NOTE

***Xenophilus azovorans* gen. nov., sp. nov., a soil bacterium that is able to degrade azo dyes of the Orange II type**

¹ Institut für Mikrobiologie,
Universität Stuttgart,
D-70569 Stuttgart,
Germany

² Institut für Bakteriologie,
Mykologie und Hygiene,
Veterinärmedizinische
Universität, A-1210 Vienna,
Austria

³ Institut für Mikrobiologie
und Genetik, Universität
Wien, A-1030 Vienna,
Austria

⁴ Institut für Angewandte
Mikrobiologie, Justus-
Liebig-Universität Giessen,
D-35392 Giessen, Germany

Silke Blümel,¹ Hans-Jürgen Busse,^{2,3} Andreas Stolz¹ and Peter Kämpfer⁴

Author for correspondence: Peter Kämpfer. Tel: +49 641 99 37352. Fax: +49 641 99 37359.
e-mail: peter.kaempfer@agr.uni-giessen.de

The taxonomy of strain KF46F^T, which was isolated previously after an aerobic enrichment with the azo compound 1-(4'-carboxyphenylazo)-2-naphthol as the sole source of energy and carbon, was investigated by a polyphasic approach. The organism contained a quinone system with ubiquinone Q-8 and 2-hydroxyputrescine and putrescine as the major polyamines, suggesting that strain KF46F^T belonged to the β -subclass of the *Proteobacteria*. The polar lipid profile consisted mainly of phosphatidylethanolamine and minor amounts of phosphatidylglycerol and diphosphatidylglycerol. Sequencing of the 16S rRNA gene supported its placement in the family *Comamonadaceae*, but the sequence similarities to the most closely related species of the genera *Hydrogenophaga*, *Acidovorax*, *Comamonas* and *Xylophilus* were only in the range 95.0 to 96.1%. Different methods for the construction of phylogenetic trees showed the separate position of strain KF46F^T 'between' the genera *Hydrogenophaga*, *Variovorax*, *Comamonas* and *Xylophilus*. Analysis of the fatty acids revealed an unusual profile, with the presence of 8:0 3-OH, 10:0 3-OH, 16:1 2-OH, 16:0 2-OH and 18:1 2-OH in addition to 17:0 cyclo, which is unique among the previously described genera of the family *Comamonadaceae*. Thus, a new taxon is proposed for strain KF46F^T, with the name *Xenophilus azovorans* gen. nov., sp. nov.

Keywords: *Xenophilus azovorans* gen. nov., sp. nov., β -*Proteobacteria*, polyphasic taxonomy

Synthetic azo compounds are used in large quantities as dyes for textiles, paper, leather, cosmetics, food and various other materials. These xenobiotic compounds are generally not degraded in conventional aerobic wastewater-treatment systems and therefore are of significant concern as pollutants of the environment (Clarke & Anliker, 1980; Pagga & Brown, 1986). Over the last 10 years, some reports have been published that demonstrated the ability of certain bacteria to decolorize azo dyes under aerobic conditions (e.g. Banat *et al.*, 1996; Blümel *et al.*, 1998; Coughlin *et al.*, 1999; Dykes *et al.*, 1994; Heiss *et al.*, 1992; Jiang & Bishop, 1994; Ogawa *et al.*, 1986; Shaul *et al.*, 1991). The adaptation of environmental bacteria to grow

with simple carboxylated azo compounds has been studied in some detail. Overney (1979) isolated a '*Flavobacterium*' that was able to grow aerobically with the simple model compound 4,4'-dicarboxyazobenzene. Later, it was shown that a mixed bacterial culture that degraded 4,4'-dicarboxyazobenzene could be adapted to the degradation of more complex azo compounds such as 1-(4'-carboxyphenylazo)-4-naphthol ('carboxy-Orange I') or 1-(4'-carboxyphenylazo)-2-naphthol ('carboxy-Orange II'). From these adaptation processes in continuous cultures, strain '*Pseudomonas*' K22 was obtained after cultivation with carboxy-Orange I and strain KF46F^T from an enrichment with carboxy-Orange II (Kulla, 1981; Kulla *et al.*, 1983, 1984; Zimmermann *et al.*, 1982, 1984). The aerobic azoreductases from both strains were purified, characterized and shown to differ significantly in their structure and substrate specificity (Zimmermann *et al.*, 1982, 1984).

The EMBL accession number for the 16S rRNA gene sequence of strain KF46F^T is AF285414.

In order to clarify the taxonomic position of bacterial strains with the ability to degrade azo compounds aerobically, we characterized strain KF46F^T from the original enrichments performed by Kulla (1981) and Kulla *et al.* (1984).

Strain KF46 was isolated from a soil inoculum after a prolonged enrichment with carboxy-Orange II as the sole source of carbon and energy (Kulla *et al.*, 1984). For the present study, strain KF46F^T was used, which is a non-mucoid variant of strain KF46, a strain that has been preserved freeze-dried for the last 25 years (T. Leisinger, personal communication). The strain was kindly provided by T. Leisinger (ETH Zürich, Switzerland).

The Gram reaction was tested as described by Gerhardt *et al.* (1994). Cell morphology was observed under a light microscope (1000×) with cells grown for 3 d at 30 °C on nutrient agar. On nutrient agar, strain KF46F^T formed visible colonies (diameter of about 1 mm) within 3 d at 30 °C. No growth was observed within 14 d at 4, 37 or 42 °C. The colonies were yellowish and opaque. Often, colonies were not detectable as single entities because of the production of extracellular slimy substances. The strain was oxidase- and catalase-positive. Cells were motile, non-spore-forming rods (about 2 µm in length) and stained Gram-negative. The strain was able to grow on various nutrient-rich media, like Luria-Bertani or nutrient broth, at 30 °C, but lost the ability to degrade Orange II rapidly under these conditions.

Physiological characterization was done as described previously (Kämpfer *et al.*, 1991). Strain KF46F^T showed no (or very weak) production of acid from various sugars and related compounds, but D-fructose, D-mannitol and D-maltitol were utilized as sole sources of carbon. In addition, several organic acids, including amino acids, and some aromatic compounds were utilized (for details, see species description below). Most of the *p*-nitrophenyl derivatives tested were not hydrolysed, but hydrolysis of L-alanine *p*-nitroanilide and 2-deoxythymidine 2'-*p*-nitrophenylphosphate were positive.

Fatty acid methyl esters were extracted and prepared by the standard protocol of the Microbial Identification System (MIDI; Microbial ID Inc.). Extracts were analysed by using a Hewlett Packard model HP6890A gas chromatograph equipped with a flame-ionization detector, an automatic sampler, an integrator and a computer, as described previously (Kämpfer & Kroppenstedt, 1996). Strain KF46F^T contained the fatty acids 16:0 (35.1%), summed feature 4 (16:1 ω 7c and iso 15:0 2-OH, 11.6%), summed feature 7 (18:1 ω 7c, 18:1 ω 9t and/or 18:1 ω 12t, 20.5%) and 17:0 cyclo (14.2%) in major amounts. Furthermore, it was interesting that several hydroxylated fatty acids were produced (8:0 3-OH, 1.2%; 10:0 3-OH, 3.7%; 16:1 2-OH, 4.4%; 16:0 2-OH, 1.5%; 18:1 2-OH, 4.0%), some in significant amounts. The fatty acids 10:0 (2.8%) and 17:0 (1.2%)

were also detected. A similar fatty acid profile has been described for the genera belonging to the family *Comamonadaceae sensu* Willems *et al.* (1991b). Willems *et al.* (1989) reported fatty acid profiles for the genera *Hydrogenophaga*, *Acidovorax*, *Variovorax* and *Comamonas*. For these genera, the major fatty acids were 16:1 ω 7c, 18:1 ω 7c, 18:1 ω 9t and/or 18:1 ω 12t, and 16:0. The presence of 8:0 3-OH and 10:0 3-OH was detected in almost all species of these genera, but 16:0 2-OH was detected only in some species of *Comamonas* and *Hydrogenophaga* (Willems *et al.*, 1989). The fatty acid 16:1 2-OH, which was found to constitute 4.4% of the total fatty acids of strain KF46F^T, has been found only in representatives of the genus *Comamonas* (Stead, 1992) and 18:1 2-OH, which also constitutes 4% in KF46F^T, has not previously been detected in representatives of the family *Comamonadaceae*.

Analysis of the respiratory quinone of KF46F^T by HPLC (Tindall, 1990) gave only one characteristic peak, which corresponded to ubiquinone Q-8. Analysis of the polyamines (Busse & Auling, 1988; Busse *et al.*, 1997) revealed a pattern for strain KF46F^T that is characteristic for taxa of the β -*Proteobacteria* (Busse & Auling, 1988). Strain KF46F^T displayed the major compounds 2-hydroxyputrescine (13.54 µmol g⁻¹ dry weight) and putrescine (11.71 µmol g⁻¹ dry weight) and moderate amounts of spermidine (3.24 µmol g⁻¹ dry weight), whereas 1,3-diaminopropane, cadaverine, homospermidine and spermine were detected only in minor amounts (0.04, 0.03, 0.03 and 0.30 µmol g⁻¹ dry weight, respectively).

Polar lipids were determined by two-dimensional TLC, as described previously (Ventosa *et al.*, 1993). The polar lipid profile of strain KF46F^T consisted of four compounds. Phosphatidylethanolamine was detected as the major lipid and phosphatidylglycerol and diphosphatidylglycerol were detected as minor spots after two-dimensional TLC. In addition, an unknown polar lipid (ninhydrin-negative, molybdenum blue-positive and alpha-naphthol-negative staining) was detected.

Isolation of genomic DNA was performed as described by Ausubel *et al.* (1996) and the determination of the G+C content was carried out by HPLC according to Mesbah & Whitman (1989). Genomic DNA of *Hydrogenophaga palleronii* DSM 63^T (G+C content 67.5 mol%; Aragno & Schlegel, 1992) and *Bacillus subtilis* DSM 618 (G+C content 43 mol%; Slepecky & Hemphill, 1992) were used as standards. The G+C content of the genomic DNA of strain KF46F^T was determined to be 70.4±0.3 mol% (mean of four measurements).

The gene for the 16S rRNA was amplified by PCR using different universal primers (Gerhardt *et al.*, 1994) and sequenced. Phylogenetic analysis was performed using the ARB software package (Ludwig & Strunk, 1997). Distance-matrix, maximum-parsimony and maximum-likelihood methods were applied for tree

Fig. 1. Phylogenetic tree showing the relationship between *Xenophilus azovorans* strain KF46F^T (= NCIMB 13707^T) and selected strains from other bacterial taxa. The tree was constructed using the maximum-parsimony method. GenBank accession numbers are given in parentheses.

construction as implemented in the ARB software package.

The 16S rRNA sequence of strain KF46F^T, containing a continuous stretch of 1484 bp (approximately positions 28–1521 according to the *Escherichia coli* numbering), was used to search the GenBank and Ribosomal Database Project libraries. Sequence searches showed that strain KF46F^T was phylogenetically most closely related to the β -subclass of the *Proteobacteria*. The results of sequence similarity calculations indicated that the nearest relatives of strain KF46F^T are *Xylophilus ampelinus* (96.1% sequence similarity), *Aquaspirillum metamorphum* (96.0%) and *Hydrogenophaga palleronii* (95.4%). Only slightly lower sequence similarities (92–95% sequence identity) were found to different species from the genera *Variovorax*, *Comamonas*, *Brachymonas*, *Delftia* and *Acidovorax*. From these results, it was evident that strain KF46F^T belonged to the family *Comamonadaceae*. Dendrograms were generated by using the maximum-parsimony, maximum-likelihood and neighbour-joining methods from the ARB program package. The results of all these trees (Fig. 1 shows the maximum-parsimony analysis) showed a separate and almost equidistant position of strain KF46F^T between the genera mentioned above.

Extrachromosomal DNA was detected by a modification of the method of Barton *et al.* (1995). Pulsed-field gel electrophoresis demonstrated that strain

KF46F^T harbours two large plasmids, of about 100 and 350 kb.

The detection of ubiquinone Q-8 as the single quinone, the presence of hydroxyputrescine and putrescine, the large amounts of 16:0 and 17:0 cyclo fatty acids and the absence of 16:0 3-OH, as well as the G + C content of the DNA and the sequence of the 16S rRNA, clearly placed strain KF46F^T in the family *Comamonadaceae*. This family consisted originally of the genera *Comamonas*, *Acidovorax*, *Variovorax*, *Hydrogenophaga* and *Xylophilus* and some misclassified *Aquaspirillum* species (Willems *et al.*, 1991b). In later studies, further species belonging to the genus *Acidovorax* were detected (Willems *et al.*, 1992; Schulze *et al.*, 1999) and also further genera were described (Hiraishi, 1994; Hiraishi *et al.*, 1995; Irgens *et al.*, 1996; Wen *et al.*, 1999). The phylogeny of the family was investigated in more detail by Wen *et al.* (1999) and, on the basis of this study, the family comprises the 10 genera included in Table 1. Strain KF46F^T is clearly different from all other previously described genera belonging to the family *Comamonadaceae*.

The main reason for creating a new genus for strain KF46F^T was the rather large degrees of sequence divergence of the 16S rRNA gene from the corresponding sequences of different genera of the family *Comamonadaceae*. Furthermore, strain KF46F^T can be clearly separated from the most closely related genera, *Hydrogenophaga*, *Comamonas* and *Xylophilus*,

Table 1. Differential characteristics of the genus *Xenophilus* and other genera in the family *Comamonadaceae*

The table was adapted 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; RQ, rhodoquinone; NA, no data available. Data for genera other than *Xenophilus* were taken from: De Vos *et al.* (1985), Palleroni (1984), Tamaoka *et al.* (1987) and Willems *et al.* (1991a) (*Delftia* and *Comamonas*); Palleroni (1984) and Willems *et al.* (1990, 1992) (*Acidovorax*); Palleroni (1984) and Willems *et al.* (1989) (*Hydrogenophaga*); Kersters & De Ley (1984), Urakami *et al.* (1995) and Willems *et al.* (1991b) (*Variovorax*); Bradbury (1984) and Willems *et al.* (1987, 1991b) (*Xylophilus*); Hiraishi *et al.* (1991) and Busse & Auling (1988) (*Rhodoferrax*); Hiraishi *et al.* (1995) (*Brachymonas*); Irgens *et al.* (1996) (*Polaromonas*); and Hamana *et al.* (1994), Krieg (1984), Pot *et al.* (1992a, b) and Sakane & Yokota (1994) (*Aquaspirillum* metamorphum).

Character	<i>Xenophilus</i>	<i>Delftia</i>	<i>Comamonas</i>	<i>Acidovorax</i>	<i>Hydrogenophaga</i>	<i>Variovorax</i>	<i>Xylophilus</i>	<i>Rhodoferrax</i>	<i>Brachymonas</i>	<i>Polaromonas</i>	[<i>Aquaspirillum</i>] <i>metamorphum</i>
Cell morphology	Rods	Rods	Rods or spirilla	Rods	Rods	Rods	Rods	Curved rods	Coccobacilli or short rods	Rods	Spirilla or curved rods
Flagella	NA	Polar or bipolar tufts	Polar or bipolar tufts	One polar	One polar	Peritrichous	One polar	One polar	—	One polar	Bipolar tufts or 1–2 flagella at only one pole
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	d	—	d	—	—	NA	NA	NA	NA	NA
β-Alanine	—	d	—	d	—	d	NA	—	NA	NA	NA
2-Aminobutyrate	NA	d	—	d	d	NA	NA	NA	—	NA	NA
3-Aminobutyrate	NA	d	d	d	d	NA	NA	NA	—	NA	NA
D-Fructose	+	+	—	+	d	NA	—	+	—	—	—
D-Glucose	—	—	—	d	+	+	+	+	—	+	—
Glycerol	(+)	d	d	+	+	+	+	—	—	+	—
Malonate	NA	d	—	d	—	d	—	NA	—	—	D
D-Mannitol	+	+	—	d	d	NA	—	+	—	NA	NA
Maleate	NA	d	—	d	—	d	—	NA	NA	NA	NA
Phenylacetate	—	d	—	—	—	d	NA	NA	NA	NA	NA
L-(+)-Tartrate	NA	d	—	—	d	d	NA	NA	—	NA	NA
D-Tryptophan	NA	d	d	—	—	NA	NA	NA	NA	NA	—
L-Tryptophan	(+)	d	—	d	d	d	NA	NA	—	NA	—
Major quinone system	Q-8	Q-8	Q-8	Q-8	Q-8	Q-8	Q-8	Q-8 + RQ-8	NA	Q-8	NA
Major cellular fatty acid(s)	16:0, 16:1, 18:1	16:0, 16:1, 18:1	16:0, 16:1, 18:1	16:0, 16:1, 18:1	16:0, 16:1	16:1, 16:0, 18:1	NA	16:0, 16:1	16:0, 16:1	16:1, 16:0, 18:1	16:0, 16:1
Major 3-OH acids	10:0, 8:0	10:0, 8:0	10:0	10:0, 8:0	8:0, (10:0)	10:0	NA	8:0	10:0	—	10:0
2-OH acids detected	16:0, 16:1, 18:1	—	16:0, 16:1	—	Traces of 16:0	14:0	NA	—	—	—	—
DNA G+C content (mol%)	70.4 (HPLC)	67–69	63–66	67–70	65–69	66–68	68–69	59–61 (HPLC)	63–65 (HPLC)	52–57	56–62

by its unique fatty acid pattern and several physiological features. On the basis of these observations, we propose strain KF46F^T as representative of a new species of a new genus, *Xenophilus azovorans* gen. nov., sp. nov.

Description of *Xenophilus* gen. nov.

Xenophilus (xe.no'phi.lus. Gr. adj. *xenos* foreign; Gr. masc. n. *philos* friend; N.L. masc. n. *Xenophilus* friend of foreign compounds, referring to the isolation of the type species by enrichment on azo dyes).

Cells are straight to slightly curved rods, 0.5–1 µm by 1–3 µm. Cells occur singly or in pairs and are motile. Gram-negative, oxidase-positive and possesses an oxidative metabolism. The G+C content of DNA is 70.4 mol% (HPLC method). *Xenophilus* belongs to the family *Comamonadaceae* and, on the basis of 16S rRNA sequences, is equidistantly related to representatives of the genera *Xylophilus* and *Hydrogenophaga*. Ubiquinone Q-8 is the only quinone type; 2-hydroxy-putrescine and putrescine are the major polyamines. The polar lipid profile is characterized by the presence of phosphatidylethanolamine (major lipid) and phosphatidylglycerol and diphosphatidylglycerol in addition to an unknown polar lipid (minor lipids). The type species is *Xenophilus azovorans*.

Description of *Xenophilus azovorans* sp. nov.

Xenophilus azovorans (a.zo.vo'rans. N.L. neut. *azo* from azo compounds; L. v. *vorare* to devour; N.L. part. adj. *azovorans* azo-devouring).

The description is the same as that given above for the genus. On nutrient agar, colonies are circular, opaque, slightly raised and pale-yellowish with entire margins. Colonies are often not detectable as single entities because of the production of extracellular slimy substances. The type strain grows at 30 °C; no growth is found at 4, 37 or 42 °C. L-Alanine *p*-nitroanilide and 2-deoxythymidine 2'-*p*-nitrophenylphosphate are hydrolysed. The following compounds are not hydrolysed: *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl β-D-glucuronide, *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-xylopyranoside, bis-*p*-nitrophenyl phosphate, bis-*p*-nitrophenyl phenylphosphonate, bis-*p*-nitrophenyl phosphorylcholine, L-aniline *p*-nitroanilide, γ-L-glutamate *p*-nitroanilide and L-proline *p*-nitroanilide. No acid production can be detected from the following sugars: glucose, lactose, sucrose, D-mannitol, dulcitol, salicin, adonitol, inositol, sorbitol, L-arabinose, raffinose, L-rhamnose, maltose, D-xylose, trehalose, cellobiose, methyl D-glucoside, erythritol, melibiose, D-arabitol and D-mannose. The following compounds are utilized as sole sources of carbon: glycerol, D-fructose, D-mannitol, maltitol, acetate, propionate, adipate, azelate, fumarate, glutarate, DL-3-hydroxybutyrate, itaconate, DL-lactate, 2-oxoglutarate, pyruvate, suberate, L-aspartate, L-histidine, L-leucine,

L-proline, L-tryptophan, 3-hydroxybenzoate and 4-hydroxybenzoate. The following compounds are not utilized as sole sources of carbon: *N*-acetylgalactosamine, *N*-acetylglucosamine, L-arabinose, L-arbutin, D-cellobiose, D-galactose, gluconate, D-glucose, D-maltose, D-mannose, α-D-melibiose, L-rhamnose, D-ribose, D-sucrose, salicin, D-trehalose, D-xylose, adonitol, *i*-inositol, D-sorbitol, putrescine, *cis*-aconitate, *trans*-aconitate, 4-aminobutyrate, citrate, mesaconate, L-alanine, β-alanine, L-ornithine, L-phenylalanine, L-serine and phenylacetate. The major non-polar fatty acids are 16:0, summed feature 4 (16:1ω7c and iso 15:0 2-OH) and summed feature 7 (18:1ω7c, 18:1ω9t and/or 18:1ω12t). In addition, 8:0 3-OH, 10:0 3-OH, 16:0 2-OH, 16:1 2-OH, 18:1 2-OH and 17:0 cyclo are detected in significant amounts.

The type strain is strain KF46F^T (= DSM 13620^T = NCIMB 13707^T). Strain KF46F^T was isolated from soil after continuous enrichment with 1-(4'-carboxyphenylazo)-2-naphthol. The 16S rRNA gene sequence has been deposited in the EMBL database under the accession number AF285414.

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