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Labrys portucalensis sp. nov., a fluorobenzene-degrading bacterium isolated from an industrially contaminated sediment in northern Portugal

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A detailed classification of a novel bacterial strain, designated F11^T, capable of degrading fluorobenzene as a sole carbon and energy source, was performed by using a polyphasic approach. This Gram-negative, rod-shaped, non-motile, non-spore-forming, aerobic bacterium was isolated from a sediment sample collected from an industrially contaminated site in northern Portugal. The predominant whole-cell fatty acids were $C_{19:0}$ cyclo ω8*c*, $C_{16:0}$, $C_{18:1}\omega$7*c*, $C_{18:0}$, $C_{18:0}$ 3-OH and $C_{16:0}$ 3-OH. The G+C content of the DNA was 62.9 mol% and the major respiratory quinone was ubiquinone 10 (UQ-10). 16S rRNA gene sequence analysis revealed that strain F11^T was a member of the class *Alphaproteobacteria* and was phylogenetically related to the genus *Labrys*, having sequence similarities of 95.6 and 93.1 % to the type strains of *Labrys monachus* and *Labrys methylaminiphilus*, respectively. DNA–DNA hybridization experiments revealed levels of relatedness of <70 % between strain F11^T and the type strains of *L. monachus* and *L. methylaminiphilus* (38.6 and 34.1 %, respectively), justifying the classification of strain F11^T as representing a novel species of the genus *Labrys*. The name *Labrys portucalensis* sp. nov. is proposed for this organism. The type strain is F11^T (=LMG 23412^T=DSM 17916^T).

The genus *Labrys* was erected by Vasil'eva & Semenov (1984) to accommodate a strain of budding prosthecate bacteria that was isolated from silt samples collected from Lake Mustijärv located in the former Estonian SSR. This organism, designated strain VKM B-1479^T, was subsequently named *Labrys monachus* (Vasil'eva & Semenov, 1985). A second species of the genus, *Labrys methylaminiphilus*, was described by Miller *et al.* (2005) to accommodate a facultatively methylotrophic bacterium (strain JLW10^T) derived from Lake Washington sediment. At the time of writing, the genus *Labrys* comprised these two recognized species.

The ability to degrade haloaromatic compounds of environmental relevance has been observed in a wide phylogenetic diversity of micro-organisms (Key *et al.*, 1997; van Pée & Unversucht, 2003). These micro-organisms play a crucial role in the detoxification of haloaromatics and they can be applied in various bioremediation strategies. During our studies on the microbial degradation of fluorobenzene (FB), a pure bacterial culture with the unique capacity to utilize this compound as a sole carbon and energy source was isolated from a sediment sample collected from an industrially polluted site in northern Portugal (Carvalho *et al.*, 2005). The isolated strain, designated F11^T, was found to belong to subgroup 2 of the class *Alphaproteobacteria* (Woese *et al.*, 1984) and to fall within the order *Rhizobiales*. In the present study, a more detailed classification of this strain is provided by using a polyphasic approach, which included a detailed analysis of its morphological and physiological characteristics, cellular fatty acid profiling, phylogenetic analysis of the 16S rRNA gene and DNA–DNA hybridization experiments. On the basis of the data presented, we suggest that strain F11^T represents a novel species of the genus *Labrys*.

Strain F11^T was isolated, as described previously (Carvalho *et al.*, 2005), from industrially contaminated sediment located at Estarreja, northern Portugal. For taxonomic

†These authors contributed equally to this work.

Abbreviation: FB, fluorobenzene.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain F11^T is AY362040.

studies, the strain was routinely cultivated at 25 °C in a minimal salts liquid medium (MM) (Caldeira *et al.*, 1999) supplemented with 0.2 % glycerol (v/v) or on nutrient agar (NA) plates. For preservation purposes, cultures of strain F11^T growing exponentially in MM containing 1 mM FB as the carbon source were supplemented with 20 % (v/v) glycerol and frozen at −80 °C.

Light microscopy was used to examine cell morphology and motility by analysing wet mounts of 72 h cultures of strain F11^T. Confirmation of cell morphology and determination of cell dimensions were accomplished in a Zeiss EM C10 electron microscope. Aliquots from pure cultures were deposited onto Formvar/carbon 400 μm mesh, 3 mm diameter grids and contrasted with 2 % (w/v) uranyl acetate. The presence of a capsule and endospores was assessed according to Creager *et al.* (1990). Gram stain, oxidase and catalase tests were performed as described by Smibert & Krieg (1994). Motility was tested as described by Alexander & Strete (2001).

The ability of strain F11^T to utilize various carbon sources was tested, in duplicate, in MM supplemented with the following single test substrates: FB, 4-fluorobenzoate, 2-fluorobenzoate, benzoate, benzene, bromobenzene, iodobenzene, chlorobenzene, 3-chloro-4-fluoroaniline, 4-chlorobenzoate, 4-chlorophenol, phenol, 4-fluorophenol and toluene (all at 0.5 mM); acetate, citrate, DL-lactate, D-glucose, D-lactose, D-mannose, maltose, methylamine and trimethylamine (20 mM); ethanol and methanol (0.1 %, v/v); glycerol (0.2 %, v/v), and yeast extract (0.2 %, w/v). Growth was monitored by measuring cell density at 600 nm of cultures incubated aerobically at 25 °C with shaking at 150 r.p.m. Negative controls, consisting of MM without the addition of a carbon source and inoculated with strain F11^T, were established for all metabolic experiments.

The utilization of additional carbon sources was tested by using the API 20NE test kit (bioMérieux) according to the manufacturer's instructions. This kit was also used to carry out the following biochemical tests: nitrate reduction, indole production, acid production from D-glucose, hydrolysis of aesculin and gelatin and to detect the presence of L-arginine dihydrolase, urease and β-galactosidase activities. The Voges–Proskauer reaction, fluorescent pigment production and starch hydrolysis were tested according to the methods described by Smibert & Krieg (1994). Hydrolysis of agarose was tested in solid medium. Poly-β-hydroxy alkanoate granules were detected as described by De Marco *et al.* (2000).

The growth temperature range was determined by incubating NA plates, streaked with strain F11^T, between 4 and 42 °C. The pH range was determined by analysing the occurrence of growth of cultures of strain F11^T in MM supplemented with 0.2 % glycerol at pH values between 4.0 and 10.0. The ability to grow under anaerobic conditions was evaluated by incubating NA plates streaked with strain F11^T in an anaerobic jar for 10 days at 30 °C. N_2 fixation was tested in triplicate by determining the growth of strain F11^T in MM without a nitrogen source but supplemented with 0.2 % glycerol. Cultures were incubated and monitored for growth as described above. To test for the ability to utilize nitrate as an electron acceptor, cultures of strain F11^T were grown in triplicate in MM supplemented with 0.2 % glycerol and 10 mM KNO_3 under anaerobic conditions. To ensure that cultures were free of oxygen, cysteine was added at a concentration of 50 mg l^{-1} to reduce all the oxygen remaining in the cultures, and resazurin (1 mg l^{-1}) was used as an oxygen indicator. Control cultures, grown aerobically, were also established.

When grown on NA plates incubated for 3–4 days at 25 °C, strain F11^T formed white, circular, convex, entire-edged, mucous, glistening colonies 1–2 mm in diameter. Light microscopy revealed rod-shaped, non-motile, capsulated, non-spore-forming cells. The Gram-stain was negative. Under the electron microscope, cells of strain F11^T were short, thick rods, 0.8–1.4 μm in width and 1.6–2.4 μm in length, with no flagella.

Strain F11^T was able to grow at temperatures of between 16 and 37 °C and had an optimum temperature range of 28–32 °C. The pH range found to support growth was between 4.0 and 8.0, with optimum pH values of between 6.0 and 8.0.

Tests for catalase and cytochrome oxidase were positive. The organism did not produce fluorescent pigments on MM supplemented with 0.2 % glycerol, while accumulation of poly-β-hydroxy alkanoate granules was observed in the same medium. The Voges–Proskauer test was positive. Indole production and D-glucose acidification tests were negative. The strain was able to hydrolyse aesculin but not gelatin, starch or agarose. It was positive for urease activity, weakly positive for β-galactosidase activity but negative for L-arginine dihydrolase activity.

The following substrates were found to support growth of strain F11^T as sole carbon and energy sources: FB, 4-fluorobenzoate, 2-fluorobenzoate, benzoate, benzene, phenol, 4-fluorophenol, acetate, citrate, DL-lactate, D-gluconate, DL-malate, D-glucose, D-lactose, D-mannose, L-arabinose, glycerol, D-mannitol, ethanol, methylamine, trimethylamine, *N*-acetylglucosamine and yeast extract. No growth was obtained with the following substrates: bromobenzene, iodobenzene, chlorobenzene, 3-chloro-4-fluoroaniline, 4-chlorobenzoate, 4-chlorophenol, toluene, methanol, caprate or phenylacetate. Maltose and adipate were found to support only weak growth of strain F11^T.

Strain F11^T was unable to grow under anaerobic conditions, indicating that it is a strict aerobe. The organism was not able to reduce nitrate nor to use it as an electron acceptor, but N_2 could be used as a nitrogen source for growth.

Antibiotic susceptibility of strain F11^T was examined by using the Difco Laboratories antibiotic disc sensitivity assay according to the manufacturer's instructions. The antibiotics tested included (μg ml^{-1}): amoxicillin (25),

cephalothin (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), kanamycin (30), sulfamethoxazole + trimethoprim (25), tetracycline (30) and vancomycin (5). Strain F11^T was resistant to all the antibiotics tested, except for sulfamethoxazole + trimethoprim.

Respiratory quinone and fatty acid analyses were performed at DSMZ, Braunschweig, Germany (http://www.dsmz.de) by HPLC and GC, respectively. The major respiratory quinone found for strain F11^T was ubiquinone 10 (UQ-10). Traces of ubiquinone 9 (UQ-9) were also found.

The main fatty acids extracted from strain F11^T were $C_{19:0}$ cyclo $\omega 8c$, $C_{16:0}$, $C_{18:1}\omega 7c$ and $C_{18:0}$ (Table 1). This profile does not match closely with that of any recognized bacterium present in the public databases, but the combination of these principal fatty acids together with the presence of 3-hydroxy fatty acids [$C_{18:0}$ 3-OH (1.9 %) and $C_{16:0}$ 3-OH (1.3 %)] is diagnostic for members of the order *Rhizobiales*. A comparison of the fatty acid profile of strain F11^T with those of *L. monachus* VKM B-1479^T and *L. methylaminiphilus* JLW10^T is given in Table 1. An unusually high proportion of lactobacillic acid (a cyclopropane fatty acid: $C_{19:0}$ cyclo $\omega 8c$) was observed for strain F11^T and for the two recognized species of the genus *Labrys*. The high content of this fatty acid in *L. monachus* had already been reported by Sittig & Schlesner (1993) and by Fritz *et al.* (2004). Lactobacillic acid tends to increase in cells with age but, according to several authors, it never becomes a main component of the fatty acid profile (Auran & Schmidt, 1972; Guckert *et al.*, 1986), indicating that the high content of this fatty acid present in these three strains is not simply related to the age of the cultures but rather represents a characteristic of the genus. Hexadecanoic acid (palmitic acid: $C_{16:0}$) was present in similar amounts in the three strains, but octadecanoic acid (stearic acid: $C_{18:0}$) and octadecenoic acid (vaccenic acid: $C_{18:1}\omega 7c$) were present in variable amounts.

The 16S rRNA gene sequence of strain F11^T has been determined previously (Carvalho *et al.*, 2005). For phylogenetic analyses, the 16S rRNA gene sequences were aligned by using the BioEdit program (version 7.0.5.2) (Hall, 1999) and analysed via the DNAML, SEQBOOT (100 iterations), DNAPARS, DNADIST (Kimura two-parameter correction), NEIGHBOR, FITCH and CONSENSE programs of the PHYLIP package (Felsenstein, 1995). 16S rRNA gene sequences were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov) (Benson *et al.*, 2007). Two alignments of 22 and 31 sequences of 1291 and 960 nt, respectively, were used. A phylogenetic tree was constructed based on the results obtained from the first alignment (Fig. 1). The 16S rRNA gene sequence of *Escherichia coli* MRE600 (GenBank accession no. J01859) was used to root the tree.

The G + C content of the genomic DNA of strain F11^T was determined by HPLC as described by Mesbah *et al.* (1989), at the BCCM/LMG Culture Collection Laboratories, University of Gent, Belgium.

DNA–DNA hybridization experiments were performed at DSMZ, Braunschweig, Germany, as described by De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983), by using a model Cary 100 Bio UV/visual spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). DNA for the hybridization experiments was extracted by using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite, according to the procedure of Cashion *et al.* (1977). For hybridization experiments, the genomic DNA of strain F11^T was hybridized with the DNA of the type strains of *L. monachus* and *L. methylaminiphilus*.

Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain F11^T was most closely related to several undescribed strains provisionally identified as members of the genus *Labrys* (GenBank accession nos DQ417335, AB236172, DQ337554, AB236170, AB236171, AB236169, AY635896, AF008564). Highest 16S rRNA gene sequence similarity to recognized species was to *L. monachus* VKM B-1479^T and *L. methylaminiphilus* JLW10^T (95.6 and 93.1 %, respectively). Phylogenetic analyses were performed by comparing strain F11^T with the type strains of members of the class *Alphaproteobacteria* and in particular with representatives of the order *Rhizobiales* (Fig. 1). Strain F11^T clustered unequivocally with species of the genus *Labrys*, consistently showing bootstrap values of 100 %. The relative positions of this cluster and of the major groups within the order *Rhizobiales* were quite variable depending on the phylogenetic method used and these branches always showed very low bootstrap values. However, the order *Rhizobiales* as a whole always formed a consistent branch with high bootstrap values (97–99 %) and the branch comprising the two recognized *Labrys* species plus strain F11^T fell within this group in all cases. When the 16S rRNA gene sequences of strains provisionally classified as representing *Labrys* species [*Labrys miyagiensis* G24103^T and G24116, *Labrys okinawensis* DSM 18385^T, *Labrys* sp. AMS5, *Labrys neptuniae* Liujia-146^T (the names *L. miyagiensis*, *L. okinawensis* and *L. neptuniae* were validly published while this study was in press) and *L. methylaminiphilus* DSM 16812 and CHNCT15] were used in the analysis, the type strains of *L. monachus* and *L. methylaminiphilus* and strain F11^T consistently formed a group with these sequences, supported in all cases by a bootstrap value of 100 % (not shown).

The G + C content of the DNA of strain F11^T was slightly lower than the values reported for *L. monachus* VKM B-1479^T and *L. methylaminiphilus* JLW10^T (Table 1).

DNA–DNA hybridization experiments revealed a level of relatedness of 38.6 % between strain F11^T and *L. monachus* VKM B-1479^T and of 34.1 % between strain F11^T and *L. methylaminiphilus* JLW10^T, indicating that strain F11^T

Table 1. Differential characteristics between strain F11^T and recognized species of the genus *Labrys*

Strains: 1, F11^T; 2, *L. monachus* VKM B-1479^T; 3, *L. methylaminiphilus* JLW10^T. +, Positive; −, negative; (+), weakly positive; NR, not reported. The three strains were Gram-negative, non-motile, capsulated, unable to hydrolyse starch, but able to utilize D-glucose, glycerol, D-mannitol, ethanol, D-mannose and yeast extract. Data for *L. monachus* and *L. methylaminiphilus* were not obtained or confirmed in the present study, so direct comparisons with the new data obtained for strain F11^T should be drawn with caution.

Characteristic	1	2	3*
Origin	Contaminated sediments	Swamp soil†	Lake sediment
Cell morphology	Short, thick rods	Flat, triangular cells with prosthecae†	Rods
Colony description	White, circular, mucous, glistening and entire-edged	Grey, circular, opaque, viscous, glistening and entire-edged†	White, circular, convex, opaque and butyrous
Catalase/oxidase	+/+	+/−‡	+/+
Growth pH range	4.0–8.0	6.0–9.0*	4.0–9.5
Optimum pH range	6.0–8.0	6.0–8.0*	5.0–7.0
Growth temperature range (°C)	16–37	20–50*	10–35
Optimum growth temperature range (°C)	28–32	28–30*	28–30
Fluorescent pigments	−	NR	−
Nitrate reduction	−	+*	+
Hydrolysis of:			
Gelatin	−	−*	(+)
Agarose	−	−*	+
Indole production	−	NR	−
Voges–Proskauer reaction	+	−†	−
Urease production	+	−†	−
N_2 fixation	+	NR	−
Utilization of:			
Acetate	+	(+)†	+
Benzoate	+	−†	NR
Citrate	+	(+)†	+
D-Gluconate	+	+†	NR
DL-Lactate	+	+†	NR
D-Lactose	+	−†	+
DL-Malate	+	(+)†	+
Maltose	(+)	+†	+
Methanol	−	(+)†	−
Methylamine	+	(+)†	+
Toluene	−	NR	−
Trimethylamine	+	−†	+
Fatty acids (% of total content)			
$C_{16:0}$	22.7	19.7§	17.7
$C_{18:1}\omega7c$	18.4	2.9§	32.8
$C_{18:0}$	3.0	13.0§	1.7
$C_{19:0}$ cyclo $\omega8c$	48.5	40.4§	49.4
$C_{20:1}\omega9$	−	11.9	−
Major ubiquinones	UQ-10	UQ-10‖	NR
DNA G+C content (mol%)	62.9	67.9†	65.7

*Data from Miller *et al.* (2005).
†Data from Vasil'eva & Semenov (1984).
‡Data from Staley & Fuerst (1989).
§Data from Fritz *et al.* (2004).
‖Data from Sittig & Schlesner (1993).

represents a separate species, based on the recommended minimum value of 70 % for strains of the same species (Wayne *et al.*, 1987).

The phenotypic, chemotaxonomic, genotypic and phylogenetic data presented in the present study indicate that strain F11^T represents a novel species of the genus *Labrys*. It

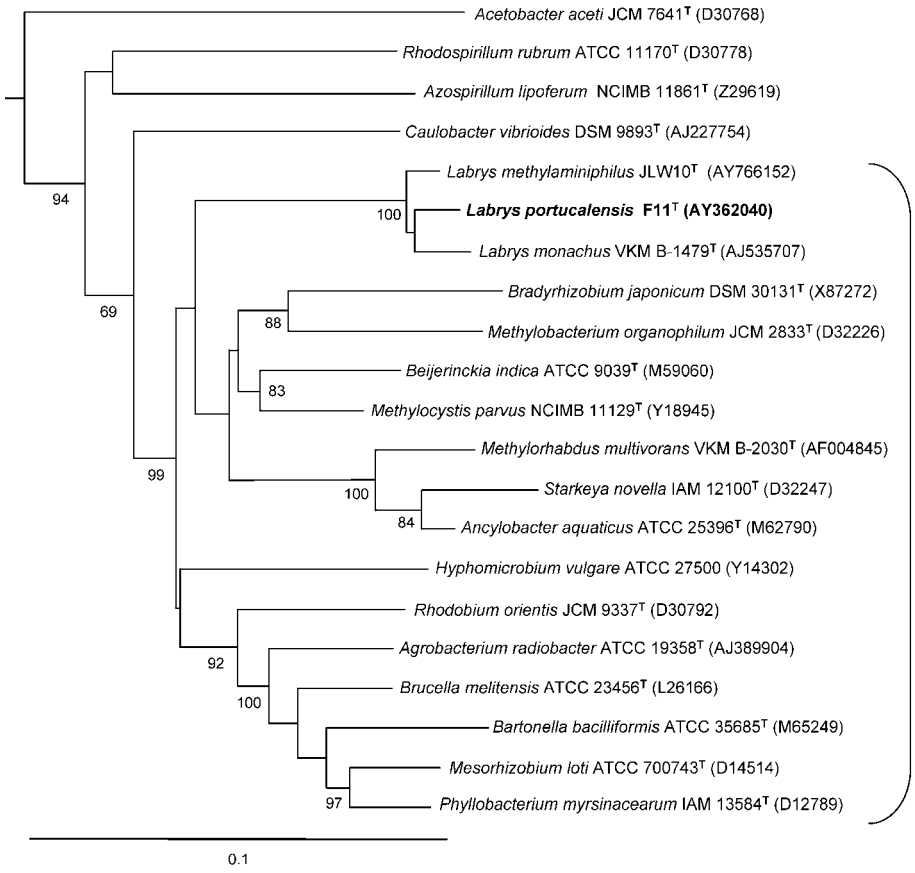

Fig. 1. Phylogenetic tree obtained by neighbour-joining analysis of 16S rRNA gene sequences. The 16S rRNA gene sequence of *Escherichia coli* MRE600 (GenBank accession number J01859) was used to root the tree (not shown). Only bootstrap values >65 % are reported at nodes. GenBank accession numbers are given in parentheses. The bracket on the right indicates the order *Rhizobiales* branch. Bar, 0.1 substitutions per site.

is clear from the phylogenetic analysis that strain F11^T and the type strains of *L. monachus* and *L. methylaminiphilus* constitute a consistent group and form a distinct branch within the order *Rhizobiales*. The very high bootstrap value (100 %) for the 16S rRNA gene sequence of strain F11^T with *L. monachus* VKM B-1479^T and *L. methylaminiphilus* JLW10^T supports the conclusion that strain F11^T is affiliated to the genus *Labrys*. However, 16S rRNA gene sequence similarity values below 97 % justify the separation of strain F11^T at the species level (Stackebrandt & Goebel, 1994).

Many of the phenotypic characteristics of strain F11^T are in accordance with this classification. For instance, UQ-10 as the major respiratory quinone of strain F11^T is in agreement with the affiliation of this micro-organism to the class *Alphaproteobacteria* (Lechner *et al.*, 1995; Yokota *et al.*, 1992). The fatty acid profile of strain F11^T is consistent with its placement within the order *Rhizobiales*.

Strain F11^T shared many taxonomic properties with *L. monachus* and *L. methylaminiphilus* (Table 1), but important differences were found with regard to both morphological traits and metabolic characteristics, again supporting strain F11^T as representing a distinct species. At the phenotypic level, strain F11^T differed from *L. monachus* and *L. methylaminiphilus* not only based on cellular morphology but also on its ability to produce urease and acetoin (positive for the Voges–Proskauer reaction) and to fix N_2, as well as its inability to reduce nitrate. Strain F11^T was able to use a wide variety of organic substrates, including methylotrophic carbon sources such as methylamine and trimethylamine (but not methanol). As shown in Table 1, the metabolic profiles of strain F11^T and of *L. methylaminiphilus* JLW10^T were very similar for the listed compounds, except for benzoate, D-gluconate and DL-lactate for which no data were available for the latter. *L. monachus* VKM B-1479^T, in contrast, was unable to grow on benzoate, D-lactose or trimethylamine and showed only weak growth on methanol. The three strains also differed in their fatty acid profiles, although they all showed an unusually high level of lactobacillic acid ($C_{19:0}$ cyclo $\omega 8c$), a characteristic that may prove to be diagnostic for the genus *Labrys*.

The genus *Labrys*, at the time comprising only *L. monachus*, was reported to have no affiliation at the family level (Fritz *et al.*, 2004). However, a phylogenetic study by Lee *et al.* (2005) on relationships within the class *Alphaproteobacteria* led to the inclusion of the genus *Labrys* in a new family designated the *Xanthobacteraceae*, within the order *Rhizobiales*. The characteristic traits of members of this family were indeed all present in strain F11^T (Lee *et al.*, 2005).

In conclusion, based on the results presented herein, we conclude that strain F11^T represents a novel species of the genus *Labrys*, for which the name *Labrys portucalensis* sp. nov. is proposed.

While this paper was in press, three additional species of the genus *Labrys* have been described, *Labrys miyagiensis* and *Labrys okinawensis* (Islam *et al.*, 2007) and *Labrys neptuniae* (Chou *et al.*, 2007).

Description of *Labrys portucalensis* sp. nov.

Labrys portucalensis (por.tu.cal.en′sis. L. adj. *portucalensis* referring to Portugal, from where the bacterium was isolated).

Cells are short, thick rods 0.5–1.0 μm in width and 0.8–1.0 μm in length, Gram-negative, non-spore-forming, capsulated, non-motile, and oxidase- and catalase-positive. When incubated on NA for 3–4 days at 25 °C, forms white colonies 1–2 mm in diameter that are circular, convex, with an entire edge and with a mucous consistency. Growth is strictly aerobic. The temperature range for growth is 16–37 °C, with optimal growth at 28–32 °C. The pH range is 4.0–8.0, with optimal growth at pH 6.0–8.0. Indole is not produced on tryptophan and acid is not produced from D-glucose. Aesculin is hydrolysed, but gelatin, starch and agarose are not. No L-arginine dihydrolase activity. Produces urease and acetoin (positive for the Voges–Proskauer reaction). No fluorescent pigments are produced. Nitrate is not reduced, but N_2 can be used as a nitrogen source for growth. A variety of organic compounds are used as sole carbon and energy sources, including: FB, 4-fluorobenzoate, 2-fluorobenzoate, benzoate, benzene, phenol, 4-fluorophenol, acetate, citrate, DL-lactate, D-gluconate, DL-malate, D-glucose, D-lactose, D-mannose, L-arabinose, glycerol, D-mannitol, ethanol, *N*-acetylglucosamine and yeast extract. Methylamine and trimethylamine (but not methanol) are also used as sole sources of carbon and energy, and therefore this species is a facultative methylotroph. The main respiratory quinone is UQ-10. The predominant fatty acids are $C_{19:0}$ cyclo $\omega 8c$, $C_{16:0}$, $C_{18:1}\omega 7c$, $C_{18:0}$, $C_{18:0}$ 3-OH and $C_{16:0}$ 3-OH. Sensitive to sulfamethoxazole + trimethoprim. The G + C content of the DNA is 62.9 mol%.

The type strain, F11^T (=LMG 23412^T=DSM 17916^T), was isolated from a sediment sample collected at a polluted site located in the industrial complex of Estarreja, northern Portugal.

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