Pseudoduganella danionis sp. nov., isolated from zebrafish (*Danio rerio*)

Peter Kämpfer,[1] Rute Irgang,[2,3] Hans-Jürgen Busse,[4] Mathías Poblete-Morales,[2,3] Tanita Kleinhagauer,[4] Stefanie P. Glaeser[1] and Ruben Avendaño-Herrera[2,3]

Correspondence
Peter Kämpfer
peter.kaempfer@umwelt.uni-giessen.de

[1]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, Giessen D-35392, Germany

[2]Laboratorio de Patología de Organismos Acuáticos y Biotecnología Acuícola, Facultad de Ciencias Biológicas, Universidad Andrés Bello, Viña del Mar, Chile

[3]Interdisciplinary Center for Aquaculture Research (INCAR), Concepción, Chile

[4]Institut für Mikrobiologie, Veterinärmedizinische Universität Wien, Wien A-1210, Austria

One beige-pigmented, Gram-staining-negative, rod-shaped bacterium, strain E3/2^T, was isolated from a zebrafish, *Daniorerio.* Phylogenetic analysis based on nearly full-length 16S rRNA gene sequences showed that the isolate shared 97.7 % 16S rRNA gene sequence similarity to the species *Pseudoduganella violaceinigra* and between 97.4 to 97.0 % to some species of the genera *Duganella* and *Massilia*, including *Duganella radicis*, *Duganella phyllosphaerae*, *Massilia dura*, *Massilia lutea*, *Duganella sacchari*, *Duganella zoogloeoides*, *Massilia albidiflava* and *Massilia umbonata.* Sequence similarities to all other species were below 97 %. The main cellular fatty acids of the strain were summed feature 3 fatty acids ($C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH), $C_{10:0}$ 3-OH, $C_{16:0}$ and $C_{12:0}$. The polyamine pattern of strain E3/2^T contained predominantly putrescine and 2-hydroxyputrescine. The major quinone was ubiquinone Q-8. Major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Based on phylogenetic, chemotaxonomic, genomic and phenotypic analyses we propose a novel species of the genus *Pseudoduganella* named *Pseudoduganella danionis* sp. nov., with strain E3/2^T (=LMG 29678^T=CCM 8698^T) as the type strain.

Duganella violaceinigra (Li *et al.*, 2004) has been reclassified into a new genus and renamed *Pseudoduganella violaceinigra* (Kämpfer *et al.*, 2012). Members of the genus *Pseudoduganella* are short, Gram-negative-staining rods that are obligately aerobic, mesophilic, chemoorganotrophic and non-spore-forming. At the time of writing, the genus *Pseudoduganella* comprised only one species with a validly published name (http://www.bacterio.net/pseudoduganella.html).

In this study a beige-pigmented bacterial strain isolated from zebrafish, *Danio rerio*, maintained in E3 medium as described by Haffter *et al.* (1996), is described as a representative of a second species of this genus. Isolation of pure colonies was obtained from plates containing tryptone yeast extract salts medium (0.4 % tryptone, 0.05 % yeast extract, 0.02 % anhydrous calcium chloride, 0.05 % magnesium sulphate heptahydrate, pH 7.2) incubated at 18 °C for 72 h.

The original colonies had a diameter of approximately 2.5 mm, with smooth edges and a beige pigmentation. The strain was subcultivated on R2A agar at 25 to 28 °C and can be maintained for several weeks at 6–8 °C. For long-term storage, the strain was cultured in R2A broth (R2A broth, Lab M) at room temperature for 5 to 7 days, mixed after growth with a final concentration of 20 % (v/v) autoclaved glycerol, and stored at −80 °C after the cultures were shock-frozen in liquid nitrogen.

The nearly full-length 16S rRNA gene was PCR-amplified from a cell lysate of strain E3/2^T with universal 16S rRNA gene targeting primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′; Lane, 1991) and sequenced by the Sanger method with primers 27F (5′-GAGTTTGATCMTGGCTCAG-3′) and E786F (5′-GATTAGATACCCTGGTAG-3′) at LGC Genomics (Berlin, Germany). The manually corrected 16S rRNA gene sequence had a length of 1448 nucleotides spanning gene termini 46 to 1505 [numbered according to the *Escherichia coli rrnB* reference sequence published by

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain E3/2^T is KX427103.

Brosius *et al.* (1978)]. The type strain of *Pseudoduganella violaceinigra* was obtained as the closest relative of strain E3/2^T with 97.8 % 16S rRNA gene sequence similarity by a first BLAST search in the EzTaxon 16S rRNA gene sequence type strain database (Kim *et al.*, 2012). The phylogenetic placement of strain E3/2^T was investigated in detail by the reconstruction of phylogenetic trees including all type strains of members of the family *Oxalobacteraceae* using the 'All species living tree project' database (LTPs; Yarza *et al.*, 2008) release LTPs123 (September 2015) and the ARB software package release 5.2 (Ludwig *et al.*, 2004). Sequences not included in the LTP database were added after alignment with the SILVA Incremental Aligner (SINA, v1.2.11; Pruesse *et al.*, 2012). After addition to the database, the alignment of the 16S rRNA gene sequences was checked and corrected manually. Phylogenetic trees were calculated using three different treeing methods: the maximum-likelihood method using RAxML version 7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis, the maximum-parsimony method using DNAPARS v3.6 (Felsenstein, 2005), and the neighbour-joining methods using ARB Neighbour-joining and the Jukes–Cantor correction (Jukes & Cantor, 1969). Pairwise sequence similarities were calculated with the ARB Neighbour-joining tool, without considering evolutionary models. All phylogenetic trees based on 100 resamplings (bootstrap analysis; Felsenstein, 1985) and sequence positions between gene termini 75 and 1443 [according to Brosius *et al.* (1978)] were considered. Gene sequences of *Noviherbaspirillum psychrotolerans* and *Janthinobacterium svalbordensis* were shorter and therefore added after tree reconstruction using the parsimony quick add marked tool of ARB without changing the overall tree topology.

Pairwise 16S rRNA gene sequence similarity of strain E3/2^T was highest to the type strain of *P. violaceinigra* (97.7 %), followed by the type strains of some species of the genera *Duganella* and *Massilia* with 97.4 to 97 %, including *Duganella radicis*, *Duganella phyllosphaerae*, *Massilia dura*, *Massilia lutea*, *Duganella sacchari*, *Duganella zoogloeoides*, *Massilia albidiflava* and *Massilia umbonata*. Sequence similarities to all other species were below 97 %. The phylogenetic tree based on the neighbour-joining method showed the clustering of strain E3/2^T with the type strain of *P. violaceinigra* (Fig. 1). The two strains were also closely related to each other in phylogenetic trees calculated based on other treeing methods, but did not form a separate cluster.

For detailed phenotypic characterization, the strain was cultured on R2A agar at 25 °C for 3 days. Gram-staining was performed by the modified Hucker method according to Gerhardt *et al.* (1994). Cell morphology was analysed by light microscopy at ×1000 magnification with a Leica DFC 3000G microscope using glass slides covered with three times washed (each time for 20 min in pure water) and autoclaved 2 % (w/v) agar (Becton Dickinson). Cytochrome oxidase activity was tested with Microbiology Bactident oxidase test strips (Merck), and catalase activity was tested by gas bubble formation after dropping 3 % H_2O_2 (v/v) onto a fresh culture grown on R2A agar.

Growth was tested in or on R2A (Oxoid), nutrient broth (Oxoid), K7 [0.1 % (w/v yeast extract, peptone and glucose, 15 g l^{-1} agar, pH 6.8], medium 65 (according to DSMZ) and PYE [0.3 % (w/v) yeast extract and 0.3 % (w/v) casein peptone, 15 g agar l^{-1}, pH 7.2], Luria Bertani (LB; Sigma-Aldrich), tryptic soy agar (TSA; Becton Dickinson), CASO agar (Carl Roth), malt agar (Merck), DEV agar (DEV; Merck), nutrient agar (NA; Becton Dickinson), marine agar 2216 (MA; Becton Dickinson), Columbia agar with sheep blood (Oxoid), glycine arginine agar, and MacConkey agar (Oxoid). Temperature-dependent growth was tested on R2A agar at 4, 10, 15, 20, 25, 28, 30, 33, 37, 45, 50 and 55 °C; salinity-dependent growth was tested at 25 °C in R2A bouillon supplemented with 0 to 11.0 % (w/v) NaCl (in 1.0 % intervals); and pH-dependent growth was tested using K7 bouillon adjusted to pH 4–9 (in 0.5 pH units intervals). The pH values were adjusted using 1 M HCl and 1 M KOH and stabilized by the addition of 5 mM phosphate buffer adjusted to the same pH. Further physiological tests were performed with API 20NE, API ZYM and API 50CH test strips (bioMérieux). For the inoculation of the API 20NE and API ZYM strips, bacterial biomass was suspended in 0.2 % (w/v) autoclaved NaCl to a McFarland standard value of 0.5 and 6.0, respectively. API 50 CH was used to test acid production, therefore a loop of fresh biomass was suspended in pure water to McFarland 4 and added to a tube of API 50 CHB/E medium for inoculation of the test panels. All panels were analysed after incubation for 3 days at 25 °C, except nitrate reduction and indole production (API 20 NE) which were analysed after 48 h. Further comparative phenotypic characterization of the strain was carried out using the 96-well physiological test panel according to Kämpfer *et al.* (1991). For inoculation of the panel, stains were suspended in 0.9 % NaCl to a McFarland value of 0.5. Analysis of acid production, carbon substrate assimilation, and enzyme activity was performed after growth for 3 days at 25 °C.

Strain E3/2^T showed a rod-shaped morphology with a cell size of 1.0 (±0.1)×2.1 (±0.6) μm. The strain grew well at 10 to 37 °C on or in R2A, NB, K7, medium 65, NA and PYE, but no growth was observed on DEV, Columbia agar with sheep blood, glycine arginine and MacConkey agar.

Good growth was obtained after three days of incubation between 4 and 37 °C (no growth at 40 °C or above), in the presence of 0–1.0 % (w/v) NaCl, and at pH 5.5 to 9.0 (no growth below pH 5.5 and above pH 9.0). Results of the test panel according to Kämpfer *et al.* (1991) showed almost no growth with the different carbon sources but acid production from several sugars and sugar-related compounds. Physiological tests performed with the API test systems confirmed these results. The differentiating physiological characteristics of strain E3/2^T compared to *Pseudoduganella violaceinigra* KCTC 12193^T are listed in Table 1. All physiological properties of the novel strain are provided in the species description.

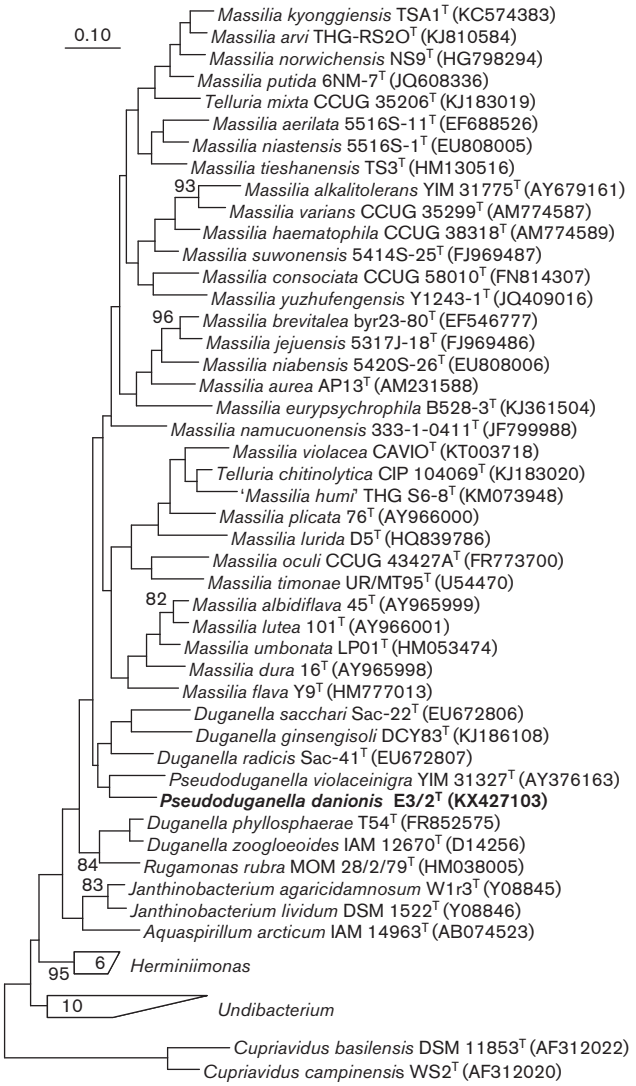


Fig. 1. Phylogenetic tree based on nearly full-length 16S rRNA gene sequences showing the placement of strain E3/2^T within the *Oxalobacteraceae.* The phylogenetic tree was generated in ARB with the neighbour-joining method and based on sequence termini 75 and 1443 [numbering according to Brosius *et al.* (1978)] and 100 replications (bootstrap support). Bootstrap values >70 % are depicted at nodes. Numbers in clusters represent number of species included in the cluster. The trees were calculated using all members of the *Oxalobacteraceae.* Less closely related genera were removed without changing the overall tree topology. The type strains of *Cupriavidus basilensis* and *Cupriavidus campinensis* were used as the outgroup. Bar, 0.1 substitutions per nucleotide positions.

Biomass for fatty acid analysis was harvested after growth on R2A at 28 °C for 48 h. The analysis was performed as described by Kämpfer & Kroppenstedt (1996). Fatty acids were separated with a 5898A gas chromatograph (Hewlett Packard), respective peaks were automatically integrated and fatty acid names and percentages were determined with the Sherlock MIDI software v.2.1 (TSBA v.4.1). The fatty acid profile of the strain was consistent with the profile described for the sole species of genus *Pseudoduganella* as shown in Table 2, with the predominant unsaturated fatty acids summed feature 3 ($C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH), $C_{10:0}$ 3-OH, $C_{16:0}$ and $C_{12:0}$ (Table 2).

Table 1. Physiological characteristics that allow differentiation strain E3/2^T and the type strain of the sole species of the genus *Pseudoduganella*

Strains: 1, E3/2^T (data from this study); 2, *Pseudoduganella violaceinigra* KCTC 12193^T (Kämpfer *et al.*, 2012). All data were obtained with exactly the same methods. Both strains were rod-shaped and negative for yellow pigmentation, urease activity, utilization of D-mannitol, and acid production from maltose, D-mannose, D-glucose, lactose and L-arabinose.

Characteristic	1	2
Optimum temperature (°C)	20–30 °C	28–30
Optimum pH	6.0	7.2
Catalase	–	+
Aesculin hydrolysis	+	–
Carbon sources utilized:		
L-Arabinose	–	+
Sucrose	–	+

Biomass of strain E3/2^T subjected to analyses of polyamines, quinones and polar lipids was cultivated in PYE broth [0.3 % (w/v) peptone from casein, 0.3 % (w/v) yeast extract, pH 7.2] and harvested at the late exponential growth phase (for polyamine analysis) or at the stationary growth phase (for quinones and polar lipids analyses). Polyamines were extracted as reported by Busse & Auling (1988) and analysed by HPLC, applying the conditions reported by Busse *et al.* (1997). Analysis of quinones and polar lipids were carried out applying the integrated procedure reported by Tindall (1990a, b) and Altenburger *et al.* (1996). For HPLC analyses, the apparatus was used described by Stolz *et al.* (2007).

The polyamine pattern of strain E3/2^T contained putrescine [43.8 μmol (g dry weight)$^{-1}$], 2-hydroxyputrescine [7.2 μmol (g dry weight)$^{-1}$], spermidine [0.4 μmol (g dry weight)$^{-1}$] and traces [<0.1 μmol (g dry weight)$^{-1}$] of 1,3-diaminopropane, cadaverine and spermine. The quinone system was composed of ubiquinones Q-8 (89 %) and Q-7 (11 %). The polar lipid profile was composed of the major compounds phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and minor amounts of an unidentified glycolipid (Fig. 2).

Based on genotypic, chemotaxonomic and physiological data, we propose a novel species of the genus *Pseudoduganella* named *Pseudoduganella danionis* sp. nov. with strain E3/2^T as the type strain.

Table 2. Fatty acid profiles of species of the genus *Pseudoduganella*

Strains: 1, *Pseudoduganella danionis* sp. nov. E3/2^T; 2, *P. violaceinigra* YIM 31327^T. Data for species 2 from Kämpfer *et al.* (2012) obtained with exactly the same methods.

Fatty acid	1	2
$C_{10:0}$	–	1.7
$C_{12:0}$	8.3	14.3
$C_{14:0}$	–	1.3
$C_{16:0}$	27.4	28.6
$C_{18:0}$	–	–
$C_{10:0}$ 3-OH	6.7	9.4
$C_{18:1}\omega7c$	4.6	2.7
Summed feature 3*	53.1	33.4

*Summed features represent groups of two or more fatty acids that could not be separated using the MIDI system. Summed feature 3 contained $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH.

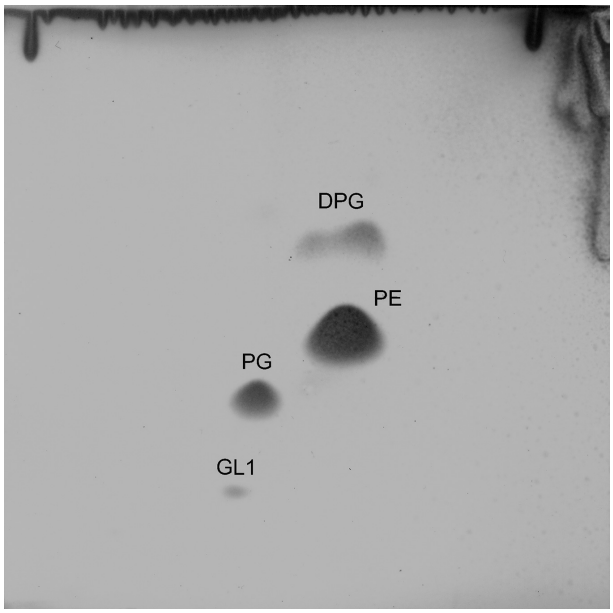


Fig. 2. Polar lipid profile of strain E3/2^T after two-dimensional thin-layer chromatography and detection with molybdatophosphoric acid. PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; GL1, unidentified glycolipid.

Description of *Pseudoduganella danionis* sp. nov.

Pseudoduganella danionis [da.ni.o′nis. N.L. gen. n. *danionis* of *Danio rerio* (zebrafish), from which the type strain was isolated].

Cells are Gram-staining-negative, rod-shaped and approximately 1.0 (±0.1)×1.9 (±0.3) μm. Colonies grown for 3 days on R2A at 25 °C are circular, shiny and beige-pigmented with a smooth border. Motile and aerobic. Negative for catalase and cytrochrome oxidase production. Good growth at 25 °C after incubation for 3 days on R2A, NA and PYE agar; no growth on Malt, LB, CASO, glycine arginine, DEV, marine agar and MacConkey agar. Optimal growth is observed between 20 and 30 °C, slightly less growth at 15 °C and 37 °C, strongly reduced growth at 10 and 4 °C, and no growth at 40 °C and above. Good growth occurs without NaCl, at pH 5.5 to 8.5, slightly at pH 7.0 and 7.5, but not at pH 5.0 or below or above pH 9.0. Negative for indole production, arginine dihydrolase, β-galactosidase, and acid production from L-arabinose, potassium gluconate, maltose, D-mannose, D-glucose, lactose, and malic acid. Negative for the assimilation of *N*-acetylglucosamine (API 20 NE), capric acid, adipic acid, trisodium citrate, D-mannitol, L-arabinose, sucrose and phenylacetic acid, negative for urease activity, but positive for aesculin hydrolysis and reduction of nitrate to nitrite. In API ZYM tests, positive for alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase, valine and cysteine arylamidase, acid phosphatase, naphthol-AS-BI-phosphate, α- and β-glucosidase. The fatty acids profile contains summed feature 3 fatty acids ($C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH), $C_{10:0}$ 3-OH, $C_{16:0}$ and $C_{12:0}$ ($C_{16:1}\omega7c$/ $C_{16:1}\omega8c$), $C_{16:0}$ and $C_{12:0}$3-OH. The polyamine pattern contains the major compounds putrescine and 2-hydroxyputrescine. Ubiquinone Q-8 is the major quinone. The polar lipid profile is composed of the predominant components phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, and minor amounts of unidentified glycolipid GL1.

The type strain E3/2^T (= LMG 29678^T = CCM 8698^T) was isolated from a Zebrafish maintained in E3 medium.

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