Note

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uni-freiburg.de*Alicycliphilus denitrificans* gen. nov., sp. nov.,
a cyclohexanol-degrading, nitrate-reducing
 β -proteobacteriumTahar Mechichi,¹ Erko Stackebrandt² and Georg Fuchs¹¹Mikrobiologie, Institut für Biologie II, Universität Freiburg, Schänzlestr. 1, D-79104 Freiburg, Germany²DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany

A facultatively denitrifying bacterium, strain K601^T, was isolated at 30 °C from a municipal sewage plant on cyclohexanol as sole carbon source and nitrate as electron acceptor. Under aerobic conditions this strain used acetate, fumarate, lactate, pyruvate, crotonate, indole, glucose, vanillate, 4-hydroxybenzoate, *m*-cresol, *o*-cresol and *p*-cresol. Under denitrifying conditions the strain used cyclohexanol, cyclohexanone, 1,3-cyclohexanedione, 2-cyclohexenone, 1,3-cyclohexanediol (*cis* and *trans*), monocarboxylic acids (C₂–C₇), adipate, pimelate, 5-oxocaproate, citrate, 2-oxoglutarate, succinate, malate, crotonate, lactate, pyruvate and fumarate. Cells were short rods, 0.6 µm wide and 1–2 µm long, motile, non-spore-forming, Gram-negative, and catalase- and oxidase-positive. Strain K601^T used nitrate, nitrite and oxygen as electron acceptors, but not sulfate, sulfite or fumarate. The DNA G+C content of strain K601^T was 66 mol%. Phylogenetic analysis, based on 16S rDNA sequencing, showed that strain K601^T represents a separate lineage of the family *Comamonadaceae* in the β -subclass of *Proteobacteria*. Based on the high 16S rDNA sequence divergence and phenotypic characteristics, the name *Alicycliphilus denitrificans* gen. nov., sp. nov. is proposed for this strain. The type strain is K601^T (=DSM 14773^T =CIP 107495^T).

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 K601^T, has been studied in greater detail. In this paper we report the description of this strain as *Alicycliphilus denitrificans* gen. nov., sp. nov.

Strain K601^T, 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 (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

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The EMBL accession number for the 16S rDNA sequence of strain K601^T is AJ18042.

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-cyclohexanedione (1), 2-hydroxycyclohexanone (1), 1,4-cyclohexanedione (1), cyclohexane (1), monocarboxylic acids (C₂–C₇) (2), adipate (2), pimelate (2), 5-oxocaproate (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-aminobenzoate (1), 2-hydroxybenzoate (1), 3-hydroxybenzoate (1), 4-hydroxybenzoate (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 (C₁–C₈) (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 λ 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 thermoprogrammer 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 & Wäger (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 $\times$ 250 mm, 5 μ 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 β -*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 β -*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 Tschek & 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 μ m wide and 1–2 μ m long, motile and stained 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 (C16:1 ω 7c, 37 %); hexadecanoic acid (C16:0, 24 %) and octadecenoic acid (C18:1 ω 7c, 21 %). The structure of the latter fatty acid is identical to *cis*-vaccenic acid (δ 11-C18:1), reported by Willems *et al.* (1989). 3-Hydroxydecanoic acid (3-OH C10:0) and saturated acids (C12:0; 4 %; C15:0, 2 %; and cycloC17:0, 2 %) occurred in smaller amounts. The

Fig. 1. 16S rDNA dendrogram displaying the relationships between strain K601^T and some other members of the family *Comamonadaceae*, β -*Proteobacteria*. Numbers at branching points refer to bootstrap values (500 resamplings, only values above 75 % shown). Scale bar, 10 nt substitutions in 100 bases.

presence of the three major components have also been reported for members of *Comamonas* and *Delftia* (Tamaoka *et al.*, 1987), as well as for *Hydrogenophaga* (Willems *et al.*, 1989).

16S rDNA sequence analysis showed that strain K601^T clusters within the family *Comamonadaceae* in the β -subclass of the *Proteobacteria* (Willems *et al.*, 1991). This family comprises the genera *Acidovorax*, *Comamonas*, *Delftia*, *Hydrogenophaga*, *Rhodoferrax*, *Brachymonas*, *Polaromonas*, *Variovorax*, *Xylophilus* (Wen *et al.*, 1999), *Xenophilus* (Blümel *et al.*, 2001) and misclassified members of *Aquaspirillum*. Strain K601^T represents an individual line of descent, showing less than 96 % sequence similarity to any other member of this family. Phylogenetically it branches between *[Aquaspirillum] psychrophilum* LMG 5408^T and a cluster consisting of *Comamonas*, *Brachymonas*, *Hydrogenophaga*, *Delftia* and *Xenophilus*. The statistical significance of most branching points is low (<75 %), hence the branching pattern should not be considered stable and some of the lineages may change position as new sequences are included. The low 16S rDNA similarities found between strain K601^T and other members of the family *Comamonadaceae* exclude a high degree of DNA–DNA reassociation similarity that would affiliate this strain to any described species of the various genera within this family (Stackebrandt & Goebel, 1994). This notion is confirmed by the low degree of DNA relatedness of 26 %, determined for strain K601^T and *Delftia acidovorans* DSM 50251^T which emerges as the phylogenetic neighbour (Fig. 1).

Analysis of the 16S rRNA gene sequences showed that strain K601^T is a member of the family *Comamonadaceae* in the β -subclass of the *Proteobacteria*. The reason for creating a new genus for strain K601^T was the high sequence divergence from related genera (more than 4 % sequence divergence). A pattern of phenotypic differences between strain K601^T was also noticed (Table 1), such as

differences in carbon source utilization, the ability to denitrify and base composition of DNA. Though members of this family are phylogenetically closely related, they are phenotypically heterogeneous. Their presence in different habitats seems to contribute to the natural biodegradation process of many natural compounds and pollutants since *Comamonadaceae* have been isolated from different environments (soil, wastewater, activated sludge, oil brine, crude oil) and on a wide variety of substrates, including carboxylic or dicarboxylic acids, aliphatic or unsaturated acids, sterols and monoaromatic compounds, as well as aromatic polymers of the lignin type (Willems *et al.*, 1992).

Description of *Alicycliphilus* gen. nov.

Alicycliphilus (a.li.cyc.li'phi.us. Gr. adj. *aliphos* fat; Gr. n. *kyklos*, circle or ring; N.L. adj. *alicyclo* referring to circular fat-like organic compounds; Gr. masc. n. *philos* friend; N.L. adj. *Alicycliphilus* alicyclic compound-liking, referring to the substrates used for the isolation of this organism).

Cells are short rods (0.6 $\times$ 1–2 μ m), 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 *Alicycliphilus denitrificans*.

Description of *Alicycliphilus denitrificans* sp. nov.

Alicycliphilus denitrificans (de.ni.tri'fi.cans. 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 *Alicyclophilus* 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.

Character	<i>Alicyclophilus</i>	<i>Xenophilus</i>	<i>Delftia</i>	<i>Comamonas</i>	<i>Acidovorax</i>	<i>Hydrogenophaga</i>	<i>Variovorax</i>	<i>Xylophilus</i>	<i>Rhodiferax</i>	<i>Brachymonas</i>	<i>Polaromonas</i>	[<i>Aquaspirillum</i>] <i>psychrophilum</i> LMG 5408 ^T
Cell morphology	Rods	Rods	Rods	Rods or spirilla	Rods	Rods	Rods	Rods	Curved rods	Coccobacilli or short rods	Rods	Spirilla or curved rods
Motility	+	+	+	+	+	+	+	+	+	–	+	+
Pigments	–	+	–	–	+	+	+	+	–	(+)	–	–
Phototrophy	–	–	–	–	–	–	–	–	+	–	–	–
Oxidase	+	+	+	+	+	+	+	–	+	+	+	+
Chemolithotrophic growth with H ₂	–	NA	–	–	d	+	d	–	NA	NA	–	NA
Psychrophilic growth	–	–	–	–	–	–	–	–	–	–	+	–
Growth factors	–	–	–	d	–	–	–	L-Glutamate	Biotin, thiamin	–	–	d
Denitrification	+	NA	–	–	d	d	–	–	–	–	–	d
Carbon source used for growth:												
Acetamide	NA	NA	d	–	d	–	–	NA	NA	NA	NA	NA
β-Alanine	NA	–	d	–	d	–	d	NA	–	NA	NA	NA
2-Aminobutyrate	NA	–	d	–	d	d	NA	NA	NA	–	NA	NA
3-Aminobutyrate	NA	NA	d	d	d	d	NA	NA	NA	–	NA	NA
D-Fructose	+	NA	+	–	+	d	NA	–	+	–	–	–
D-Glucose	+	+	–	–	d	+	+	+	+	–	+	–
Glycerol	+	–	d	d	+	+	+	+	–	–	+	–
Malonate	+	(+)	d	–	d	–	d	–	NA	–	–	D
D-Mannitol	NA	NA	+	–	d	d	NA	–	+	NA	NA	NA
Maleate	NA	+	d	–	d	–	d	–	NA	NA	NA	NA
Phenylacetate	–	NA	d	–	–	–	d	NA	NA	–	NA	NA
L-(+)-Tartrate	NA	–	d	–	–	d	d	NA	NA	NA	NA	NA
D-Tryptophan	–	NA	d	d	–	–	NA	NA	NA	–	NA	–
L-Tryptophan	–	NA	d	–	d	d	d	NA	NA	NA	NA	–
DNA G+C content (mol%)	66	70·4	67–69	63–66	67–70	65–69	66–69	68–69	59–61	63–65	52–57	56–62

cyclohexanol, cyclohexanone, 1,3-cyclohexanedione, 2-cyclohexenone, 1,3-cyclohexanediol (*cis* and *trans*), monocarboxylic acids (C₂–C₇), adipate, pimelate, 5-oxocaproate, 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-xylose and aliphatic alcohols (C₁–C₈). Under aerobic conditions the following compounds are used: propionate, L-malate, 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).

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