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

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The Clostridium-like organisms TO-931T 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-931T 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-931T 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-931T (= JCM 10541T = DSM 13275T).

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

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 cocarcinogens (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-931T and HD-17, isolated from human faeces, were reported to exhibit high levels of bile acid 7α-dehydroxylation activity (Doerner et al., 1997; Hirano et al., 1981). Most of the strains that have high levels of bile acid 7α-dehydroxylation activity were identified as Clostridium scindens (Kitahara et al., 2000). Strains TO-931T 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-931T and HD-17 and C. scindens are noted as being highly bile acid 7α-dehydroxylation 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-dehydroxylation activity of strains TO-931T 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-931T and HD-17, which possess high levels of bile acid 7α-dehydroxylation 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-dehydroxylation activity, and other reference strains, were used in this study (Table 1). Strain TO-931T was
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 Eggert–Gagnon (EG) agar (Eiken) plates with 5% horse blood for 2 d at 37 °C in anaerobic jars (Hirayama Manufacturing Corporation) and strain HD-17 was isolated from the faeces of a healthy human by F. Takamine, using previously described procedures (Doerner et al., 1997).

Two strains were cultured on Eggert–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, 10 g NaHCO3, 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 NaOH.

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'-dAGAGTTTGTATCCTGGCTCAG-3' (designated primer 27F) and 5'-dGGTTACGACTT-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-931T 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-931T and HD-17 have been deposited in the DDBJ database. The following 16S rRNA/rDNA sequences were used for the phylogenetic analysis: C. hiranonis TO-931T DSM 2634 (X76161), C. bifermentans DSM 638 (X75906), C. butyricum DSM 8082 (X68178), C. difficile DSM 11209 (X73450), C. felsineum DSM 794 (X77851), C. formicaceticum DSM 92 (X77836), C. glycolicum DSM 10636 (X73451), C. glycolicum DSM 1288T (X76750), C. halophilum DSM 5387 (X77837), C. irregularae DSM 2635 (X73447), C. lituseburense DSM 5388T (X78845), C. limosum DSM 25799 (M59107), C. mangenotii DSM 92 (X76939), C. sordellii DSM 9174 (M59105), C. sticklandii DSM 11403 (L04167), C. testudinum DSM 51058 (L11304), C. thioalkaliphilum DSM 25533 (M59118), C. butyricum DSM 92 (X73452) and Peptostreptococcus anaerobius ATCC 27337T (D14150). C. butyricum NCIB 8012 was used as an outgroup organism.

DNA base composition. DNA was extracted from the cells harvested from EGF broth [24 g lab-lemco powder (Oxoid), 10 g proteose pepton No. 3 (Difco), 5 g yeast extract, 4 g NaHPO4, 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

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Source</th>
<th>Bile acid 7α-dehydroxylating activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. hiranonis TO-931T JCM 10541T</td>
<td>Faeces of a healthy adult</td>
<td>+</td>
<td>Doerner et al. (1997)</td>
</tr>
<tr>
<td>C. hiranonis HD-17 JCM 10542</td>
<td>Faeces of a healthy adult</td>
<td>+</td>
<td>Hirano et al. (1981)</td>
</tr>
<tr>
<td>C. bifermentans JCM 1386</td>
<td>Soil</td>
<td>+</td>
<td>Archer et al. (1982)</td>
</tr>
<tr>
<td>C. difficile JCM 1996</td>
<td>Faeces of a newborn infant</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>C. glutinum JCM 1400T</td>
<td>Post-operative human peritonitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. glycolicum JCM 1401T</td>
<td>Mud</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. irregularae JCM 1425T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. limeae JCM 1409T</td>
<td>Mud</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. mangenotii JCM 1428T</td>
<td>African soil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. sordellii JCM 3814T</td>
<td>Gangrene in humans</td>
<td>+</td>
<td>Hayakawa &amp; Hattori (1970)</td>
</tr>
</tbody>
</table>
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 nm, 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 1296², C. bifermentans JCM 1386², C. sordellii JCM 3814² and C. glycolicum JCM 1401³ 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, glycogen, inositol, inulin, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose,
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Filifactor villosus

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

\begin{table}[h]
\centering
\caption{DNA base composition and levels of DNA–DNA hybridization among \textit{Clostridium hiranonis} and related strains}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Strain & \textit{G} + \textit{C} content (mol\%) & DNA–DNA complementarity (\%) with labelled DNA from: \\
& & TO-931\textsuperscript{T} & HD-17 & JCM 1386\textsuperscript{T} & JCM 3814\textsuperscript{T} & JCM 1401\textsuperscript{T} \\
\hline
\textit{C. hiranonis} TO-931\textsuperscript{T} JCM 10541\textsuperscript{T} & 31.1 & 100 & 84 & 30 & 28 & 24 \\
\textit{C. hiranonis} HD-17 JCM 10542 & 31.9 & 82 & 100 & 32 & 30 & 24 \\
\textit{C. bifermentans} JCM 1386\textsuperscript{T} & 33.1 & 21 & 28 & 100 & 39 & 26 \\
\textit{C. sordellii} JCM 3814\textsuperscript{T} & 32.2 & 19 & 29 & 41 & 100 & 25 \\
\textit{C. glycolicum} JCM 1401\textsuperscript{T} & 29* & 18 & 22 & 23 & 23 & 100 \\
\textit{C. difficile} JCM 1296\textsuperscript{T} & 28* & 17 & 16 & 19 & 26 & 20 \\
\hline
\end{tabular}
\end{table}


salicin, sorbitol, sorbose, starch, trehalose or xylose. Strains TO-931\textsuperscript{T} and HD-17 exhibit gas formation, do not produce indole or \(\text{H}_{\text{2}}\text{S}\), do not reduce nitrate and do not hydrolyse aesculin, gelatin or starch.

\textbf{Enzymic activity}

The enzymic activity tests, using AN-IDENT, of strains TO-931\textsuperscript{T} and HD-17 are shown in Table 3. Strains phylogenetically related to \textit{C. hiranonis} in cluster XI were tested, as reference strains, at the same time. Strains TO-931\textsuperscript{T} and HD-17 gave positive reactions on \(N\)-acetyl-\(\beta\)-\(D\)-glucosaminidase and proline aminopeptidase, but the reference strains used in this test gave negative reactions on \(N\)-acetyl-\(\beta\)-\(D\)-glucosaminidase. The enzymic activity of \(N\)-acetyl-\(\beta\)-\(D\)-glucosaminidase is useful as a distinguishing characteristic of \textit{C. hiranonis}.
Table 3. Enzymic activity of Clostridium strains

<table>
<thead>
<tr>
<th>Test</th>
<th>C. hiranonis TO-931T</th>
<th>C. hiranonis HD-17</th>
<th>C. bifermantans JCM 1386T</th>
<th>C. difficile JCM 1296T</th>
<th>C. gnhii JCM 1400T</th>
<th>C. glycolicum JCM 1401T</th>
<th>C. irregular JCM 1425T</th>
<th>C. mangerentii JCM 1428T</th>
<th>C. sordelli JCM 3814T</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-β-glucosamidase</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>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>Proline aminopeptidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</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-931T 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 1–2–20 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, erythitol, aesculin, galactose, glycerol, 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-glucosaminidase 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-931T is 31-1 mol%. The type strain is strain TO-931T, isolated from the faeces of a healthy human, and has been deposited in the Japan Collection of Microorganisms, Japan, as strain JCM 10541T, and also in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany, as strain DSM 13275T.

REFERENCES


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