Siphonobacter aquaeclarae gen. nov., sp. nov., a novel member of the family 'Flexibacteraceae', phylum Bacteroidetes

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A Gram-negative bacterium, designated P2^T, was isolated from the biofilm developed on the inner surface of an ultrapure cooling water system in a Hungarian power plant and was characterized by a polyphasic approach. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain P2^T was affiliated with the family 'Flexibacteraceae' in the phylum Bacteroidetes. Its closest relative was *Flectobacillus lacus* CL-GP79^T (88.7 % 16S rRNA gene sequence similarity) followed by *Arcicella rosea* TW5^T (86.5 %), *Arcicella aquatica* NO-502^T (86.4 %), *Flectobacillus roseus* GFA-11^T (86.3 %) and *Flectobacillus major* DSM 103^T (85.4 %). Cells of strain P2^T were facultatively anaerobic, non-motile rods. The major fatty acids were C_{16:1}ω5c (42.5 %), iso-C_{15:0} 2-OH (17.2 %), iso-C_{17:0} 3-OH (16.1 %) and iso-C_{15:0} (8.5 %). The major menaquinone was MK-7 and the predominant polar lipid was phosphatidylethanolamine. The DNA G + C content was 54.5 mol%. Thus, the phenotypic and genotypic analyses clearly showed that strain P2^T is considerably different from members of other genera in the family 'Flexibacteraceae'. Based on these results, it is concluded that strain P2^T represents a novel species in a new genus, for which the name *Siphonobacter aquaeclarae* gen. nov., sp. nov. is proposed, with type strain P2^T (=DSM 21668^T =NCAIM B 02328^T).

Bacteria that are affiliated with the phylum Bacteroidetes are widely distributed in nature, especially in aquatic ecosystems (Kirchman, 2002; Zwart *et al.*, 2002), and they play a significant role in biogeochemical cycling and in the degradation of complex biopolymers (Cottrell & Kirchman, 2000). Some of them take part in freshwater biofilm formation, like species of the genera *Adhaeribacter* (Rickard *et al.*, 2005) and *Arcicella* (Furuhata *et al.*, 2008). Biofilm formation in industrial water systems (e.g. in cooling water systems) is an undesirable process because it can result in microbially induced corrosion of pipe surfaces or obturation of the pipes (Branda *et al.*, 2005). Therefore, the investigation of formation and microbial composition of freshwater biofilms has been in focus and novel species have been isolated from a highly eutrophic pond (*Flectobacillus lacus*; Hwang & Cho, 2006), freshwater (*Flectobacillus major* and *F. roseus*; Gromov, 1963; Sheu *et al.*, 2009), a freshwater neuston film (*Arcicella aquatica*; Nikitin *et al.*, 2004) and tap water (*Arcicella rosea* TW5^T; Kämpfer *et al.*, 2009). A novel bacterium, strain P2^T, was isolated from a biofilm sample

obtained from the inner surface of an ultrapure cooling water pipeline. Based on the 16S rRNA gene sequence, the isolate was affiliated with the family 'Flexibacteraceae' but showed a considerably low level of sequence similarity with strains of previously described species of this family.

A biofilm sample was taken from the inner surface of a cooling water system in a Hungarian power plant. The sampled pipeline system contained ultrapure, desalinated water, of which the temperature varied between 23 and 25 °C. The sample was serially diluted in physiological saline (0.9 %, w/v, NaCl), plated on R2A agar (medium 830, DSMZ) and incubated at 28 °C for 1 week under aerobic conditions. Colonies were selected randomly and purified twice on R2A agar at 28 °C. Growth was tested on R2A agar, nutrient agar (medium 1, DSMZ), tryptic soy agar (TSA; Difco) and on MacConkey agar (Difco).

Colony morphology of strain P2^T was tested on R2A agar by direct observation of single colonies. Cell morphology and motility were studied by native preparation and by Gram-staining according to Claus (1992). Growth in the absence of oxygen was tested using R2A agar slants incubated in an anaerobic chamber (Forma Scientific). Oxidase activity was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P2^T is FJ177421.

studied by the method of Tarrand & Gröschel (1982). Catalase production and Voges–Proskauer reaction were demonstrated by the methods of Cowan & Steel (1974). Acid production from D-glucose was checked by the OF test according to Hugh & Leifson (1953). Growth at 4–60 °C was determined using R2A agar. Tolerance to 0, 2.5, 5 and 10 %, w/v, NaCl and pH 3–11 (in increments of one pH unit) were determined using R2A broth. Urease activity, reduction of NO₃[−], starch hydrolysis, caseinase, gelatinase, phosphatase activity and hydrolysis of Tween 80 were studied according to Smibert & Krieg (1994). Acid production from different carbon sources and enzymic activities of strain P2^T were investigated with API 50 CH and API ZYM kits (bioMérieux) at 28 °C, according to the manufacturer's instructions. The API ZYM tests were read after 4 h and the API 50 CH tests were read after 24–48 h.

For isoprenoid quinone analysis, cells were cultivated in liquid Rich medium (Yamada & Komagata, 1972). Quinones were extracted according to the method of Collins *et al.* (1977) and the profile was analysed by HPLC (HP

9001; Groth *et al.*, 1997). For polar lipid and fatty acid analysis, cells were cultivated in R2A broth and TSA, respectively, for 24 h. Cellular fatty acids were extracted by the method of Stead *et al.* (1992) and analysed by GC (Groth *et al.*, 1996). Summed features were analysed later by mass spectrometry. Polar lipids were determined according to the method described by Minnikin *et al.* (1979).

The DNA base composition was determined from bacterial cells disrupted by using a French press. After purification of the DNA on hydroxyapatite according to the procedure of Cashion *et al.* (1977), DNA was degraded to nucleosides using P1 nuclease and bovine intestinal mucosa alkaline phosphatase, as described by Mesbah *et al.* (1989). The nucleosides were separated by reversed-phase HPLC, as described by Tamaoka & Komagata (1984). The G+C content of the DNA was calculated from the ratio of deoxyguanosine to thymidine.

To perform PCR amplification of the 16S rRNA gene, genomic DNA was isolated using the G-spin Genomic DNA

Table 1. Phenotypic characteristics differentiating strain P2^T from related taxa

Strains: 1, *Siphonobacter aquaeclarae* sp. nov. P2^T; 2, *Flectobacillus lacus* CL-GP79^T (Hwang & Cho, 2006); 3, *F. major* DSM 103^T (Hwang & Cho, 2006); 4, *F. roseus* GFA-11^T (Sheu *et al.*, 2009); 5, *Arcicella aquatica* NO-502^T (Nikitin *et al.*, 2004); 6, *A. rosea* TW5^T (Kämpfer *et al.*, 2009). +, Positive; v, variable; w, weakly positive; −, negative; ND, no data available.

Characteristic	1	2	3	4	5	6
Cell dimensions (µm)						
Length	1.3–2.7	4.7–10.0	2.0–5.0	1.5–2.5	2.5–3.0	ND
Width	0.3–0.4	0.3–0.6	0.6–1.0	0.3–0.5	0.5–0.75	ND
Temperature for growth (°C)						
Range	4–37	10–35	4–30	10–40	4–40	15–36
Optimum	20–30	25–30	20–30	30	28–30	ND
pH for growth						
Range	5–9	6–9	6–8	7–8	ND	ND
Optimum	7	7	7	7	7	ND
Biochemical tests						
Catalase	+	+	w	+	+	ND
Gelatinase	+	+	+	ND	ND	ND
Oxidase	−	+	+	+	ND	−
Nitrate reductase	−	v	−	−	−	ND
Urease	−	−	v	−	−	ND
Voges–Proskauer test	−	−	−	ND	ND	ND
Glucose fermentation	−	+	+	−	−	ND
Indole production	−	−	+	−	ND	ND
Acid production from:						
L-Arabinose	−	−	+	v	+	−
L-Fucose	+	−	+	v	ND	ND
D-Lyxose	+	−	+	−	ND	ND
D-Mannose	+	−	+	+	ND	−
D-Tagatose	+	−	−	−	ND	ND
D-Arabitol	−	+	−	−	ND	ND
D-Mannitol	−	−	−	−	+	−
Amygdalin	+	−	+	v	ND	ND
Arbutin	+	−	+	+	ND	−
DNA G+C content (mol%)	54.5	38.3	39.5	39.7	34.5	ND

Extraction kit (iNtRON Biotechnology). Amplification was performed using the primers 27F and 1492R (Lane, 1991). PCR products were purified with the PCRquick-spin PCR Purification kit (iNtRON Biotechnology). The almost-complete 16S rRNA gene sequence of the strain was determined by using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), according to the manufacturer's instructions. Sequencing products were separated on a model 3100 Genetic Analyzer (Applied Biosystems). The sequences were aligned with MEGA4 software (Tamura *et al.*, 2007) against sequences from the GenBank database (Altschul *et al.*, 1997). A phylogenetic tree was reconstructed using the neighbour-joining method (Saitou & Nei, 1987) and bootstrap analysis for 1000 replications was also conducted.

Strain P2^T grew well on R2A agar, nutrient agar and TSA but no growth was observed on MacConkey agar. Colonies were initially mucoid and light yellow but became light brown after 1 week of incubation. Cells were Gram-stain-negative, facultatively anaerobic, straight, non-motile rods. Good growth was observed between 4 and 37 °C (optimum 20–30 °C). No growth was detected on R2A agar supplemented with 2.5–10 % (w/v) NaCl. Growth was observed at pH 5–9 (optimum pH 7). With the API ZYM tests, strain P2^T showed high activities of amino acid-hydrolysing enzymes. More detailed characteristics are given in Table 1 and the species description.

Some results of the chemotaxonomic analysis of strain P2^T were similar to those of its closest relatives. The major isoprenoid quinone of strain P2^T was MK-7 and a trace amount of MK-5 was also observed. Investigation of the polar lipid profile showed that the main component was phosphatidylethanolamine. At the same time, the fatty acid profile of strain P2^T, C_{16:1}ω5c (42.5 %), summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 17.2 %), iso-C_{17:0} 3-OH (16.1 %) and iso-C_{15:0} (8.5 %), showed characteristic differences from the closest relatives (Table 2). Although similar fatty acid profiles were observed for strains of species belonging to the genus *Flectobacillus*, in which C_{16:1}ω5c is the major fatty acid, differences in the proportions of individual fatty acids were observed. The complete lack of summed feature 4 (one or more of C_{16:1}ω7c, C_{16:1}ω7t and iso-C_{16:1}ω7c 2-OH), which is a major component in *Arcicella* strains, and the presence of summed feature 3 clearly indicated that strain P2^T is separate from the genus *Arcicella*. In the case of strain P2^T, summed feature 3 was identified as iso-C_{15:0} 2-OH.

The phylogenetic analysis showed that strain P2^T belonged to the family 'Flexibacteraceae' and it was clearly separated from other genera of this family (Fig. 1). The analysis of the almost-complete 16S rRNA gene sequence (1351 bp) revealed that strain P2^T was a member of the phylum *Bacteroidetes* and had highest 16S rRNA gene sequence similarity with *F. lacus* CL-GP79^T (88.7 %), which was isolated from a highly eutrophic pond. Other close relatives were *A. rosea* TW5^T (86.5 %), *A. aquatica* NO-502^T (86.4 %), *F. roseus* GFA-11^T (86.3 %) and *F. major* DSM 103^T (85.4 %).

Table 2. Cellular fatty acid profiles of strain P2^T and related taxa

Strains: 1, *Siphonobacter aquaeclarae* sp. nov. P2^T; 2, *F. lacus* CL-GP79^T (Hwang & Cho, 2006); 3, *F. major* DSM 103^T (Hwang & Cho, 2006); 4, *F. roseus* GFA-11^T (Sheu *et al.*, 2009); 5, *A. aquatica* NO-502^T (Kämpfer *et al.*, 2009); 6, *A. rosea* TW5^T (Kämpfer *et al.*, 2009). Values are percentages of total fatty acids. ECL, Equivalent chain-length; tr, trace amount (<1 %); –, not detected.

Fatty acid	1	2	3	4	5	6
C _{14:0}	tr	1.7	1.0	5.7	1.4	–
C _{15:0}	–	4.3	tr	tr	2.0	4.5
C _{16:0}	1.5	1.3	1.0	6.5	5.6	4.5
C _{15:1} ω6c	–	4.2	tr	tr	–	–
C _{16:1} ω5c	42.5	26.9	36.4	40.2	23.5	14.6
C _{17:1} ω6c	–	1.5	tr	–	–	–
iso-C _{15:0}	8.5	12.1	9.5	15.0	8.5	16.7
anteiso-C _{15:0}	tr	2.4	2.5	tr	1.0	–
C _{15:0} 3-OH	–	3.5	–	–	–	–
iso-C _{15:0} 3-OH	4.1	3.8	3.7	6.9	5.6	6.3
C _{16:0} 2-OH	–	–	tr	1.7	–	–
C _{16:0} 3-OH	2.8	2.9	2.9	7.0	3.5	4.4
iso-C _{16:0} 3-OH	1.5	–	–	–	–	–
iso-C _{17:0} 3-OH	16.1	3.6	4.6	3.9	2.4	2.6
Summed features*						
2	–	1.4	tr	1.3	–	–
3	17.2	19.2	10.8	8.9	–	–
4	–	–	–	–	44.2	38.8
5	–	tr	2.5	–	–	–
ECL 13.565	1.29	tr	tr	–	–	–
ECL 14.959	1.51	5.0	1.6	–	–	–
ECL 15.731	–	–	14.7	–	–	–
ECL 16.582	1.45	tr	tr	–	–	–

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System (MIDI). Summed feature 2 consisted of C_{14:0} 3-OH and/or iso-C_{16:1} I. Summed feature 3 consisted of iso-C_{15:0} 2-OH and/or C_{16:1}ω7c. Summed feature 4 consisted of one or more of C_{16:1}ω7c, C_{16:1}ω7t and iso-C_{16:1}ω7c 2-OH. Summed feature 5 consisted of anteiso-C_{17:1} B and/or iso-C_{17:1} I.

The DNA G + C content of strain P2^T was remarkably higher than its closest relatives (54.5 mol%) (Table 1).

Therefore, on the basis of the low 16S rRNA gene sequence similarity between strain P2^T and recognized bacterial relatives and the results of the chemotaxonomic, biochemical and physiological analyses, strain P2^T is considered to represent a novel species in a new genus, for which the name *Siphonobacter aquaeclarae* gen. nov., sp. nov. is proposed.

Description of *Siphonobacter* gen. nov.

Siphonobacter (Si.pho.no.bac'ter. Gr. n. *siphō* –ônos tube, pipe; N.L. masc. n. *bacter* rod; N.L. masc. n. *Siphonobacter* rod-shaped bacterium from a pipeline, referring to the source of isolation of the type strain of the type species).

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain P2^T among various representative members of the family 'Flexibacteraceae'. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Bar, 0.01 substitutions per site.

Gram-negative, facultatively anaerobic, non-motile rods occurring singly or in pairs. Positive for catalase and negative for oxidase and nitrate reduction. Major whole-cell fatty acids are C_{16:1}ω5c, iso-C_{15:0} 2-OH, iso-C_{17:0} 3-OH and iso-C_{15:0}. The major isoprenoid quinone is menaquinone MK-7. The main polar lipid is phosphatidylethanolamine. On the basis of 16S rRNA gene sequence analysis, the genus is affiliated to the family 'Flexibacteraceae' in the phylum Bacteroidetes. The type species is *Siphonobacter aquaeclarae*.

Description of *Siphonobacter aquaeclarae* sp. nov.

Siphonobacter aquaeclarae (a.qua.e.cla'ra.e. L. n. *aqua* –ae water; L. adj. *clarus* –a –um clear, bright; N.L. gen. n. *aquaeclarae* of/from a clear water).

In addition to properties described for the genus, the species has the following characteristics. Grows well on R2A agar, nutrient and TSA, but not on MacConkey agar. Cells are 1.3–2.7 μm long and 0.3–0.4 μm wide. No gliding motility observed. Grows at 4–37 °C and pH 5–9, but not with 2.5 % NaCl. Methyl red test is positive but Voges–Proskauer test is negative. Hydrolyses gelatin, starch and Tween 80, but not casein or urea. For sole carbon sources, produces acid from cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, D-glucose, lactose, maltose, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, starch, sucrose, D-tagatose, trehalose, turanose, N-acetylglucosamine, amygdalin, arbutin and inulin, but not from D-fucose, D-ribose, L-sorbose, D-adonitol, D-arabitol, dulcitol, erythritol, glycerol, inositol, D-mannitol, D-sorbitol, xylitol or gluconate. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase and α-fucosidase. The genomic DNA G + C content of the type strain is 54.5 mol%.

The type strain, P2^T (=DSM 21668^T =NCAIM B 02328^T), was isolated from biofilm developed on the inner surface of an ultrapure cooling water pipeline system in a Hungarian power plant.

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