Chitinivorax tropicus gen. nov., sp. nov., a chitinolytic bacterium isolated from a freshwater lake

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A facultatively anaerobic, chitinolytic bacterium, strain KL-9⁷, was isolated from a freshwater lake in Taiwan and characterized by using a polyphasic taxonomic approach. Cells of strain KL-9⁷ were Gram-negative, rod-shaped, motile by means of a single polar flagellum and non-spore-forming. Growth occurred at 15–40 ºC (optimum, 30–37 ºC), at pH 7.0–9.0 (optimum, pH 8.0) and with 0–1.0 % NaCl (optimum, 0 %). The predominant fatty acids were summed feature 3 (comprising C₁₆ : ₁₀, C₁₆ : ₁₀ and/or C₁₆ : ₁₀(6,c)) and C₁₆ : ₁₀. The major isoprenoid quinone was Q-8. The DNA G+C content of strain KL-9⁷ was 64.6 mol%. The polar lipid profile consisted of a mixture of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidyl[dimethyl]ethanolamine and several uncharacterized phospholipids and aminolipids. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain KL-9⁷ formed a distinct lineage with respect to closely related genera within the class Betaproteobacteria, being most closely related to members of the genera Leeia, Chitinimonas, Silvimonas and Andreprevotia. Levels of 16S rRNA gene sequence similarity with respect to the type strains of type species of these genera were below 91 %. On the basis of genotypic and phenotypic data, strain KL-9⁷ is thus considered to represent a novel species of a new genus within the class Betaproteobacteria, for which the name Chitinivorax tropicus gen. nov., sp. nov. is proposed. The type strain of Chitinivorax tropicus is KL-9⁷ (=BCRC 80168T=LMG 25530T).

During investigations of the biodiversity of micro-organisms associated with Jin-Shih Lake (22° 39' 56" N 120° 19' 34" E; pH 8.6, 32 ºC), Kaoshiung County, Taiwan, several cream-coloured bacterial colonies were isolated on R2A agar (Difco). Strains showing similar colony morphology were selected and one representative, strain KL-9⁷, was selected for detailed analysis. Strain KL-9⁷ was maintained on R2A agar at 25 ºC. Subcultivation was performed on R2A agar at 25 ºC for 48–72 h. On this medium, strain KL-9⁷ was able to grow at 15–40 ºC, but not at 10 or 45 ºC. The isolate was preserved at –80 ºC in R2A broth with 20 % (v/v) glycerol or by lyophilization.

Genomic DNA was isolated by using a bacterial genomic kit and the 16S rRNA gene sequence was analysed as described by Chen et al. (2001). Primers FD1 (5'-AGAGTTGTATCTCGGCTCAG-3') and RD1 (5'-AAAGGTTGATCCAGCC-3') were used for amplification of bacterial 16S rRNA genes by PCR. These primers correspond to nucleotide positions 8–27 and 1524–1540 of the Escherichia coli 16S rRNA gene, respectively, and can be used for amplifying nearly full-length 16S rRNA genes. The PCR product was purified, and direct sequencing was performed by using sequencing primers FD1, RD1, 520F and 800R (Weisburg et al., 1991; Anzai et al., 1997) with a DNA sequencer (ABI Prism 3730; Applied Biosystems). The almost-complete 16S rRNA gene sequence (1454 nt) of strain KL-9⁷ was compared against 16S rRNA gene sequences available from the EzTaxon server (Chun et al., 2007), the Ribosomal Database Project (Maidak et al., 2001) and GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Analysis of the sequence data was performed by using the software package BioEdit (Hall, 1999) and MEGA version 3.1 (Kumar et al., 2004), after multiple alignments of the data by CLUSTAL X (Thompson et al., 1997). The resulting multiple sequence alignment was corrected manually and gaps at the 5' and 3' ends of the alignment were omitted from further
analyses. Distances (corrected according to Kimura’s two-parameter model; Kimura, 1983) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). The maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were generated by using the treeing algorithms contained in the PHYLIP software package (Felsenstein, 1993).

In each case, bootstrap values were calculated based on 1000 replications.

The phylogenetic analyses based on 16S rRNA gene sequences showed that strain KL-9T formed a distinct lineage with respect to the closely related genera *Leeia* (Lim et al., 2007), *Chitinimonas* (Chang et al., 2004), *Silvimonas* (Yang et al., 2005) and *Andreprevotia* (Weon et al., 2007) within the class *Betaproteobacteria* in the neighbour-joining tree (Fig. 1). The overall topologies of the phylogenetic trees obtained with the maximum-likelihood and maximum-parsimony methods were similar. 16S rRNA gene sequence similarity calculations (over 1400 bp) indicated that strain KL-9T was closely related to *Leeia oryzae* HW7T (90.7% similarity), *Silvimonas terrae* KM-45T (89.9%), *Chitinimonas taiwanensis* cfT (89.7%) and *Andreprevotia chitinilytica* JS11-7T (88.8%).

The above four type strains were used as reference strains and evaluated together under identical experimental conditions to those for strain KL-9T. *L. oryzae* HW7T (=DSM 17879T) and *A. chitinilytica* JS11-7T (=DSM 18519T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *C. taiwanensis* cfT (=BCRC 17210T) was obtained from the Bioresource Collection and Research Center (BCRC). *S. terrae* KM-45T (=NBRC 100961T) was obtained from the NITE Biological Research Center (NBRC).

Cell morphology was observed by phase-contrast microscopy (Leica DM 2000) and by scanning electron microscopy (S-3500N; Hitachi) (see Fig. S1 in IJSEM Online) by using cells grown on R2A agar at 30 °C for 6 h (lag growth phase), 18 h (exponential phase) and 36 h (stationary phase). Cellular motility was tested by the hanging drop method (Murray et al., 1994). The Gram Stain Set S (Difco) kit and the Ryu non-staining KOH method (Powers, 1995) were used for testing the Gram reaction. Colony morphology was observed on R2A agar by using a stereoscopic microscope (SMZ 800; Nikon).

The pH range for growth was determined by measuring the optical densities (at 600 nm) of R2A broth cultures. pH was adjusted prior to sterilization to pH 4.0–9.0 (at intervals of 1.0 pH unit) by using the appropriate biological buffers (Breznak & Costilow, 1994): citrate/Na₂HPO₄ buffer, pH 4.0–5.0; phosphate buffer, pH 6.0–7.0; Tris buffer, pH 8.0–9.0. Verification of pH after autoclaving revealed only minor changes. The temperature range for growth was determined on R2A agar at 4, 10, 15, 20, 25, 30, 35, 37, 40, 45 and 50 °C. To investigate tolerance to NaCl,
R2A broth was prepared according to the formula of the Difco medium with NaCl concentration adjusted to 0, 0.5 and 1.0-6.0% (w/v, at intervals of 1.0%). Growth under anaerobic conditions was determined after incubating strain KL-9T on R2A agar in the Oxoid AnaeroGen system. Strain KL-9T was examined for a broad range of phenotypic properties. Activities of catalase, oxidase, DNase, urease and lipase (corn oil), and hydrolysis of starch, casein, gelatin and Tween 20, 40, 60 and 80 were determined by using standard methods (Smibert & Krieg, 1994). Catalase production was demonstrated on a slide by the production of bubbles from a drop of a 3% hydrogen peroxide solution. DNase activity was read after 48 h of incubation by flooding DNase agar (Difco) plates with a 0.1% (w/v) aqueous solution of toluidine blue. Heavy inoculation of standard Christensen urea agar medium was used for demonstration of urease production. The gelatin liquefaction test was performed by using R2A broth supplemented with 4% gelatin and incubated at the optimum growth temperature. Chitin hydrolysis activity was determined on a chitinase-detection agar plate (CDA plate). Chitin hydrolysis was visualized by the formation of a clear zone around the colonies on CDA plates. The CDA plate was prepared as described by Wen et al. (2002). Hydrolysed chitin (hydrolysate) was analysed by MS after the culture supernatant of microbial cells was added to phosphate buffer with 0.5% colloidal chitin for 12 h at 37 °C. Mass spectra were recorded by using a quadrupole time-of-flight mass spectrometer (Q-TOF; Micromass). The quadrupole mass analyser was scanned over a mass-to-charge ratio range of 100–1500 Da (m/z) with a scan step of 2 s and inter-scan interval of 0.1 s per step. Additional, biochemical tests were performed by using API ZYM, API 20E and API 20NE kits (all from bioMérieux) and carbon source utilization was evaluated by using GN2 microplates (Biolog). All commercial phenotypic tests were performed according to the manufacturers' recommendations.

For analysis of whole-cell fatty acids, all strains were grown on R2A agar at 30 °C for 2 days. Fatty acid methyl esters were prepared and separated according to the standard protocol described in the Microbial Identification System (Microbial ID), and were identified by MIDI version 6.0 and the RTSSBA6.00 database. The major cellular fatty acids of strain KL-9T (>5%) were summed feature 3 (comprising C16:1ω7c and/or C16:1ω6c (39.4%), C16:0 (26.3%), C12:0 (6.7%) and C18:1ω7c (61.1%). The detailed fatty acid compositions of strain KL-9T and its closest phylogenetic neighbours are shown in Table 1. The fatty acid profile of strain KL-9T was similar to those of its closest phylogenetic neighbours, although there were differences in the proportions of some components. Like its closest relatives, L. oryzae HW7T, C. taiwanensis cf5, C. terrae KM-45T and A. chitinilytica JS11-7T, strain KL-9T had a high proportion of C16:0 and summed feature 3. However, the much lower proportion of C16:0 and presence of C17:0 cyclo in strain KL-9T allowed good discrimination of the novel strain from L. oryzae HW7T. Strain KL-9T contained from C. taiwanensis cf5 based on a much lower proportion of C10:0 3-OH, and it could be distinguished from S. terrae KM-45T and A. chitinilytica JS11-7T by its much lower proportion of C18:1ω7c. Furthermore, the presence of some minor fatty acids such as C15:0ω6c and C16:1ω7c alcohol is helpful in separating the novel strain from its four closest phylogenetic neighbours.

Polar lipids were extracted and analysed by two-dimensional TLC according to Embley & Wait (1994). Molybdo-phosphoric acid was used for detection of all lipids, ninhydrin reagent for lipids containing free amino groups, Zinzadze reagent for phosphorus-containing lipids and x-naphthol reagent for glycolipids. Strain KL-9T exhibited a complex polar lipid profile consisting of phosphatidyl-ethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidyldimethylethanolamine and several uncharacterized phospholipids and aminolipids (Fig. S2). Like its closest relatives, L. oryzae HW7T, C. taiwanensis cf5, C. terrae KM-45T and A. chitinilytica JS11-7T, strain KL-9T contained

<table>
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<tr>
<th>Fatty acid</th>
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<th>4</th>
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<td>30.9</td>
</tr>
<tr>
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<td>2.3</td>
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</tr>
<tr>
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<td>1.3</td>
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<td>2.5</td>
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<td>39.4</td>
<td>37.7</td>
<td>36.3</td>
<td>10.2</td>
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</table>

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 comprises C14:0 3-OH and/or iso-C15:0 and summed feature 3 comprises C16:1ω7c and/or C16:1ω6c.

Table 1. Cellular fatty acid compositions of strain KL-9T and its closest phylogenetic neighbours

Strains: 1, KL-9T; 2, Leeia oryzae HW7T; 3, Chitinimonas taiwanensis cf5; 4, Silvimonas terrae KM-45T; 5, Andreprevotia chitinilytica JS11-7T. All data are from this study. All strains were grown on R2A agar at 30 °C for 2 days. Values are percentages of the total fatty acids; fatty acids that make up <1% of the total are not shown or are indicated by a dash. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. The cis isomer is indicated by the suffix c.
phosphatidylethanolamine, phosphatidylglycerol and di-
phosphatidylglycerol. However, phosphatidylmethylth-
nolamine was detected in strain KL-9T and Phosphatidyldimethyleth-
aldehyde, phosphatidylethanolamine, phosphatidylglycerol and di-
phosphatidylglycerol. However, phosphatidylmethylth-
nolamine was detected in strain KL-9T and Phosphatidyldimethyleth-

Isoprenoid quinones were extracted and purified according
to the method of Collins (1985) and were analysed by
HPLC. Both strain KL-9T and C. taiwanensis Cl1 had Q-8
as their major respiratory quinone, which is the same as
those of the other three closest relatives (Lim et al., 2007;
Weon et al., 2007; Yang et al., 2005). The DNA G+C
content of strain KL-9T, determined by HPLC according
to Mesbah et al. (1989), was 64.6 ± 1.0 mol% (mean ± SD of
3 determinations).

MS examination of the chitinolytic products revealed that
they contained monosaccharide and disaccharide
(GlcNAc and (GlcNAc)2; Fig. S3). Detailed results of physiological,
biochemical and morphological characterization of strain
KL-9T are provided in the genus and species descriptions
below and in Table 2. Phenotypic examination revealed
many common traits between the novel strain and its closest
phylogenetic neighbours, L. oryzae HW7T, C. taiwanensis Cl2,
S. terrae KM-45T and A. chitinilytica JS11-7T. However,
strain KL-9T could be differentiated clearly from these four
taxa by its temperature range for optimal growth (30–37
°C), its pH for optimal growth (pH 8.0), the presence of
α-fucosidase activity and its inability to assimilate arabinose
or N-acetylglucosamine (Table 2). Strain KL-9T could also be
differentiated from L. oryzae HW7T by the inability to
ferment glucose, ability to hydrolyse starch, corn oil and
Tweek 40, inability to assimilate gluconate, adipate or
malate, and presence of C14 lipase, valine arylamidase,
cystine arylamidase, trypsin and α-chymotrypsin activities. It
could be distinguished from C. taiwanensis Cl2 based on the
ability to hydrolyse DNA and Tweek 40, inability to
hydrolyse gelatin or ascucin, inability to assimilate mannitol,
and absence of urease, β-galactosidase, β-glucosidase and N-
acetyl-β-glucosaminidase activities. Some features of strain
KL-9T, such as the inability to ferment glucose, ability to
hydrolyse casein and corn oil, inability to hydrolyse ascucin,
inability to assimilate mannitol or glucosace, ability to
assimilate citrate, and absence of β-galactosidase, β-glucosi-
dase and N-acetyl-β-glucosaminidase activities, may be
helpful for separating the novel strain from S. terrae KM-
45T. In addition, strain KL-9T could be differentiated from A.
chitinilytica JS11-7T by the inability to ferment glucose,
ability to reduce nitrate, ability to hydrolyse DNA and casein,
inability to hydrolyse Tweek 80 or gelatin, inability to
assimilate mannitol or glucosace, ability to assimilate citrate,
presence of C8 esterase lipase, C14 lipase, valine arylamidase,
cystine arylamidase, trypsin and α-chymotrypsin, and
absence of N-acetyl-β-glucosaminidase activities.

It is now generally accepted that 16S rRNA gene sequence
similarities between two bacterial strains of less than 95 %
are an indication of affiliation to different genera (Ludwig
et al., 1998). Strain KL-9T most likely represents a species of
a new genus, as levels of 16S rRNA gene sequence similarity
to its closest recognized relatives, L. oryzae HW7T, S. terrae
KM-45T, C. taiwanensis Cl2 and A. chitinilytica JS11-7T, were
90.7, 89.9, 89.7 and 88.8 %, respectively. Moreover, strain
KL-9T could be readily distinguished from these phylo-
genetic neighbours based on fatty acid composition and
physiological and biochemical characteristics. Therefore,
based on phenotypic and phylogenetic criteria, we suggest
that strain KL-9T represents a novel species of a new genus,
for which the name Chitinivorax tropicus gen. nov., sp. nov.
is proposed.

Description of Chitinivorax gen. nov.

Chitinivorax (Chi.ti.ni.vo’rax. N.L. n. chitinum chitin; L.
adj. vorax devouring, ravenous, voracious; N.L. masc. n.
Chitinivorax chitin-devouring).

Cells are Gram-negative, rod-shaped, motile by means of a
single polar flagellum, non-spore-forming and facultatively
anaerobic. Oxidase- and catalase-positive. The predominant
quinone is Q-8. The major cellular fatty acids (>5 %)
are summed feature 3 (comprising C16:1ω7c and/or C16:1ω6c),
C16:0, C12:0 and C18:1ω7c. Phosphatidylethanolamine, phospha-
idylglycerol, diphosphatidylglycerol, phospha-
}
urease and \( \beta \)-galactosidase activities, and assimilation of arabinose, mannitol, \( N \)-acetylglucosamine, gluconate, caprate, adipate, malate and phenylacetate. In the API ZYM kit, positive for alkaline phosphatase, C4 esterase, C14 lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, \( \alpha \)-chymotrypsin, \( \alpha \)-glucosidase and \( \alpha \)-fucosidase activities, but negative for (Table 2. Differential characteristics of strain KL-9\textsuperscript{T} and its closest phylogenetic neighbours

<table>
<thead>
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<th>Characteristic</th>
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<tr>
<td>Cell size (( \mu )m)</td>
<td>0.3–0.4×2.5–3.5</td>
<td>0.5–0.8×1.1–1.8</td>
<td>0.3–0.4×1.2–1.8</td>
<td>0.5–0.8×1.3–2.0</td>
<td>0.7–0.9×2.0–3.0</td>
</tr>
<tr>
<td>pH range for growth (optimal)</td>
<td>7.0–9.0 (8.0)</td>
<td>5.0–8.0 (6.0)</td>
<td>4.0–9.0 (6.0–8.0)</td>
<td>6.0–8.0 (7.0)</td>
<td>5.0–8.0 (6.0–7.0)</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adipate</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Malate</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Polar lipids*</td>
<td>PE, PG, DPG, PDE, PL1, PL2, AL1, AL2, AL3, AL4</td>
<td>PE, PG, DPG, GL, PL1, PL2, PL3, AL3</td>
<td>PE, PG, DPG, PL1, PL2, PL3, AL3</td>
<td>PE, PG, DPG, PL2, AL3</td>
<td>PE, PG, DPG, PL2, PE, PG, DPG, PDE, AL3</td>
</tr>
<tr>
<td>DNA G+C content (mol%)†</td>
<td>64.6</td>
<td>56\textsuperscript{a}</td>
<td>62.8\textsuperscript{b}</td>
<td>58\textsuperscript{c}</td>
<td>62\textsuperscript{d}</td>
</tr>
</tbody>
</table>

*PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PDE, phosphatidyldimethylethanolamine; PL1–PL4, uncharacterized phospholipids; GL, uncharacterized glycolipid; AL1–AL4, uncharacterized aminolipids.

†Data from: a, Lim et al. (2007); b, Chang et al. (2004); c, Yang et al. (2005); d, Weon et al. (2007).
z-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase and z-mannosidase activities. The following compounds are utilized as sole carbon sources in GN2 microplates: glycogen, Tweens 40 and 80, adonitol, d-arabitol, d-fructose, gentiobiose, z-D-glucose, z-lactose, maltose, D-mannose, melibiose, D-psicose, turanose, cis-aconitic acid, citric acid, D-galacturonic acid, D-gluconic acid, itaconic acid, z-ketoglutaric acid, succinic acid, succinamic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, L-leucine, L-ornithine, L-serine, L-threonine, inosine, x-D-glucose 1-phosphate and d-glucose 6-phosphate. Does not utilize any of the other substrates in the GN2 microplate.

The type strain, KL-9T (= BCRC 80168T = LMG 25530T), was isolated from a freshwater sample collected from Jin-Shih Lake, Kaoshiung County, Taiwan. The DNA G+C content of the type strain is 64.6 mol%.

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References


