A bacterial strain named IGB-41T was isolated from a soil sample from an ant hill near Stuttgart, Germany. The strain was Gram-negative, rod-shaped, motile and facultatively anaerobic. Phylogenetic analysis based on 16S rRNA grouped the strain IGB-41T within the class Betaproteobacteria into the family Neisseriaceae together with Silvimonas amyloymylica NBRC 103189T, Silvimonas iriomotensis NBRC 103188T and Silvimonas terrae KM-45T as the closest relatives with sequence similarities of 96.7, 96.6 and 96.1 %, respectively. The G+C content of the genomic DNA was determined to be 61.5 mol% and quinone analysis revealed Q-8 as the only detectable quinone. Major cellular fatty acids were identified as C16:0, summed feature 3 (iso-C15:0 2-OH and/or C16:1ω7c) and C18:1ω7c. Strain IGB-41T was unique in harbouring phosphoaminolipids, aminolipids and glycoaminolipids when compared with Silvimonas amyloymylica NBRC 103189T in polar lipid analysis. On the basis of the physiological, phenotypic and genotypic characteristics of strain IGB-41T, we suggest that the novel strain should be assigned to a new genus Amantichitinum and novel species Amantichitinum ursilacus. The type species of the genus Amantichitinum is Amantichitinum ursilacus and the type strain is IGB-41T (=DSM 23761T=CIP 110167T).

Chitin is the second most abundant polymer on earth, synthesized for use as structural scaffold by a variety of organisms including fungi, crustaceans and insects (Patil et al., 2000). Due to its omnipresence, many organisms are able to degrade chitin and use it as a carbon and energy source in metabolic processes (Campbell & Williams, 1951). Habitats characterized by a natural enrichment of chitinous polymer, such as ant hills and other agglomerations of arthropoda, are more likely to harbour chitinolytic micro-organisms than other habitats. Therefore, screening for chitin-metabolizing micro-organisms in such habitats is highly promising. During a research project focused on the identification and characterization of new chitinolytic bacteria for use in industrial processes, strain IGB-41T was isolated from a soil sample taken near Stuttgart, Germany.

A soil sample with a temperature of 18 °C was taken from an abandoned ant hill located near Lake Bärensee in Stuttgart, Germany. Chitinolytic organisms were enriched by sequential use of different media with chitin of rising complexity. In a first and second step, acidic pretreated chitin was used as carbon source, which in the third step was supplemented with protein-free milled chitin. The latter was the sole carbon source for the fourth culture step. Three grams of the soil sample were transferred into 100 ml screening medium (pH 4.2) containing 0.05 M potassium phosphate, 5 mg Ca(NO3)2·4H2O l⁻¹, 20 mg MgSO4·7H2O l⁻¹, 100 mg (NH4)2SO4 l⁻¹, 1 mg Fe(III)NH4-citrate l⁻¹, 100 μg ZnSO4·7H2O l⁻¹, 30 μg MnCl2·4H2O l⁻¹, 300 μg H3BO3 l⁻¹, 200 μg CoCl2·6H2O l⁻¹, 10 μg CuCl2·2H2O l⁻¹, 20 μg NiCl2·6H2O l⁻¹, 30 μg Na2MoO4·2H2O l⁻¹, 25 mg tryptone l⁻¹, 12.5 mg yeast extract l⁻¹, 25 mg NaCl l⁻¹ and 0.5 g wet mass colloidal chitin l⁻¹. The suspension was incubated at 20 °C. After 10 days, a 10 ml aliquot of the suspension was added to 90 ml fresh culture medium. This procedure was repeated twice with tryptone, yeast extract and NaCl omitted and 1 g chitin (Senn Chemicals) added for the third step. In a fourth step, colloidal chitin was also omitted.

The micro-organism suspension was diluted 1 : 10⁶, spread on Luria–Bertani (LB)-agar pH 4.2 and incubated for 5 days at 20 °C. Morphologically distinguishable colonies
were purified by transfer onto new plates for repeated incubation under equivalent conditions.

The basic medium for growth determination was modified LB (LBmod) containing 10 g tryptone l⁻¹ and 5 g yeast extract l⁻¹, 2 % agar, buffer or NaCl was added, if necessary. Growth at different temperatures was analysed on LBmod-agar plates at pH 6.85 at temperatures between 4 and 40 °C (4, 10, 15 20 25, 30, 35, 37 and 40 °C). For the determination of pH-dependent growth, LBmod-agar plates were supplemented with either 0.05 M citric acid/NaOH or ammonium chloride/ammonia buffer to yield pH values between 4 and 10 (pH 4.14, 4.85, 5.73, 6.61, 6.98, 8.19 and 9.2). The final pH was determined prior to sterilization. Salt tolerance was determined by growth at NaCl concentrations between 0 and 4 % (0, 1, 2, 3 and 4 %). Growth was determined after 3, 5 and 10 days and evaluated by colony size. Isolate IGB-41T was maintained as glycerol stock (50%, by vol.) at −80 °C.

Gram reaction was conducted as described by Bast (2001) with Lactobacillus plantarum and Escherichia coli as controls. Cell morphology was examined by light microscopy and scanning electron microscopy after fixation with glutaraldehyde and dehydration by sequential washing with rising ethanol concentrations.

Growth under anaerobic conditions, lysis by KOH, aminopeptidase-, oxidase- and catalase activities as well as use or hydrolysis of different compounds such as adipate, citrate, malate, phenylacetate, gelatin, DNA, Tween 80 and starch were tested by the Deutsche Stammssammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) according to standard procedures (Reddy et al., 2007).

For the determination of G+C content, genomic DNA was purified according to the procedure of Cashion et al. (1977). DNA was hydrolysed and prepared for measurement according to the protocol of Mesbah et al. (1989). Analysis of G+C content was done by HPLC according to the methods of Tamaoka & Komagata (1984) and Mesbah et al. (1989). The reaction was controlled with genomic DNA of Bacillus subtilis DSM 402 (43.5 mol% G+C), Xanthomonas campestris DSM 3586T (65.1 mol% G+C) and Streptomyces violaceoruber DSM 40783 (72.1 mol% G+C). The determination of G+C content was performed in commission by the DSMZ.

DNA–DNA hybridization experiments were performed with isolate IGB-41T, Silvimonas amyloxytica NBRC 103189T, Silvimonas iromotensis NBRC 103188T and Silvimonas terrae DSM 18233T by the DSMZ. DNA was isolated using the procedure of Cashion et al. (1977). DNA–DNA hybridization was carried out as previously described (Huß et al., 1983; De Ley et al., 1970) using a Cary 100 Bio UV–VIS-spectrophotometer equipped with a Peltier-thermostat-regulated 6 × 6 multichannel changer and a temperature controller with an in situ temperature probe (Varian). Results were obtained in 2 × SSC, 5 % formamid at 70 °C. Genomic DNA was isolated after mechanical cell disruption according to Ausubel et al. (1987). Nearly full-length 16S rRNA (1492 bp) of isolate IGB-41T was amplified using standard PCR primers (for-16S: AGAGTTTGATCMTGGCTCAG; rev-16S: TACGYYTACCTTGTACGAC- TT). PCR was performed under the following cycling conditions: initial denaturation at 94 °C for 3 min, 94 °C for 30 s, annealing for 45 s at 56 °C and elongation at 72 °C for 2 min. After 30 cycles, final elongation was allowed for 10 min at 72 °C. Phylogenetic trees were reconstructed based on 16S rRNA using the ARB software package (Ludwig et al., 2004). 16S rRNA gene sequence quality of isolate IGB-41T was checked for sequence anomalies as described by Ashelford et al. (2006). 16S rRNA sequences were aligned locally using CLUSTAL_X v2.1 (Larkin et al., 2007). The resulting alignment was used for tree reconstruction and is available as a supplementary FASTA file in IJSEM Online. The neighbour-joining algorithm (Saitou & Nei, 1987) with Jukes–Cantor correction was applied to reconstruct a phylogenetic tree with 1000-fold resampling. Tree topology was evaluated using Randomized Axelerated Maximum-likelihood RAxML v7.0.3 implemented into ARB (Stamatakis, 2006).

Fatty acid methyl esters were obtained from 40 mg cell mass, grown on trypticase soy broth agar (TSA) for two days at 28 °C. Cells were scraped from Petri dishes followed by saponification, methylation and extraction using minor modifications of the method described by Miller (1982) and Kuykendall et al. (1988). The fatty acid methyl ester mixtures were separated by the DSMZ using the Sherlock Microbial identification System (MIS) (MIDI, Microbial ID).

Polar lipid analysis and analysis of respiratory quinones was performed by the Identification Service of the DSMZ and Dr B. J. Tindall, DSMZ. Cells were grown in LBmod without NaCl at 25 °C prior to analysis. Lipid extraction was done from 100 mg of freeze-dried cells using the two stage method described by Tindall (1990a, b). Respiratory quinones were extracted using methanol : hexane (2 : 1, by vol.) followed by phase separation into hexane (Tindall, 1990a, b). Separation of lipoquinones into their different classes was conducted by TLC on silica gel (Macherey-Nagel) using hexane : tert-butylnethyl ether [9 : 1 (v/v)] as the solvent. UV-absorbing bands corresponding to different quinone classes were further analysed by HPLC. This step was carried out on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey–Nagel, 2 mm × 125 mm, 3 μm, RP18) using methanol : heptane [9 : 1 (v/v)] as the eluent. Respiratory lipoquinones were detected at 269 nm.

Polar lipids were extracted by adjusting the remaining methanol:0.3 % aqueous NaCl phase to give a chloroform : methanol : 0.3 % aqueous NaCl mixture 1 : 2 : 0.8 (by volume). The extraction solvent was stirred overnight and cell debris was pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the

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sequence similarity of recognized species of the genus *Silvimonas* which is in a range of 98.8–99.2 %. This suggests that strain IGB-41^T^ forms an independent phylum in proximity to species of the genus *Silvimonas*.

The level of DNA–DNA relatedness as determined by DNA–DNA hybridization between isolate IGB-41^T^ and the type strains of *Silvimonas amylolytica*, *Silvimonas iriomotensis* and *Silvimonas terrae* was found with values of 18.4, 13.1 and 9.9 %, respectively. This is far below the proposed threshold for species delineation of 70 % (Wayne et al., 1987), which indicates that strain IGB-41^T^ is a representative of a new genus and not a member of the genus *Silvimonas*.

The predominant fatty acids of strain IGB-41^T^ were identified as C\textsubscript{16:0} (26.1 %), summed feature 3 (iso-C\textsubscript{15:0} 2-OH and/or C\textsubscript{16:1ω7c} (26.1 %) and C\textsubscript{18:1ω7c} (22.4 %). Differences between the fatty acid composition of species of the genus *Silvimonas* and isolate IGB-41^T^ were evident, especially for short-chain and hydroxylated fatty acids. In this comparison, strain IGB-41^T^ was the only representative with the metabolic compounds C\textsubscript{14:0} 2-OH (2.2 %), C\textsubscript{15:0} (1.4 %) and C\textsubscript{18:1ω7c} 2-OH (1.5 %) (Table S1), whereas the other fatty acids were found with similar patterns and concentration ranges. Therefore, to further discriminate between the organisms, we performed polar lipid analysis.

Fig. 1. Rooted neighbour-joining tree showing phylogenetically close neighbours of isolate IGB-41^T^ within the family *Neisseriaceae*. Filled circles indicate branching patterns also recovered using RAxML. Bootstrap values ≥50 % are shown. Bar, 1 nt substitution per 100 bases.
Table 1. Differential characteristics of strain IGB-41^T and the closest phylogenetic neighbours

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (μm)</td>
<td>0.7–0.8 × 1.5–3.0</td>
<td>0.4–0.5 × 0.8–1.3</td>
<td>0.3–0.5 × 1.0–1.5</td>
<td>0.5–0.8 × 1.3–2.0</td>
<td>0.3–0.7 × 0.7–2.5</td>
<td>0.7–0.9 × 2.0–3.0</td>
<td>0.5 × 1.0–2.0</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pH range</td>
<td>6–9</td>
<td>4–9</td>
<td>4–9</td>
<td>6–8</td>
<td>≥5</td>
<td>5–8</td>
<td>7–9</td>
</tr>
<tr>
<td>pH optimum</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>7</td>
<td>7–8</td>
<td>6–7</td>
<td>7</td>
</tr>
<tr>
<td>Temp. range (°C)</td>
<td>10–35</td>
<td>5–40</td>
<td>5–40</td>
<td>15–40</td>
<td>10–30</td>
<td>5–35</td>
<td>15–37</td>
</tr>
<tr>
<td>Temp. optimum (°C)</td>
<td>20–25</td>
<td>30</td>
<td>30</td>
<td>25–30</td>
<td>30</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>NaCl tolerance (w/v, %)</td>
<td>0</td>
<td>0–1</td>
<td>0–3</td>
<td>0–2</td>
<td>0–3.0</td>
<td>0–2</td>
<td>0–1</td>
</tr>
<tr>
<td>Optimal NaCl Concentration</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>0–1.0</td>
<td>ND</td>
<td>0–0.5</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>61.5</td>
<td>57.5</td>
<td>59.9</td>
<td>58</td>
<td>63.8</td>
<td>62</td>
<td>63.0</td>
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<tr>
<td>Major quinone</td>
<td>Q-8</td>
<td>Q-8</td>
<td>Q-8</td>
<td>Q-8</td>
<td>Q-8</td>
<td>Q-8</td>
<td>ND</td>
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<tr>
<td>Assimilation of mannose</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitin</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>(+)</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Aesculin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DNA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*According to original description.
from the TLC data images given in a format defined by Tindall et al. (2010) (Fig. S2).

Further differences were observed in biochemical and physiological characteristics. Strain IGB-41T was able to grow with pH values ranging from 6 to 9. However, growth occurred only in the absence of NaCl. A growth temperature range from 10 to 35 °C with an optimum at 20–25 °C was observed. In contrast, species of the genus Silvimonas show growth at up to 40 °C, are characterized by higher salt tolerance and are reported to be positive for mannose assimilation, while the latter was not found for isolate IGB-41T. Silvimonas terrae KM-455 has been reported to be positive for chitinolytic activity (Yang et al., 2005). However, this was not confirmed in a more recent study (Muramatsu et al., 2010) suggesting that none of the species of the genus Silvimonas known so far is positive for chitinolytic activity. Thus, strain IGB-41T can be clearly distinguished from these species based on its chitinolytic and mannose-assimilating activities. The G+C content of strain IGB-41T was found to be 61.5 mol% and this is outside the threshold of 57–60 mol% given in the emended genus definition of Silvimonas produced by Muramatsu et al. (2010). Further fundamental differences between the strains discussed are summarized in Table 1.

According to the physiological data observed, the highly reproducible clustering of strain IGB-41T, which was never observed with species within the genus Silvimonas, and the assumptions on the assignment of new genera and species made in previous works (Stackebrandt & Goebel, 1994; Wayne et al., 1987), we propose the assignment of isolate IGB-41T to a new genus and a novel species. We suggest the name Amantichitinum ursilacus gen. nov., sp. nov. with isolate IGB-41T as type strain.

Description of Amantichitinum gen. nov.

Amantichitinum [A.man.iti.chi.tin.um. L. part. adj. amans –antis loving; N.L. chitinum chitin; N.L. neut. n. Amantichitinum loving-chitin (bacterium)].

Cells of this genus are Gram-negative, motile, rod-shaped (1.5–3.0 μm × 0.7–0.8 μm) and facultatively anaerobic. They occur singly or in pairs (Fig. S1). Colonies on LBmod are milky white to beige, circular, convex and about 1 mm in diameter. Representatives are catalase- and oxidase-positive. The only detectable quinone is Q-8. Major fatty acids (>10 %) are C16:0 summed feature 3 (comprising iso-C15:0 2-OH and/or C16:1ω7c) and C18:1ω7c. Based on 16S rRNA sequence analysis, Amantichitinum belongs to the Betaproteobacteria. The type species is Amantichitinum ursilacus.

Description of Amantichitinum ursilacus sp. nov.

Amantichitinum ursilacus [ur.si.la’cus. L. n. ursus –i a bear; L. n. lacus a lake; N.L. gen. n. ursilacus of Bear Lake (Bärensee), a lake nearby Stuttgart, Germany, on the edge of which the strain was isolated].

Exhibits the following properties in addition to those given in the genus description. Growth is observed between 10 and 35 °C and at pH values of 6–9 (optimum pH 7). Growth occurs in the absence of NaCl. Gives a positive result in tests for nitrate reduction and acid formation from glucose. Hydrolyses aesculin, Tween 80, starch and chitin, but not gelatin or DNA. According to the API 50 CH panel, positive for fermentation of L-arabinose, D-ribose, DL-xylose, galactose, glucose, fructose, D-mannose, D-mannitol, N-acetylgalactosamine, amygdalin, arbutin, aesculin, ferric citrate, salicin, D-cellobiose, D-maltose, starch, glycogen and D-arabitol. A weak reaction is observed for L-rhamnose, D-lactose (bovine), D-sucrose, gentiobiose and D-lyxose metabolism. The reactions for glycerol, erythritol, D-arabinose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranose, D-melibiose, D-trehalose, inulin, D-melezitose, D-raf finose, xylitol, D-turanose, D-tagatose, D-fucose, L-fucose, L-arabitol, potassium gluconate, potassium-2-ketogluconate and potassium-5-ketoglucurate are negative. Assimilation of adipate, citrate, malate and phenylacetate is negative. The major fatty acids (>5 %) are C16:0 (26.1 %), summed feature 3 (iso-C15:0 2-OH and/or C16:1ω7c) (26.1 %), C18:1ω7c (22.4 %), C17:0cyclo (6.0 %) and C14:0 (5.5 %).

The type strain, IGB-41T (=DSM 23761T=CIP 110167T), was isolated from soil from an abandoned ant hill located near lake Bärensee in Stuttgart, Germany. The DNA G+C content of the type strain is 61.5 mol%.

Acknowledgements

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References


