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

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The **Clostridium**-like organisms TO-93¹ and HD-17, isolated from human faeces, have high levels of bile acid 7α-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 7α-dehydroxylating activity. DNA–DNA hybridization experiments with the isolates, TO-93¹ 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 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 (= JCM 10541¹ = DSM 13275⁵).

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

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**INTRODUCTION**

Some intestinal species of the genus *Clostridium* exhibit bile acid 7α-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 7α-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 7α-dehydroxylation is an important physiological reaction in the intestinal ecosystem.

Strains TO-93¹ and HD-17, isolated from human faeces, were reported to exhibit high levels of bile acid 7α-dehydroxylating activity (Doerner et al., 1997; Hirano et al., 1981). Most of the strains that have high levels of bile acid 7α-dehydroxylating activity were identified as *Clostridium scindens* (Kitahara et al., 2000). Strains TO-93¹ 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 and HD-17 and *C. scindens* are noted as being highly bile acid 7α-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 7α-dehydroxylating activity of strains TO-93¹ 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 and HD-17, which possess high levels of bile acid 7α-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 7α-dehydroxylating activity, and other reference strains, were used in this study (Table 1). Strain TO-93¹ was

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

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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), 40 ml salts solution, 40 ml Fildes solution and 10 g trypticase (BBL; Becton Dickinson), 10 g yeast extract (Difco), 40 ml Fildef solution and 0.5 g L-cysteine.HCl.H₂O. 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 Fildef solution contained 150 ml physiological saline, 6 ml concentrated HCl, 50 ml horse blood and 1 g peptone (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, 5, 5, 6, 6, 7, 7, 7, 8, 8, 8, 8 and 9. The pH was adjusted by adding HCl or Na₂CO₃.

**16S rDNA sequence analysis.** The sequences of strains TO-931T and HD-17 have been deposited in the DDBJ database. The following 16S rDNA sequences were used for the phylogenetic analysis: Clostridium butyricum DSM 2634 (X76161), Clostridium bifermentans ATCC 638T (X75906), Clostridium butyricum NCIMB 8082 (X68178), Clostridium difficile DSM 11209 (X73450), Clostridium felsineum DSM 794T (X77851), Clostridium formicaceticum DSM 92T (X77836), Clostridium ghetonii DSM 10636 (X73451), Clostridium glycolicum DSM 1288T (X76750), Clostridium halophilum DSM 5387T (X77837), Clostridium irregularum DSM 2635T (X73447), Clostridium litorale DSM 5388T (X78485), Clostridium lituseburense ATCC 25759T (M59107), Clostridium mangenotii ATCC 25761T (M59098), Clostridium paradoxum DSM 7308T (Z69939), Clostridium sordelli ATCC 9714T (M59105), Clostridium sticklandii VPI 14603 (L04167), Clostridium thermo-alkalophilum ATCC 51508T (L11304), Eubacterium terreum ATCC 25553T (M59118), Filifactor villosus DSM 1645T (X73452) and Peptostreptococcus anaerobius ATCC 27337T (D14150). 16S rDNA sequences from the newly isolated strains were compared with the sequences of reference organisms from the GenBank database.

**Phylogenetic analysis.** 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-931T and HD-17 were deposited in the DDBJ database. The following 16S rDNA sequences were used for the phylogenetic analysis: Clostridium butyricum DSM 2634 (X76161), Clostridium bifermentans ATCC 638T (X75906), Clostridium butyricum NCIMB 8082 (X68178), Clostridium difficile DSM 11209 (X73450), Clostridium felsineum DSM 794T (X77851), Clostridium formicaceticum DSM 92T (X77836), Clostridium ghetonii DSM 10636 (X73451), Clostridium glycolicum DSM 1288T (X76750), Clostridium halophilum DSM 5387T (X77837), Clostridium irregularum DSM 2635T (X73447), Clostridium litorale DSM 5388T (X78485), Clostridium lituseburense ATCC 25759T (M59107), Clostridium mangenotii ATCC 25761T (M59098), Clostridium paradoxum DSM 7308T (Z69939), Clostridium sordelli ATCC 9714T (M59105), Clostridium sticklandii VPI 14603 (L04167), Clostridium thermo-alkalophilum ATCC 51508T (L11304), Eubacterium terreum ATCC 25553T (M59118), Filifactor villosus DSM 1645T (X73452) and Peptostreptococcus anaerobius ATCC 27337T (D14150).
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-931ᵀ were straight or slightly curved rods that occurred in pairs or as single cells (Fig. 1). Spores were observed in strains TO-931ᵀ and HD-17. Strain TO-931ᵀ 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-931ᵀ 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-931ᵀ and HD-17 formed a single cluster and a new and distinct line of descent. Strain TO-931ᵀ 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-931ᵀ 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 7α-dehydroxylating activity (Archer et al., 1982; Hayakawa & Hattori, 1970), but their activities were very low compared with those of strains TO-931ᵀ and HD-17 (Doerner et al., 1997). It is interesting that strains TO-931ᵀ 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 7α-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 7α-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 1296ᵀ, *C. bifermentans* JCM 1386ᵀ, *C. sordellii* JCM 3814ᵀ and *C. glycolicum* JCM 1401ᵀ were used as the reference strains because they were closely related to strains TO-931ᵀ and HD-17 on the phylogenetic tree and because *C. bifermentans* and *C. sordellii* showed bile acid 7α-dehydroxylating activity. The G + C contents of strains TO-931ᵀ and HD-17 were 31·1 and 31·9 mol%, respectively. The levels of DNA–DNA hybridization between strains TO-931ᵀ and HD-17 were greater than 82 %, whereas the levels between strain TO-931ᵀ and the reference strains were less than 39 %. These data clearly revealed that strains TO-931ᵀ 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-931ᵀ and HD-17 should belong to a new species of the genus *Clostridium*. We propose the name *Clostridium hiranonis* for strains TO-931ᵀ and HD-17.

Now, the bile acid 7α-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-931ᵀ and HD-17 produce acid from fructose, glucose, mannose and sucrose, but not from adonitol, arabinose, amygdalin, cellobiose, dulcitol, aesculin, erythritol, aesculin, galactose, glycerol, glycophenyl toluidine, inositol, inulin, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, adonitol, amygdalin, arbutin, arabinose, aesculin, dulcitol, erythritol, galactose, glycerol, glycophenyl toluidine, inositol, inulin, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose,
salicin, sorbitol, sorbose, starch, trehalose or xylose. Strains TO-931$^T$ and HD-17 exhibit gas formation, do not produce indole or H$_2$S, 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*.

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

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

*According to Bergey’s Manual of Systematic Bacteriology (Cato et al., 1986).*
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. lituseburensense JCM 1404^T, the test below were all negative.

<table>
<thead>
<tr>
<th>Test</th>
<th>C. hiranonis TO-931^T</th>
<th>C. hiranonis HD-17</th>
<th>C. bifermentans JCM 1386^T</th>
<th>C. difficile JCM 1296^T</th>
<th>C. ghoni TO-1480^T</th>
<th>C. glycolicum JCM 1401^T</th>
<th>C. irregular JCM 1425^T</th>
<th>C. mangenotii JCM 1428^T</th>
<th>C. sordellii JCM 3814^T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>N-acetyl-β-D-glucosaminidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>α-Glucosidase</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Alkaline phosphatase</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Proline aminopeptidase</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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</table>

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 × 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-glucosaminidase and proline aminopeptidase in the ANI-DENT test. Moderate amounts of acetate 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-butrylic 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


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