Alicyclic compounds, naturally made by plant cells as secondary metabolites and occurring in fossil fuels, are widespread in nature. They are also natural intermediates in the anaerobic degradation of aromatic compounds. In chemical industries they serve as insecticides, herbicides and as intermediates or solvents in many chemical reactions. Micro-organisms have the ability to use a variety of these xenobiotic compounds and convert them to cellular metabolites under aerobic or anoxic conditions.

The aerobic degradation of alicyclic compounds has been extensively studied in several aerobic bacterial genera such as Acinetobacter (Donoghue & Trudgill, 1975), Pseudomonas (Tanaka et al., 1977) and Xanthobacter (Trower et al., 1985). The aerobic degradation of alicyclic compounds requires molecular oxygen and monooxygenases for the cleavage of the ring. Anaerobic degradation of alicyclic compounds has been less well studied and little information on this field is available (Evans, 1977; Trudgill, 1984; Dangel et al., 1988, 1989; Foss & Harder, 1998; Foss et al., 1998). Three isolates growing under denitrifying conditions with alicyclic compounds such as cyclohexanol have been obtained (Dangel et al., 1988) and two of them were studied (Dangel et al., 1988, 1989). Since the isolates were rather similar to each other, only one isolate, referred to as strain K601T, has been studied in greater detail. In this paper we report the description of this strain as Alicycliphilus denitrificans gen. nov., sp. nov.

Strain K601T, previously identified as a Pseudomonas species, was isolated from a waste water treatment plant with cyclohexanol as sole carbon source and nitrate as electron acceptor (Dangel et al., 1988). The medium used for enrichment, isolation and routine cultivation contained (1 l-1 distilled water): 1.08 g KH2PO4, 5.6 g K2HPO4, 0.54 g NH4Cl, 0.15 g CaCl2 .2H2O, 0.2 g MgSO4 .7H2O, 1.27 g NaNO3, 1 ml trace element solution SL-10 (Widdel et al., 1983), 1 ml selenite/tungstate solution (Tschech & Pfennig, 1984), 1 ml vitamin solution VL-7 (Pfennig, 1978) and carbon source (1 mM cyclohexanol). The final pH was 7.2–7.4. The medium was made anaerobic by applying several cycles of vacuum and flushing with oxygen-free nitrogen gas at room temperature. For aerobic growth, the same medium composition was used except that it did not contain NaNO3. Cultures were routinely grown at 30 °C and aerobically grown cultures...
were shaken at 120 r.p.m. To determine the substrate spectrum of strain K601 \(^T\) the following compounds (concentrations in mM in parentheses) were tested under aerobic and anoxic conditions in the mineral medium described above: cyclohexanol (1), cyclohexanone (1), 1,3-cyclohexanedione (1), 2-cyclohexenone (1), 1,3-cyclohexanediol (cis and trans) (1), 1,2-cyclohexanediol (1), 1,2-cyclohexanediol (1), 2-hydroxycyclohexanone (1), 1,4-cyclohexanedione (1), cyclohexane (1), monocarboxylic acids (C\(_2\)-C\(_7\)) (2), adipate (2), pimelate (2), 5-oxo-caproate (2), citrate (2), 2-oxoglutarate (2), succinate (2), malate (2), crotonate (2), lactate (2), pyruvate (2), fumarate (2), phenol (1), aniline (1), malate (2), propionate (2), benzoate (1), 2-amino-benzoate (1), 2-hydroxy-benzoate (1), 3-hydroxy-benzoate (1), 4-hydroxy-benzoate (1), resorcinol (1), hydroxyquinol (1), m-cresol (1), o-cresol (1), p-cresol (1), vanillate (1), indole (1), 4-aminobenzoate (1), resorcinol (1), 2-naphthoic acid (1), biphenyl 2-carboxylic acid (1), gentisate (1), protocatechuate (1), 3-fluorobenzoate (1), 3-chlorobenzoate (1), formate (2), glucose (2), fructose (2), xylose (2) and aliphatic alcohols (C\(_1\)-C\(_8\)) (2). The electron acceptors tested were nitrate, nitrite, sulfate, sulfite, fumarate (all at 5 mM) and oxygen. All substrates were anaerobically prepared and were autoclaved or filter-sterilized.

For fatty acid analysis the strain was grown on R2A medium and cells were harvested after 3 days by centrifugation. About 40 mg (w/w) of the cells was saponified, methylated, extracted and analysed by using the Microbial Identification System (MIS) described by Miller (1982).

For determination of DNA base composition, the DNA was isolated and purified by chromatography on hydroxyapatite and the G+C content was determined by HPLC (Mesbah et al., 1989). Non-methylated \(\lambda\) DNA (Sigma) was used as standard. For DNA–DNA hybridization, DNA was isolated by the method of Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970), with the modifications described by Escara & Hutton (1980) and Huss et al. (1983) using a Gilford System model 2600 equipped with a Gilford model 2527-R thermostepper and plotter. Reassociation was carried out in 2 \(\times\) SSC containing 10 % dimethylsulfoxide at 68 °C. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke & Bahnweg, 1986; Jahnke, 1992).

Phase-contrast microscopy was performed by using a Zeiss microscope equipped with a camera. Wet mounts for photomicrographs of the micro-organisms were made according to Pfennig & Wagener (1986).

Growth was measured spectrophotometrically at 580 nm using cuvettes with a 1 cm light path. Aromatic compounds were measured using an HPLC system equipped with a UV detector set at 280 nm. Separation was achieved using a Beckman Ultrasphere column (4.6 × 250 mm, 5 \(\mu\)m particle size) maintained at room temperature. The mobile phase, consisting of a mixture of two solvents (water and 0.01%, v/v, acetic acid in 50 %, v/v, acetonitrile) was used at a flow rate of 1 ml min \(^{-1}\). For separation of aromatic compounds, 25 % acetonitrile solvent phase was initially held for 20 min, then the concentration was increased to 50 % over a period of 5 min and held for 5 min. The column was re-equilibrated with 25 % methanol for at least 5 min before the next injection. Nitrate and nitrite were estimated using the Quantofix test (Macherey–Nagel).

Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and sequencing of PCR products were carried out as described by Rainey et al. (1996). Purified PCR products were sequenced directly using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer’s instructions. The Applied Biosystems 310 DNA Genetic Analyser was used for the electrophoresis of the sequence reaction products. The almost complete 16S rDNA sequences of the isolates were aligned manually with those of all currently available nucleotide sequences of representatives of the \(\beta\)-Proteobacteria retrieved from GenBank and EMBL databases using the ae2 editor (Maidak et al., 1999). The method of Jukes & Cantor (1969) was used to calculate evolutionary distances on the basis of 1298 nt. Phylogenetic dendrograms were reconstructed according to the method of DeSoete (1983) and the neighbour-joining and maximum-likelihood methods contained in the PHYLIP package (Felsenstein, 1993). Following determination of the phylogenetic position within the \(\beta\)-Proteobacteria the dendrogram was restricted to the nearest neighbours. The accession numbers of these reference organisms are indicated in Fig. 1.

Strain K601 \(^T\) was anaerobically enriched and isolated with cyclohexanol as sole carbon source and with nitrate as electron acceptor (Dangel et al., 1988). The inoculum used for the enrichment was from a municipal sewage plant in Konstanz (Germany). The medium used was as described by Tschech & Fuchs (1987). The growth conditions were 28–30 °C and pH 7.2–7.4. Cells of strain K601 \(^T\) were short rods, 0.6 µm wide and 1–2 µm long, motile and stains Gram-negative. Catalase and oxidase reactions were positive. Strain K601 \(^T\) was a facultative anaerobe. The metabolism was strictly oxidative. Nitrate, nitrite and oxygen were used as electron acceptors while sulfate, sulfite and fumarate were not reduced. Strain K601 \(^T\) was capable of aerobic and anaerobic growth on a large range of organic substrates. Physiological reactions observed under anoxic denitrifying conditions and under aerobic conditions are indicated in the species description. The G+C content of strain K601 \(^T\) was 66 mol% as determined by HPLC.

The major fatty acids were hexadecenoic acid (C\(_{16}\):1\(\omega\)7\(\omega\), 37 %); hexadecanoic acid (C\(_{16}\):0, 24 %) and octadecenoic acid (C\(_{18}\):1\(\omega\)7\(\omega\), 21 %). The structure of the latter fatty acid is identical to cis-vaccenic acid (C\(_{18}\)-1\(\omega\)7\(\omega\), 1 reported by Willems et al. (1989). 3-Hydroxydecanoic acid (3-OH C\(_{10}\):0) and saturated acids (C\(_{12}\):0; 4 %; C\(_{15}\):0, 2 %; and cycloC\(_{17}\):0; 2 %) occurred in smaller amounts. The
The following substrates are used under anoxic conditions: hydrocarbons, aromatic polymers of the lignin type (Willems et al., 1992), and a wide variety of substrates, including carboxylic or dicarboxylic acids, aliphatic or unsaturated carboxylic acids, aliphatic or unsaturated carboxylic acids, and aromatics and monoaromatic compounds, as well as aromatic polymers of the lignin type (Willems et al., 1992). Cells are short rods (0.6 m×1–2 μm), motile, Gram-negative, catalase- and oxidase-positive. Optimal growth occurs at 28–30 °C and pH 7.2–7.4 under aerobic or anoxic conditions. Metabolism is strictly oxidative. Electron acceptors nitrate, nitrite and oxygen are used; sulfate, sulfite and fumarate are not reduced. G+C content is near 66 mol%. Phylogenetically related to the family Comamonadaceae. Type species is Alicyciphilus denitrificans.

Description of Alicyciphilus denitrificans sp. nov.

Alicyciphilus denitrificans (de.ni.trifi.can.s. L. prep. de away from; L. n. nitrum soda; N.L. n. nitrum nitrate; N.L. v. denitrifico to denitrify; N.L. part. adj. denitrificans denitrifying).

The description is the same as given above for the genus. The following substrates are used under anoxic conditions:
Table 1. Characteristics differentiating the genus *Alicycliphilus* and other genera in the family *Comamonadaceae*

This table was adapted from Blümel *et al.* (2001) and from Wen *et al.* (1999). +, Present in all species; −, absent from all species; (+), weak reaction; d, 11–89 % of strains positive; D, variable reaction depending on the method used; NA, no data available.

<table>
<thead>
<tr>
<th>Character</th>
<th><em>Alicycliphilus</em></th>
<th>Xenophilus</th>
<th>Delftia</th>
<th>Comamonas</th>
<th>Acidovorax</th>
<th>Variovorax</th>
<th>Xylophilus</th>
<th>Hydrogenophaga</th>
<th>Rhodoferax</th>
<th>Brachymonas</th>
<th>Polaromonas</th>
<th><em>Aquaspirillum</em> psychrophilum LMG 54087</th>
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<tr>
<td>Cell morphology</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods or spirilla</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Curved rods or short rods</td>
<td>Rods</td>
<td>Spirilla or curved rods</td>
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<tr>
<td>Motility</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>−</td>
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<td>NA</td>
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<td>l-Glutamate</td>
<td>Biotin, thiamin</td>
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<td>Denitrification</td>
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<td>d</td>
<td>d</td>
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<td>d</td>
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cyclohexanol, cyclohexanone, 1,3-cyclohexanediol, 2-cyclohexenone, 1,3-cyclohexanediol (cis and trans), monocarboxylic acids (C_2-C_7), adipate, pimelate, 5-oxopropanoate, citrate, 2-oxoglutarate, succinate, L-malate, propionate, crotonate, L-lactate, pyruvate and fumarate. The following compounds are not used: aniline, phenol, benzoate, 2-aminobenzoate, 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, resorcinol, hydroxyquinol, m-cresol, o-cresol, p-cresol, vanillate, naphthoate, indole, 1,2-cyclohexanediol, 1,2-cyclohexanedione, 2-hydroxycyclohexanone, 1,4-cyclohexanedione, cyclohexane, formate, D-glucose, D-fructose, D-xylene and aliphatic alcohols (C_1-C_8). Under aerobic conditions the following compounds are used: propionate, L-lactate, aniline, fumarate, indole, vanillic acid, acetate, 4-hydroxybenzoate, m-cresol, o-cresol, p-cresol, crotonate, D-glucose, L-lactate and pyruvate. The following compounds are not used: 4-aminobenzoate, benzoate, resorcinol, 2-naphthoate, biphenyl 2-carboxylate, 2-aminobenzoate, 3-hydroxybenzoate, gentisate, protocatechuate, hydroxyquinol, 3-fluorobenzoate and 3-chlorobenzoate. G+C content is 66 mol%. Isolated from an enrichment culture inoculated with a liquid sample from a municipal sewage plant in Konstanz (Germany). Type strain is K601^T (═DSM 14773^T =CIP 107495^T).

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der chemischen Industrie.

References


