Brachymonas chironomi sp. nov., isolated from a chironomid egg mass, and emended description of the genus *Brachymonas*

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A Gram-negative, aerobic, chemo-organotrophic, catalase- and oxidase-positive strain, designated AIMA4^T, was isolated from a chironomid (Diptera; Chironomidae) egg mass which was sampled from a waste stabilization pond in northern Israel. Phylogenetic analysis based on the 16S rRNA gene placed strain AIMA4^T in the genus *Brachymonas* (class *Betaproteobacteria*). Strain AIMA4^T shared 16S rRNA gene sequence similarity values of 96.9% with *Brachymonas denitrificans* JCM 9216^T and <95.7% with the type strains of other genera. Strain AIMA4^T was found to be a non-motile coccobacillus or rod that did not grow under anaerobic conditions and did not produce acid from carbohydrates. Growth was observed at salinities from 0 to 2.5% (w/v) NaCl, at pH values from 5.0 to 9.0 and at temperatures from 18 to 37 °C. The major fatty acids (>10%) of strain AIMA4^T were C_{16:1}ω7c, C_{16:0} and C_{18:1}ω7c. Phosphatidylethanolamine, phosphatidylglycerol and phosphatidylserine were the predominant components of the polar lipids. The major ubiquinone was Q-8. On the basis of phenotypic properties and phylogenetic distinctiveness, strain AIMA4^T (=LMG 24400^T=DSM 19884^T) was classified in the genus *Brachymonas* as the type strain of a novel species for which the name *Brachymonas chironomi* sp. nov. is proposed.

The genus *Brachymonas* was created by Hiraishi *et al.* (1995a) while characterizing rhodoquinone-containing bacteria that had been isolated from soybean crude waste sludge. At present, the genus comprises only one species: *Brachymonas denitrificans* (Hiraishi *et al.*, 1995a, b). In this study, a new member of the genus *Brachymonas* (family *Comamonadaceae*, class *Betaproteobacteria*) that was isolated from a freshwater insect egg mass is proposed on the basis of a taxonomic study using a polyphasic approach.

Chironomids (*Diptera*; *Chironomidae*) are the most abundant insects in freshwater aquatic habitats. Females of the genus *Chironomus* lay egg masses at the water's edge. Each egg mass contains hundreds of eggs embedded in a thick, gelatinous matrix. Chironomid egg masses harbour *Vibrio cholerae* and provide a natural reservoir for this bacterium (Broza & Halpern, 2001; Halpern *et al.*, 2004).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AIMA4^T is EU346912.

An electron micrograph of negatively stained cells of strain AIMA4^T, polar lipid profiles of *Brachymonas denitrificans* DSM 15123^T and strain AIMA4^T, and a table detailing the fatty acid contents of *Brachymonas denitrificans* DSM 15123^T and strain AIMA4^T are available as supplementary material with the online version of this paper.

All isolates of *V. cholerae* have the ability to degrade chironomid egg masses and prevent the eggs from hatching (Halpern *et al.*, 2003, 2006). Strain AIMA4^T, an aerobic, non-motile Gram-negative short rod, was isolated during a study into the diversity of the culturable bacteria present in chironomid egg masses. Strain AIMA4^T did not possess the ability to degrade chironomid egg masses.

Chironomid egg masses were sampled as previously described (Halpern *et al.*, 2007). To determine the diversity of culturable bacteria, the egg masses were thoroughly washed with sterile saline water. Homogenates from the masses were then diluted and cultured directly on m-Aeromonas medium (Himedia), a selective agar that enhances the growth of members of the genus *Aeromonas*. Strain AIMA4^T was isolated in July 2006 from an egg mass that was sampled from a waste stabilization pond in northern Israel, cultured on m-Aeromonas agar and incubated at 30 °C for 48 h in the dark. The 16S rRNA gene sequence of strain AIMA4^T was analysed to determine its phylogenetic position. The universal bacterial primers 8f and 1512r (Felske *et al.*, 1997) were used to amplify internal fragments of the 16S rRNA gene. Purified PCR products were sequenced with 8f, 534r, 968f and 1512r primers as described in detail by Raats & Halpern (2007).

This resulted in a sequence of approximately 1500 bp. The identification of phylogenetic neighbours was initially carried out by the BLAST (Altschul *et al.*, 1997) and FASTA (Pearson & Lipman, 1988) programs against the database of type strains with validly published prokaryotic names (Chun *et al.*, 2007). The 50 sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using a global alignment algorithm, which was implemented using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Sequence alignment was performed using the CLUSTAL W program and a phylogenetic tree (Fig. 1) was generated using the neighbour-joining method with MEGA version 4 software (Tamura *et al.*, 2007). The 1500 bp sequence of strain AIMA4^T shared highest sequence similarity of 96.9 % with that of *B. denitrificans* JCM 9216^T and <95.7 % with the type strains of other genera (Fig. 1).

For electron microscopy, bacteria in Luria–Bertani (LB) agar medium were suspended in saline. The samples were fixed to a carbon-coated grid and stained with 2 % uranyl acetate and photographed under a JEM-1200EX electron microscope (JEOL). Electron microscopy showed that the cells were coccobacilli or rods measuring 0.75–1.2 µm in width and 1.0–3.0 µm in length (see Supplementary Fig. S1 in IJSEM Online).

For phenotypic characterization, LB agar was used as the basal growth medium. Salt tolerance was determined at 30 °C on LB agar containing 0–5 % (w/v) NaCl. Growth at 10, 14, 18, 25, 30, 33, 37, 40 and 45 °C was measured using LB agar. The pH range for growth was determined using LB broth adjusted to pH 4.0–8.0 at 1.0 unit intervals. The pH was adjusted prior to sterilization by the addition of HCl or NaOH and measured again after sterilization. For alkaline medium, LB broth was adjusted to pH 9.0, 9.5 and 10.0 with 0.1 M Na₂CO₃ or 0.1 M NaHCO₃ and then filtered through a 0.2 µm pore size

filter (Corning) to obtain a sterilized medium. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on LB agar supplemented with 0.5 % (w/v) glucose or 0.1 % (w/v) potassium nitrate. Biochemical tests were performed by using API 20E, API 20NE, API 50CH and API ZYM identification systems (bioMérieux), according to the manufacturer's instructions, except that the incubation temperature was 30 °C. Catalase activity was evidenced by bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined using 1 % *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma-Aldrich, T3134). Growth was tested on MacConkey agar (Difco) and cetrimide agar (Himedia). The phenotypic traits of the novel isolate are given in the species description and in Table 1.

For cellular fatty acid analysis, strain AIMA4^T and *B. denitrificans* DSM 15123^T were cultured on tryptic soy agar (Difco) for 24 h at 28 °C. Fatty acids were then extracted, methylated and analysed by GC using the Sherlock Microbial Identification system (MIDI) as described by Kämpfer & Kroppenstedt (1996). The identity of the major components was confirmed by GC/MS by using a Singlequad 320 instrument (Varian). Strain AIMA4^T and *B. denitrificans* DSM 15123^T had a similar fatty acid profile with respect to the occurrence of C_{16:1}ω7c, C_{16:0} and C_{18:1}ω7c as the major fatty acids. Strain AIMA4^T had a higher content of C_{14:0} and a lower content of C_{12:0} (see Supplementary Table S1 in IJSEM Online).

For polar lipid and isoprenoid quinone analyses, strains AIMA4^T and *B. denitrificans* DSM 15123^T were cultivated on complex PB medium (Hiraishi & Komagata, 1989) at 28 °C for 24 h (strain DSM 15123^T) or 48 h (strain AIMA4^T) until the necessary amount of biomass was obtained. The polar lipids of strains AIMA4^T and *B. denitrificans* DSM 15123^T were extracted according to

Fig. 1. Neighbour-joining dendrogram of 16S rRNA gene sequences obtained from *Brachymonas chironomi* sp. nov. AIMA4^T and related species and genera. Bootstrap values (>50%) resulting from 1000 replicates are indicated at branching nodes. *Propionivibrio dicarboxylicus* was used as an outgroup. Asterisks indicate the branches of the tree that were also formed by using the maximum-parsimony method. Bar, 0.01 substitutions per nucleotide position.

Table 1. Characteristics that differentiate strain AIMA4^T and *B. denitrificans* DSM 15123^TStrains: 1, AIMA4^T; 2, *B. denitrificans* DSM 15123^T. +, Positive; −, negative; w, weak.

Characteristic	1	2
Cell size (μm)	0.75–1.2 × 1.0–3.0	0.6–1.0 × 1.2–2.0
DNA G + C content (mol%)	60	63–65
Pigment	Beige that turns to light brown (after few days of incubation)	Cream or pale yellow
Quinones	Q-8, Q-7, Q-9	Q-8
Rhodoquinones	None	RQ-8
Anaerobic growth	−	+
NaCl range for growth (% w/v)	0–2.5	0–3
Growth temperature (°C)		
Range	18–37	10–40
Optimum	30	30–35
MacConkey agar	w (after 96 h)	−
Tryptophan deaminase	−	+
Acetoin production	+	−
Nitrate reduction to nitrite	+	−
Nitrate reduction to N ₂	−	+
Assimilation of:		
N-Acetyl-glucosamine	−	+
Adipate	−	+
Arabinose	−	+
Citrate	−	+
Gluconate	−	+
Glucose	−	+
Malate	−	+
Maltose	−	+
Mannitol	−	+
Mannose	−	+

Minnikin *et al.* (1979) and analysed by two-dimensional TLC (Collins & Jones, 1980). Strains AIMA4^T and *B. denitrificans* DSM 15123^T contained phosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine. *B. denitrificans* DSM 15123^T also contained diphosphatidylglycerol, two unknown phospholipids and an unknown aminophospholipid (see Supplementary Fig. S2 in IJSEM Online). Isoprenoid quinone analysis of strain AIMA4^T was performed by HPLC as described by Groth *et al.* (1996). Strain AIMA4^T contained the ubiquinones Q-8, Q-7 and Q-9 in an approximate molar ratio of 96:2:1 (Table 1). In addition to the major component ubiquinone Q-8, *B. denitrificans* DSM 15123^T also contained rhodoquinone RQ-8. Rhodoquinone RQ-8 gave rise to a purple band migrating around 75 % slower than the yellow band of Q-8 on silica gel TLC plates when benzene was used as the developing solvent (Hiraishi & Hoshino, 1984). The mass spectrum of RQ-8 recorded after elution from the TLC plate by using a Singlequad 320 MS equipped with a direct insertion probe device (Varian) showed the molecular ion at *m/z* 711 and fragment ions at *m/z* 235 and 220. Since additional peaks at *m/z* 698, 205 and 167 (instead of the fragment ion at *m/z* 182 as reported by Hiraishi & Hoshino, 1984) were recorded, it appears likely that demethylated RQ-8 was also present. The quinone extract

of strain AIMA4^T did not contain any detectable traces of rhodoquinones.

DNA was isolated after disruption of cells by using a French pressure cell and purified by hydroxyapatite chromatography (Cashion *et al.*, 1977). DNA was degraded to nucleosides by P1 nuclease and bovine intestinal mucosa alkaline phosphatase (Roche Diagnostics) as described by Mesbah *et al.* (1989). The nucleosides were separated by reversed-phase HPLC by using the method described by Tamaoka & Komagata (1984). Lambda phage DNA and three bacterial DNAs (*Bacillus subtilis* DSM 402, *Xanthomonas campestris* pv. *campestris* DSM 3586^T and *Streptomyces violaceoruber* DSM 40783) with published genome sequences were used as standards. The G + C content of the DNA of strain AIMA4^T (60 mol%) was calculated from the ratio of deoxyguanosine to thymidine.

The novel isolate, being aerobic, chemo-organotrophic, non-motile, coccobacilli or rods, catalase- and oxidase-positive, giving no acid production from sugars including glucose, containing C_{16:0} as the dominant fatty acid, showing an absence of lysine and ornithine decarboxylases, being negative for indole production and for urea and gelatin hydrolysis, shared the main characteristics of *B. denitrificans*. However, it differed from *B. denitrificans* by

means of several phenotypic traits, as listed in Table 1, and especially by its inability to grow under anaerobic conditions, its ability to reduce nitrate only to nitrite (and not to nitrogen) and by the absence of rhodoquinones.

On the basis of the phenotypic characterization and a 16S rRNA gene sequence similarity of <97% to its closest phylogenetic neighbour *B. denitrificans* (Stackebrandt & Goebel, 1994), we propose that the description of the genus *Brachymonas* should be emended and strain AIMA4^T should be classified as the type strain of a novel species of the genus *Brachymonas* species, for which the name *Brachymonas chironomi* sp. nov. is proposed.

Emended description of genus *Brachymonas* Hiraishi *et al.* 1995

Brachymonas (Bra.chy.mo'nas. Gr. adj. *brachy* short; Gr. n. *monas* a unit, monad; M.L. fem. n. *Brachymonas* a small short unit).

The description is as given by Hiraishi *et al.* (1995a) with the following amendments. Nitrate is reduced to nitrite or nitrogen. Neutrophilic and non-halophilic. The major cellular fatty acids are C_{16:1}ω7c, C_{16:0} and C_{18:1}ω7c and phosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine occur as polar lipids. The major quinone is ubiquinone Q-8. Rhodoquinone RQ-8 may occur in some species. The DNA G+C values are in the range of 60 to 65 mol%. The type species is *Brachymonas denitrificans*.

Description of *Brachymonas chironomi* sp. nov.

Brachymonas chironomi [chi.ro'no.mi. N.L. gen. n. *chironomi* of *Chironomus*, named after the non-biting midge insect from the genus *Chironomus* (*Chironomidae*: *Diptera*) from which the type strain was isolated].

Cells are Gram-negative, catalase and oxidase-positive, non-motile coccobacilli or rods measuring 0.75–1.2 µm in width and 1.0–3.0 µm in length. Cells occur singly or in pairs, sometimes in chains. After 48 h incubation on LB agar at 30 °C, beige coloured colonies are circular, smooth, convex and opaque with entire margins. After a few days incubation, the colony colour changes to brown–beige. Good growth occurs under aerobic conditions. Growth is not observed under anaerobic conditions even after 8 days incubation. Growth is observed after 48 h incubation at 18–37 °C (optimum 30 °C), with 0–2.5% (w/v) NaCl (optimum 0.5% NaCl) and at pH 5.0–9.0 (optimum pH 6.0–8.0). Growth is observed on Zobell marine (Himedia) and MacConkey (weak growth) agars, but not on cetrimide agar. Catalase and oxidase activities are present. The following results are obtained from API 20NE after 48 h of incubation at 30 °C: nitrate is reduced to nitrite; phenylacetic acid is assimilated. The following results are obtained from API 20E strips after 48 h of incubation at 30 °C: nitrate is reduced to nitrite; acetoin is

produced; H₂S and indole are not produced; urea and gelatin are not hydrolysed; citrate is not utilized; and β-galactosidase, arginine dihydrolase, lysine- and ornithine decarboxylases and tryptophan deaminase activities are absent. In API 50CH strips incubated for 48 h at 30 °C, acid is not produced from any of the carbohydrate substrates. In API ZYM strips incubated for 4.5 h at 30 °C, weakly positive activities are observed for the following enzymes: alkaline and acid phosphatases, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase. The dominant cellular fatty acids of the type strain are C_{16:1}ω7c, C_{16:0} and C_{18:1}ω7c. The main isoprenoid quinone is Q-8. Phosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine occur as polar lipids.

The type strain, AIMA4^T (=LMG 24400^T=DSM 19884^T), was isolated from a chironomid egg mass in Israel. The DNA G+C content of the type strain is 60 mol% (HPLC).

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