**Chitinibacter tainanensis** gen. nov., sp. nov., a chitin-degrading aerobe from soil in Taiwan

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Five strains with strong chitinolytic activity were isolated from a soil sample collected from southern Taiwan. The strains shared more than 92% DNA–DNA similarity, indicating membership of the same genospecies. This close relationship was supported by high similarities in fatty acid composition and biochemical characteristics. A 16S rRNA gene sequence analysis indicated that the isolates were members of the class ‘Betaproteobacteria’, in which they formed an individual subline of descent that was distantly related (<94% similarity) to lineages defined by *Formivibrio citricus* DSM 6150T and *Iodobacter fluviatilis* DSM 3764T. On the basis of the phylogenetic and phenotypic distinctness of these novel chitin-degrading organisms, a new genus, *Chitinibacter*, is proposed, with *Chitinibacter tainanensis* (type strain, S1T = BCRC 17254T = DSM 15459T) as the type species.

Next to cellulose, chitin is the second most-abundant polysaccharide on Earth, as it is a major component of most fungal cell walls, insect exoskeletons and the shells of crustaceans (Flach et al., 1992). Derivatives of chitin, including polysaccharides, oligosaccharides and monosaccharides, have been shown to play a role in the organogenesis of plants and the embryogenesis of invertebrates (Bakkers et al., 1997). Recently, chitin oligosaccharides have been demonstrated to enhance functions of the immunological system in host animals, possibly functioning as tumoricidal agents (Suzuki et al., 1986). To prepare functional chitin derivatives, micro-organisms were isolated from soils and screened for chitinase activities. This report deals with the detailed characterization of five strains of bacteria with strong chitinolytic activity and with the proposal of *Chitinibacter tainanensis* gen. nov., sp. nov.

Chitinolytic bacteria were isolated from various soil samples collected from Tainan Prefecture in the southern part of Taiwan, using agar medium containing 0.2% colloidal chitin (Hsu & Lockwood, 1975) as a selective medium. The reduced sugars formed in the medium, indicating the degradation of chitin, were detected according to a modified procedure of Imoto & Yagishita (1971). Colonies with surrounding clear zones were selected and incubated at 30°C in Luria–Bertani (LB) medium (Life Science).

Micro-organisms used for comparison with the isolated chitin-degrading strains were *Alcaligenes latus* ATCC 29712T [obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA], *Iodobacter fluviatilis* DSM 3764T and *Formivibrio citricus* DSM 6150T [both obtained from the Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ), Braunschweig, Germany]. Growth media for *I. fluviatilis* and *A. latus* were nutrient agar (Difco) and brain–heart infusion medium (Difco), while *F. citricus* was cultivated on *Formivibrio* medium (medium DSM 505; DSMZ, 2001). Cultivation was at 30°C. Ten strains of chitin-degrading micro-organisms were isolated from soil samples collected in the southern part of Taiwan. Strains isolated from Tainan grew at 30°C aerobically with shaking, and showed the strongest chitin-degrading potential, as indicated by determination of the reducing power in the medium. Strain S1T, which was deposited at the Bioresource Collection and Research Centre, Food Industry Research and Development Institute, Taiwan as *C. tainanensis* (as BCRC 17254T) and at the DSMZ (as DSM 15459T), and strains S5, S6, S9 and S12 were selected for further studies.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Chitinibacter tainanensis* S1T is AY264287. The phenotypic characteristics (Table A) and cellular fatty acid compositions (Table B) of the novel chitin-degrading strains, and DNA–DNA hybridization results (Table C), are available as supplementary data in IJSEM Online.
All tests on the novel bacteria were performed using LB medium at 30 °C unless stated otherwise. The cell morphology of the strains was observed using a Zeiss light microscope. To visualize flagella, a drop of bacterial suspension was gently transferred to a copper grid (square 100-mesh) which was cleaned with acetone, coated with colloïdion, dried in a desiccator overnight and then stabilized with carbon in a high-vacuum coating unit (HUS-SGB; Hitachi). Excess water was removed by absorption with filter paper from the side of the grid. Bacteria retained on the grid were negatively stained with 1% phosphotungstic acid (pH 7) for 60 s. After the stain solution had been removed from the grid with filter paper, the images were viewed and photographed with a Hitachi H-600 transmission electron microscope. A Gram stain was performed using the Gram-colour set (Merck). Growth at various temperatures, ranging from 5 to 50 °C, using 5 °C increments, was determined on LB medium. The diameters of colonies were measured after incubation at 30 °C for 3 days. The effect of pH on growth was tested with 0·5 × LB medium buffered with 0·2 M citric acid and Na2HPO4 and adjusted to pH values 3, 4 and 5–10 (using increments of 0·5) with NaOH or HCl. Aliquots (5 ml) of the pH-buffered medium in 13 × 100 mm screw-capped test tubes were inoculated with 0·25 ml bacterial suspension (concentration equal to McFarland 1) prepared from a 2-day-old culture. Four replicates were used for each pH test. After incubation under shaking for 7 days, growth was indicated by a change in optical density at 400 nm, measured using a spectrophotometer (Walden Precision Apparatus). The pH was stable during the incubation. The oxygen requirement was tested by using growth distribution following a shake-tube inoculation method (Cappuccino & Sherman, 1987). The presence of cytochrome oxidase was tested using oxidase reagent droppers (Difco); catalase activity was determined using H2O2 solution as described by Koneman et al. (1979). The abilities of the strains to utilize various substrates as sole carbon sources were evaluated using the API 20NE panel (bioMérieux) and Vitek NFC cards (bioMérieux) according to the manufacturer’s instructions.

Hydrolysis of chitin by the isolated strains was tested using agar medium supplemented with colloidal chitin as described above. Cytophaga sp. DSM 3657 and Serratia marcescens DSM 30121T were used as chitinase-positive control strains. I. fluviatilis DSM 3764T and F. citricus DSM 6150T were also tested, as they were phylogenetic neighbours of the novel isolates. The preparation of colloidal chitin was according to the description of Monreal & Reese (1969).

The agar medium contained the following (l–l) according to the description of Monreal & Reese (1969). The preparation of colloidal chitin was also tested, as they were phylogenetic neighbours of the novel isolates. The optimal pH was around pH 7–5.

While the chitinase-positive reference strains Cytophaga sp. DSM 3657 and S. marcescens DSM 30121T, as well as the novel isolate SI1, were strongly positive, the reaction of I. fluviatilis DSM 3764T was very weak and delayed, producing a small clear zone of about 2 mm around the large colonies of 2·5 cm in diameter. F. citricus DSM 6150T did not grow at all on the chitin agar, while the control grew on the same medium in which chitin had been replaced by trisodium acetate. This strain should therefore be considered chitin-negative.

The ubiquinone system of the novel chitin-degrading bacteria was determined by using the method described by Collins & Jones (1981). The TLC-purified quinones were analysed with a Nova-Pak C18 (15 × 3·9 cm) column (Waters). Peaks were detected at 275 nm after elution with methanol/2-propanol (2:1) at flow rate of 1 ml min⁻¹. Q-6, Q-7, Q-9 and Q-10 standards were purchased from Sigma. Q-8 was extracted from the A. latus ATCC 29712 culture. Q-8 was found to be the major quinone in strain SI1 (98% of the total quinones) and I. fluviatilis DSM 3764T (95%); this is quite common among members of the ‘Betaproteobacteria’.

Cellular fatty acid compositions of the novel isolates and I. fluviatilis DSM 3764T and F. citricus DSM 6150T were determined using the Sherlock Microbial Identification System (MIDI). Extracts of the methylated fatty acids were prepared according to the protocol provided by the manufacturer and analysed with a 5890 gas chromatograph equipped with a flame ionization detector and an automatic injector with a G1512A controller (Hewlett Packard). Identification of the peaks was made by comparing the results with the built-in TSBA 40 database (MIDI). The major fatty acids (>1% of total fatty acids) of the chitin-degrading strains were as follows: 16:1ω7c/15 iso 2-0H (48–45–51 83 %), 18:1ω7c (17–72–19 85 %), 16:0 (15–36–17 57 %), 12:0 3-OH (3–87–4–21 %), 12:0 3–OH (2–45–3–09 %), 10-methyl 19:0 (1–37–2–03 %) and 16:0 3-OH (1–24–1–44 %). A complete listing of the fatty acids is available in IJSEM Online (Table B). The differences in fatty acid profiles of phylogenetically related species are listed in Table 1.
Isolation of genomic DNA for sequence analysis was done as described by Franke et al. (1999). PCR amplification of the 16S rRNA gene and the subsequent cycle sequencing reaction were carried out using the MicroSeq 16S rRNA gene kit (Applied Biosystems). The products were analysed with a PRISM 310 genetic analyser (Applied Biosystems). Phylogenetic analyses was performed by using different treeing algorithms [distance matrix (De Soete, 1983; Felsenstein, 1993); neighbour joining and maximum parsimony (Felsenstein, 1993)].

Analysis of the 16S rRNA gene sequence of strain S1T, consisting of 1514 nt, revealed its closest phylogenetic neighbours to be F. citricus DSM 6150T (Y17602) and I. fluviatilis ATCC 33051T (M22511), members of the ‘Betaproteobacteria’, with similarity values of 93 %–4 % and 90 %–6 %, respectively. Partial 16S rRNA gene sequence analysis (positions 13–518 according to the Escherichia coli numbering system; Brosius et al., 1978) of the other four chitin-degrading isolates revealed 100 % identity with the homologous stretch of strain S1T. Different treeing algorithms gave consistent results by placing the chitin-degrader as a new lineage between the Formivibrio and Iodobacter lineages (Fig. 1). The order of branching points within this cluster is not stable as judged from the bootstrap value of 64 %.

A commercial kit (Genomic-tips; Qiagen) was used for the preparation of genomic DNA for determination of the base composition of the DNA (mol% G + C content) and DNA–DNA reassociation. The G + C content was determined by using reversed-phase HPLC (Tamaoka & Komagata, 1984) with slight modifications. The nucleotides were separated using a Cosmosil 5C18 column (4.0 × 150 mm) (Waters) in a mobile phase composed of 0.2 M NH4H2PO4/acetonitrile (20:1, v/v) at a flow rate of 1 ml min−1 at room temperature. The nucleotides were detected and quantified by absorption at 270 nm. DNA similarity values between the chitin-degrading strains, and between two of these strains and the phylogenetically nearest type strains of other species, were determined using the fluorometric hybridization method in microdilution wells (Ezaki et al., 1989). The fluorescence intensity of each well was measured with a Fluoroskan II microplate fluorometer (Labsystems) at a wavelength of 360 nm for excitation and at 450 nm for emission. The chitin-degrading

### Table 1. Differential characteristics of C. tainanensis from phylogenetically related species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>C. tainanensis S1T</th>
<th>F. citricus DSM 6150T*</th>
<th>I. fluviatilis NCTC 11159T†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic growth</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Chitin hydrolysis</td>
<td>+</td>
<td>−</td>
<td>Weak (delayed)</td>
</tr>
<tr>
<td>Cellular fatty acids</td>
<td>12:0 3-OH 3:87–4:21 %</td>
<td>−/Trace</td>
<td>2:3 %</td>
</tr>
<tr>
<td></td>
<td>14:0 Trace 2:2 %</td>
<td></td>
<td>8:4 %</td>
</tr>
<tr>
<td></td>
<td>14:0 3-OH 9:5 %</td>
<td></td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>10-methyl 19:0 1:37–2:03 %</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Growth at 40 °C</td>
<td>+</td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>55:9–56:2</td>
<td>61</td>
<td>50–52</td>
</tr>
<tr>
<td>Major quinone</td>
<td>Q-8</td>
<td>ND</td>
<td>Q-8</td>
</tr>
</tbody>
</table>

ND, Not determined.
*Data for F. citricus were taken from Logan (1989), except for chitin hydrolysis and cellular fatty acids.
†Data for I. fluviatilis were taken from Tanaka et al. (1991) and Hippe et al. (1999), except for chitin hydrolysis and cellular fatty acids.
‡All five strains of C. tainanensis were tested.

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the nearest neighbours of the novel chitin-degrading bacterium in the class ‘Betaproteobacteria’. The bar corresponds to a 10 % difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting any two organisms. The numbers shown at the branch points indicate percentage bootstrap values from 1000 datasets. Only bootstrap values greater than 60 % are shown.
strains had a DNA G+C content ranging from 55·9 to 56·2 mol%. This value is significantly lower than the 61 mol% determined for \textit{F. citricus} DSM 6150\textsuperscript{T} (Hippe et al., 1999) and higher than the 50–52 mol% of \textit{I. fluviatilis} NCTC 11159\textsuperscript{T} (Logan, 1989). The DNA similarity test revealed that the five strains shared high DNA similarity values (92–100%), indicating membership of the same genospecies. DNA similarity values between strain S1\textsuperscript{T} or strain S12 and \textit{F. citricus} DSM 6150\textsuperscript{T} and \textit{I. fluviatilis} DSM 3764\textsuperscript{T} were below 10%.

The five isolates from Taiwanese soil were identical with respect to morphology, physiology, chemotaxonomy and genomic properties, and should be considered members of the \textit{Betaproteobacteria}, with \textit{Formivibrio} and \textit{Iodobacter} as the closest (albeit remote) neighbours. Strain S1\textsuperscript{T} and the four similar strains (S5, S6, S9 and S12) can be clearly distinguished from the two phylogenetic neighbours which form two clearly separate genomic entities, as judged from the low sequence-similarity values. The most salient property is the degradation of chitin, a characteristic that is weak or absent in the type strains of the phylogenetic neighbours. Other characteristics separating the chitinolytic taxon from \textit{Formivibrio} and \textit{Iodobacter} are listed in Table 1. Although some chitinolytic bacteria have been shown to be insect pathogens (Sampson & Gooday, 1998), the question of whether these new Taiwanese strains are pathogenic to insects remains to be investigated.

**Description of the genus Chitinibacter gen. nov.**

\textit{Chitinibacter} (chi.ti.ni.bac\textsuperscript{ter}. N.L. n. \textit{chitinum} chitin; N.L. masc. n. \textit{bacter} rod; N.L. masc. n. \textit{Chitinibacter} rod that degrades chitin).

Strictly aerobic, Gram-negative rods that are straight to slightly curved (Fig. 2a) with round ends, 1·3–2·6 μm in length and 0·5–0·9 μm in width. Highly motile by means of one polar flagellum or two polar flagella (Fig. 2b, c). Oxidase- and catalase-positive. No acid from glucose. Q-8 is the predominant isoprenoid quinone. Major fatty acids (>5%) are 16:1ω7c/15 iso 2-ΟΗ (48·45–51·83%), 18:1ω7c (17·72–19·85%) and 16:0 (15·36–17·57%). Phylogenetically, a member of the class ‘\textit{Betaproteobacteria}’. The G+C content of the DNA is 56 mol%.

The type species is \textit{Chitinibacter tainanensis}.

**Description of Chitinibacter tainanensis sp. nov.**

\textit{Chitinibacter tainanensis} (tai.na.nen’sis. N.L. adj. \textit{tainanensis} referring to Tainan, a town in Southern Taiwan, the origin of the soil sample from which the type strain was isolated).

In addition to the properties defining the genus, strains of the species form milky white, circular, entire, convex colonies on LB agar, measuring 0·5–2·0 mm in diameter. Growth occurs at temperatures from 10 to 40°C, with the optimum at 30–37°C, and at pH values from 5·5 to 9·0, with the optimum at pH 7·5. Negative for nitrate reduction, indole production, arginine dihydrolase, urease, β-glucosidase, β-galactosidase and protease. On the basis of API and Vitek tests, glucose, N-acetylglucosamine and gluconate are utilized. The following are not utilized: arabinose and D-mannose (strain S6 gave a weak reaction for both in API tests), D-mannitol, maltose and citrate. API tests were negative for maltose, caprate, adipate, malate and phenyl acetate. Vitek tests were negative for myoinositol, D-galactose, sucrose, trehalose, D-xylose, DL-lactate, sodium acetic acid, propionic acid, adipic acid, itaconic acid, suberic acid, sebacic acid, azelaic acid, heptanoic acid, L-aspartic acid, L-alanine, L-histidine and L-proline. Isolated from Taiwan, near Tainan Prefecture (120°16′21″E 23°13′09″N).

Type strain is S1\textsuperscript{T} (=BCRC 17254\textsuperscript{T} = DSM 15459\textsuperscript{T}).

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References


