<i>C. difficile</i> JCM 1296 ^T	<i>C. ghonii</i> JCM 1400 ^T	<i>C. glycolicum</i> JCM 1401 ^T	<i>C. irregulare</i> JCM 1425 ^T	<i>C. manganotii</i> JCM 1428 ^T	<i>C. sordellii</i> JCM 3814 ^T
Indole	–	–	–	–	–	–	–	–	+
<i>N</i> -acetyl- β -D-glucosamidase	+	+	–	–	–	–	–	–	–
α -Glucosidase	–	–	–	–	+	–	–	–	–
Alkaline phosphatase	–	–	–	–	–	+	–	–	+
Proline aminopeptidase	+	+	+	+	–	+	+	+	+

Description of *Clostridium hiranonis* sp. nov

Clostridium hiranonis (hi.ra.no'nis. N.L. masc. gen. n. *hiranonis* of Hirano, after the Japanese microbiologist Seiju Hirano for his contributions to the study of the isolate).

The description of the characteristics given below is based on the results of studies with the two strains, TO-931^T and HD-17.

Cells cultivated on EG blood agar plates are Gram-positive, spore-forming and non-motile. The straight or slightly curved rod-shaped cells are 1.6–10 μm $\times$ 0.8 μm in size and occur singly or in pairs. Colonies on EG blood agar plates are approximately 1.0–2.0 mm in diameter, disc-shaped and greyish white to grey. The optimum temperature for growth is approximately 37 °C, and the optimum pH for growth is between 7.5 and 8.0. Obligate anaerobe. All strains produce acid from glucose, fructose, mannose and sucrose. Neither strain produces acid from adonitol, amygdalin arabinose, cellobiose, dulcitol, erythritol, aesculin, galactose, glycerol, glycogen, inositol, inulin, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sorbose, starch, trehalose or xylose. Nitrate and sulfate are not reduced. Aesculin, gelatin and starch are not hydrolysed. Indole is not produced. Acid and gas are produced. All strains give positive reactions on *N*-acetyl- β -D-glucosamidase and proline aminopeptidase in the ANI-DENT test. Moderate amounts of acetic acid and iso-valeric acid are produced as the end products in peptone–yeast-extract medium supplemented with glucose; minor amounts of propionic acid and iso-butyric acid are also produced. The G+C content of strain TO-931^T is 31.1 mol%. The type strain is strain TO-931^T, isolated from the faeces of a healthy human, and has been deposited in the Japan Collection of Microorganisms, Japan, as strain JCM 10541^T, and also in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany, as strain DSM 13275^T.

REFERENCES

- Archer, R. H., Maddox, I. S. & Chong, R. (1982). Transformation of cholic acid by *Clostridium bifermentans*. *J Appl Bacteriol* **52**, 49–56.
- Cato, E. P., George, W. L. & Fingold, S. M. (1986). Genus *Clostridium*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1141–1200. Baltimore: Williams & Wilkins.
- Cheah, P. Y. & Bernstein, H. (1990). Modification of DNA by bile acids: a possible factor in the etiology of colon cancer. *Cancer Lett* **49**, 207–210.
- Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J., Fernandez-Garayzaval, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- Doerner, K. C., Takamine, F., La Voie, C. P., Mallonee, D. H. & Hylemon, P. B. (1997). Assessment of fecal bacteria with bile acid 7 α -dehydroxylating activity for the presence of *bai*-like genes. *Appl Environ Microbiol* **63**, 1185–1188.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in microdilution 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.
- Hayakawa, S. & Hattori, T. (1970). 7 α -Dehydroxylation of cholic acid by *Clostridium bifermentans* strain ATCC 9714 and *Clostridium sordellii* strain NCIB 6929. *FEBS Lett* **6**, 131–133.
- Hill, M. J. (1975). The role of colon anaerobes in the metabolism of bile acids and steroids, and its relation to colon cancer. *Cancer* **36**, 2387–2400.
- Hirano, S., Nakama, R., Tamaki, M., Matsuda, N. & Oda, H. (1981). Isolation and characterization of thirteen intestinal microorganisms capable of 7 α -dehydroxylating bile acids. *Appl Environ Microbiol* **41**, 737–745.
- Holdeman, L. V., Cato, E. P. & Moore, W. E. C. (1977). *Anaerobe Laboratory Manual*, 4th edn. Blacksburg: Virginia Polytechnic Institute and State University.
- Kitahara, M., Takamine, F., Imamura, T. & Benno, Y. (2000). Assignment of *Eubacterium* sp. VPI 12708 and related strains with high bile acid 7 α -dehydroxylating activity to *Clostridium scindens* and proposal of *Clostridium hylemonae* sp. nov.,

isolated from human faeces. *Int J Syst Evol Microbiol* **50**, 971–978.

Mastromarino, A., Reddy, B. S. & Wynder, E. L. (1976). Metabolic epidemiology of colon cancer: enzymic activity of fecal flora. *Am J Clin Nutr* **29**, 1455–1460.

Masuda, N. & Oda, H. (1983). 7 α -Dehydroxylation of bile acids by resting cells of an unidentified, gram-positive, nonspore-forming anaerobic bacterium. *Appl Environ Microbiol* **45**, 456–462.

Narushima, S., Itoh, K., Takamine, F. & Uchida, K. (1999). Absence of cecal secondary bile acids in gnotobiotic mice associated with two human intestinal bacteria with the ability to dehydroxylate bile acids *in vitro*. *Microbiol Immunol* **43**, 893–897.

Reddy, B. S., Watanabe, K., Weisburger, J. H. & Wynder, E. L. (1977). Promoting effect of bile acids in colon carcinogenesis in germ-free and conventional F344 rats. *Cancer Res* **37**, 3238–3242.

Reddy, B. S., Simi, B., Patel, N., Aliaga, C. & Rao, C. V. (1996). Effect of amount and types of dietary fat on intestinal bacterial 7 α -dehydroxylase and phosphatidylinositol-specific phospholipase C and colonic mucosal diacylglycerol kinase and PKC activities during stages of colon tumour promotion. *Cancer Res* **56**, 2314–2320.

Saito, H. & Miura, K. (1963). Preparation of transforming

deoxyribonucleic acid by phenol treatment. *Biochim Biophys Acta* **72**, 619–629.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Stellwag, E. J. & Hylemon, P. B. (1979). 7 α -Dehydroxylation of cholic acid and chenodeoxycholic acid by *Clostridium leptum*. *J Lipid Res* **20**, 325–333.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

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.

Wells, J. E. & Hylemon, P. B. (2000). Identification and characterization of a bile acid 7 α -dehydroxylation operon in *Clostridium* sp. strain TO-931, a highly active 7 α -dehydroxylating strain isolated from human feces. *Appl Environ Microbiol* **66**, 1107–1113.

Wells, J. E., Berr, F., Thomas, L. A., Dowling, R. H. & Hylemon, P. B. (2000). Isolation and characterization of cholic acid 7 α -dehydroxylating fecal bacteria from cholesterol gallstone patients. *J Hepatol* **32**, 4–10.