Effluviibacter roseus gen. nov., sp. nov., isolated from muddy water, belonging to the family 'Flexibacteraceae'

K. Suresh, S. Mayilraj and T. Chakrabarti

Correspondence

T. Chakrabarti
tapan@imtech.res.in

Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Sector 39A, Chandigarh – 160 036, India

A Gram-negative bacterial isolate (designated SRC-1^T) was isolated from an occasional drainage system and characterized by a polyphasic approach to determine its taxonomic position. Phylogenetic analysis based on 16S rRNA gene sequences affiliated strain SRC-1^T with the family 'Flexibacteraceae' of the phylum *Bacteroidetes*. It showed greatest sequence similarity to *Pontibacter actinarum* KMM 6156^T (95.5%) followed by *Adhaeribacter aquaticus* MBRG1.5^T (89.0%) and *Hymenobacter roseosalivarius* DSM 11622^T (88.9%), but it differed from these micro-organisms in many phenotypic characteristics. Strain SRC-1^T was an obligate aerobe and its cells were non-motile, irregular rods. The major fatty acids included mainly unsaturated and hydroxy fatty acids, including 17 : 1 iso I/anteiso B (36.7%), 15 : 0 iso (15.8%) and 17 : 0 iso 3-OH (10.3%), and the DNA G + C content was 59.5 mol%. From the phenotypic and genotypic analyses it was clear that strain SRC-1^T was quite different from members other genera in the family 'Flexibacteraceae'. Therefore we conclude that strain SRC-1^T represents a novel genus, for which the name *Effluviibacter* gen. nov., containing a single species *Effluviibacter roseus* sp. nov., is proposed. The type species of the genus is *Effluviibacter roseus*, the type strain of which is strain SRC-1^T (=MTCC 7260^T =DSM 17521^T).

The *Cytophaga*–*Flavobacterium*–*Bacteroides* group of bacteria, now classified as the phylum *Bacteroidetes* (Garrity & Holt, 2001), are widely distributed in diverse ecological niches. Although they are dominant in marine environments (Bowman *et al.*, 1997), they are also found in soil, fresh water, in plants and in air (Buczolits *et al.*, 2002). This phylum includes Gram-negative heterotrophic bacteria with the capacity to degrade complex polysaccharides such as agar, cellulose and chitin, and they play an important role in carbon cycling in the environment. This phylum includes three classes, one of which, the 'Sphingobacteria', includes five families: *Sphingobacteriaceae*, 'Saprospiraceae', 'Flexibacteraceae', 'Flammeovirgaceae' and *Crenotrichaceae* (Garrity & Holt, 2001). The family 'Flexibacteraceae' is the largest of these, containing 10 genera with validly published names (Ludwig & Klenk, 2001). A number of genera, including *Pontibacter*, *Adhaeribacter*, *Algoriphagus*, *Belliella*, *Hongiella* and *Reichenbachiella* (Nedashkovskaya *et al.*, 2003, 2005; Rickard *et al.*, 2005; Bowman *et al.*, 2003; Bretter *et al.*, 2004; Yi & Chun, 2004), have been described recently as belonging to this family. In the present study, we have characterized a novel bacterium, strain SRC-1^T, by using a polyphasic approach. On the basis of its morphological,

biochemical, chemotaxonomic and genotypic characteristics, we conclude that the strain is quite distinct from existing genera within the family 'Flexibacteraceae' and merits classification within a novel genus.

Strain SRC-1^T was isolated from muddy water from an occasional drainage system of a residential area in Chandigarh, India. The sample was serially diluted in physiological saline (NaCl at 0.9%, w/v), plated on nutrient agar medium (Hi-media) and incubated at 30 °C. A small mucoid colony was observed upon prolonged incubation and it was purified. The strain was Gram-negative and could grow at temperatures between 4 and 37 °C, the optimum being 30 °C. On nutrient agar at 30 °C, colonies were circular, slightly shiny, mucoid and deep pink in colour. Cells of strain SRC-1^T were observed under a phase-contrast microscope (Axiophot; Zeiss) using an oil-immersion objective (× 100) to ascertain the shape and motility of the cells.

Physiological tests, including analysis of growth at different temperatures, pH values and salt concentrations, were done using nutrient agar medium. For the various biochemical analyses listed in Table 1 and in the species description, the culture was grown at 30 °C in nutrient broth and the tests were performed as described by Lanyi (1987) and Smibert & Krieg (1994). In addition, the ability of strain SRC-1^T to utilize carbon compounds as sole carbon and energy sources

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain SRC-1^T is AM049256.

Table 1. Comparison of biochemical characteristics of strain SRC-1^T with those of closely related bacteria

Strains: 1, strain SRC-1^T; 2, *P. actiniarum* KMM 6156^T (data from Nedashkovskaya *et al.*, 2005); 3, *Adhaeribacter aquaticus* MBRG1.5^T (Rickard *et al.*, 2005); 4, *H. roseosalivarius* DSM 11622^T (Hirsch *et al.*, 1998; Buczolits *et al.*, 2002). NR, Not reported; W, weakly positive. All four strains are negative for nitrate reduction.

Characteristic	1	2	3	4
Colony colour	Pink	Pink	Pink	Rose
Cell shape	Irregular rod	Rod	Rod	Rod
Gliding motility	—	+	—	—
Growth at:				
4 °C	+	—	+	+
42 °C	—	+	—	NR
Growth on 8.0 % NaCl	+	+	—	—
Methyl red test	+	NR	NR	NR
Casein hydrolysis	+	—	NR	+
Starch hydrolysis	+	—	—	+
Carbohydrate utilization				
Adonitol	—	—	+	—
D-Fructose	+	—	+	—
<i>myo</i> -Inositol	—	—	+	—
Lactose	+	—	—	W
Acid production from glucose	+	—	—	NR
Amino acid assimilation				
Alanine	—	+	+	—
Histidine	+	—	+	—
Isoleucine	+	NR	+	NR
Lysine	+	NR	+	NR
Methionine	—	NR	+	NR
Glycine	+	NR	+	NR
Antibiotic sensitivity*				
Gentamicin	S	R	S	NR
Streptomycin	S	R	NR	NR
DNA G + C content (mol%)	59.5	48.7	40.0	56.0
Phospholipids†	PG, DPG, UK1	NR	NR	PE, DPG (trace)

*S, Sensitive; R, resistant.

†PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; UK1, unknown phospholipid.

was determined in medium [1.05 % (w/v) K₂HPO₄, 0.45 % (w/v) KH₂PO₄, 0.1 % (w/v) (NH₄)₂SO₄ and 1.5 % (w/v) agarose] supplemented with 0.5 % (w/v) of each filter-sterilized carbon compound. Assimilation of various amino acids as sole nitrogen sources was determined in a medium containing 1 % (w/v) D-glucose, 0.05 % (w/v) MgSO₄·7H₂O, 0.05 % (w/v) NaCl, 0.001 % (w/v) Fe₃SO₄·7H₂O, 0.1 % (w/v) K₂HPO₄ and 1.5 % (w/v) agarose, supplemented with the amino acid (0.2 %, w/v) being tested. Nutrient agar with antibiotic discs (Hi-Media) was used for checking the sensitivity of strain SRC-1^T to different antibiotics. Cellular fatty acid methyl esters were extracted and analysed using the Microbial Identification System (MIDI) as described previously (Pandey *et al.*, 2002). Isoprenoid quinones were extracted

according to the method described by Collins *et al.* (1977) and identified as described by Tamaoka *et al.* (1983) and Suresh *et al.* (2004b). Extraction of polar lipids and analysis of phospholipids were done as described previously (Suresh *et al.*, 2004a). DNA was isolated as described by Shivaji *et al.* (1989). The G + C content of genomic DNA was estimated spectrophotometrically (Lambda 35; Perkin Elmer) as described by Mandel & Marmur (1968).

Details of the phenotypic and chemotaxonomic characteristics of SRC-1^T are given in the genus and species descriptions. Strain SRC-1^T was strictly aerobic and the cells were Gram-negative, non-motile, irregular rods (Table 1). The fatty acid profile was dominated by unsaturated and hydroxy fatty acids, including 17 : 1 iso I/anteiso B (summed feature

4) (36.7%), 15:0 iso (15.8%) and 17:0 iso 3-OH (10.3%) (Table 2). The presence of the fatty acid 15:0 iso has been reported for many genera belonging to the family 'Flexibacteraceae'; in strain SRC-1^T, it constituted the second largest component of the total cellular fatty acid content. The polar lipids in strain SRC-1^T were phosphatidylglycerol, diphosphatidylglycerol and an unknown phospholipid. The DNA G + C content was 59.5 mol%.

The 16S rRNA gene sequence of strain SRC-1^T was amplified by a PCR using primers 8-27f (5'-AGAGTTTGATCCTG-GCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACG-ACTT-3'), as described by Pandey *et al.* (2002); the PCR product was purified using the QIAquick PCR purification kit (Qiagen). The purified amplicon was sequenced using the BigDye Terminator cycle sequencing kit and an ABI PRISM model 310 automatic DNA sequencer (Applied Biosystems). The 16S rRNA gene sequences of type species of genera of the 'Flexibacteraceae' with validly published names were retrieved from the EMBL database, aligned with

the almost-complete sequence (1401 bp continuous stretch) of the 16S rRNA gene of strain SRC-1^T using CLUSTAL_X (Thompson *et al.*, 1997) and edited manually. The confidence value for the aligned dataset was obtained by bootstrap analysis of 1000 replications using SEQBOOT (PHYLIP package; Felsenstein, 1993). Pairwise evolutionary distances were computed using the DNADIST (distance-matrix) program with the Kimura two-parameter model (Kimura, 1980). Phylogenetic trees were constructed using the neighbour-joining and UPGMA methods in the NEIGHBOR program and also by using the TREECONW package (Van de Peer & De Wachter, 1997). A consensus tree was constructed from multiple trees by using the CONSENSE program.

Phylogenetic analyses, based on 16S rRNA gene sequences, indicated that strain SRC-1^T belonged to the family 'Flexibacteraceae', showing greatest similarity (95.5%) to *Pontibacter actiniarum* KMM 6156^T (Nedashkovskaya *et al.*, 2005), followed by *Adhaeribacter aquaticus* MBRG1.5^T (89.0%; Rickard *et al.*, 2005), *Hymenobacter roseosalivarius* DSM 11622^T (88.9%; Hirsch *et al.*, 1998) and *Hymenobacter actinosclerus* CCUG 39621^T (88.5%; Collins *et al.*, 2000). The 16S rRNA gene sequence of two species of *Hymenobacter* mentioned above and that of strain SRC-1^T showed significant dissimilarity between nucleotides 188 and 231 (nucleotide numbering according to *Escherichia coli*), and this stretch of the sequence was not considered in similarity comparisons. The phylogenetic tree created by using the neighbour-joining method placed strain SRC-1^T in a clade with *P. actiniarum* (with a bootstrap value of 100), forming a cluster with *Adhaeribacter*, *Hymenobacter* and *Taxeobacter* species (Fig. 1). Irrespective of the methods used, the overall topologies of the trees were very similar.

The DNA–DNA relatedness among these genera is assumed to be quite low, as none of the most closely related species showed a high level of 16S rRNA gene sequence similarity, and, according to accepted criteria, differences of more than 3% at the 16S rRNA gene sequence level represent similarities of less than 70% at the whole-genome level (Stackebrandt & Goebel, 1994). Strain SRC-1^T showed 16S rRNA gene sequence similarity of 95.5% to *P. actiniarum* KMM 6156^T, which is close to the proposed threshold (95%) for the description of a novel genus (Ludwig *et al.*, 1998); however, there are significant phenotypic differences (Table 1). The cell morphology of SRC-1^T differed from that of the closely related species *P. actiniarum* in having an irregular rod shape; in addition, cells of SRC-1^T are non-motile. *P. actiniarum* and strain SRC-1^T also differed regarding the hydrolysis of casein and starch. The fatty acid compositions also differed, with strain SRC-1^T containing significant quantities of 16:0 iso (5.9%) and 18:1 iso H (3.1%), which were absent in *P. actiniarum*. One of the predominant fatty acids of *P. actiniarum* was summed feature 3 (15:0 iso 2-OH, 16:1 ω 7c and/or 16:1 ω 7t) (14.7%), which was absent in strain SRC-1^T (Table 2). These two bacteria also differed in antibiotic sensitivity and DNA G + C content (Table 1). Although strain SRC-1^T shares some biochemical

Table 2. Fatty acid profiles of strain SRC-1^T and closely related strains

Strains: 1, strain SRC-1^T; 2, *P. actiniarum* KMM 6156^T (data from Nedashkovskaya *et al.*, 2005); 3, *Adhaeribacter aquaticus* MBRG1.5^T (Rickard *et al.*, 2005); 4, *H. roseosalivarius* DSM 11622^T (Buczolits *et al.*, 2002). –, Absent; NR, not reported.

Fatty acid	1	2	3	4
14:0 iso	0.8	NR	NR	NR
15:0 iso	15.8	28.8	22.5	8.3
15:0 anteiso	3.5	0.1	4.4	NR
15:0 iso 2-OH	—	NR	16.5	NR
15:0 iso 3-OH	2.4	3.0	3.1	2.7
16:0	1.7	0.2	NR	1.1
16:1 ω 5c	2.1	0.8	16.9	23.3
16:0 3-OH	1.3	NR	NR	1.2
16:0 iso	5.9	NR	NR	2.1
16:0 iso 3-OH	1.2	NR	NR	1.0
16:1 iso H	4.4	0.5	NR	2.7
17:0 iso	1.3	2.2	NR	1.7
17:0 anteiso	1.3	NR	NR	NR
17:0 iso 3-OH	10.3	6.5	12.1	5.8
17:1 ω 6c	5.6	1.4	5.1	1.1
17:1 iso ω 9c	1.0	NR	NR	NR
18:1 iso H	3.1	NR	NR	NR
Summed feature 3*	—	14.7	NR	29.8†
Summed feature 4*	36.7	31.3	11.2‡	18.5‡

*Summed features contain two or three fatty acids that could not be separated by the MIDI system. Summed feature 3 consists of 15:0 iso 2-OH, 16:1 ω 7c and/or 16:1 ω 7t; summed feature 4 consists of 17:1 iso I/anteiso B.

†Reported as summed feature 4.

‡Reported as summed feature 5.

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationship between strain SRC-1^T and genera of the family 'Flexibacteraceae' of the phylum *Bacteroidetes*. Bootstrap values greater than 50 % are given at nodes. *Salinibacter ruber* M-31^T and *Arthrobacter globiformis* DSM 20124^T were used as outgroups. Bar, 0.1 substitutions per site.

characteristics with *Adhaeribacter aquaticus*, the 16S rRNA gene sequence similarity was quite low (89.0 %). Moreover, they differed from each other in genomic G + C content (Table 1). Thus, on the basis of phenotypic, chemotaxonomic and phylogenetic characteristics, it is proposed that strain SRC-1^T be recognized as belonging to a novel genus within the family 'Flexibacteraceae', for which the name *Effluviibacter* gen. nov. is proposed. The genus contains a single species, the type species *Effluviibacter roseus* sp. nov., of which SRC-1^T is the type strain.

Description of *Effluviibacter* gen. nov.

Effluviibacter (Ef.flu.vi.i.bac'ter. L. neut. n. *effluvium* outflow; N.L. masc. n. *bacter* rod; N.L. masc. n. *Effluviibacter* rod from an outflow, referring to the source of isolation of the first strain).

Cells are Gram-negative, non-motile, irregular rods. Obligate aerobes. Oxidase- and catalase-positive. The predominant whole-cell fatty acids are 17:1 iso I/anteiso B (summed feature 4) (36.7 % in the type strain of the type species), 15:0 iso (15.8 %) and 17:0 iso 3-OH (10.3 %). On the basis of 16S rRNA gene sequence analysis, the genus belongs to the family 'Flexibacteraceae' of the phylum *Bacteroidetes*. *Effluviibacter roseus* is the type species.

Description of *Effluviibacter roseus* sp. nov.

Effluviibacter roseus (ro'se.us. L. masc. adj. *roseus* rose-coloured, pink).

Exhibits the following features in addition to those listed in the description of the genus. Cells are non-sporulating, irregular rods, 1.0–3.0 µm in length and 0.3–0.5 µm in width. Colonies on nutrient agar are dark pink, circular, convex, smooth and 2–3 mm in diameter after incubation for 6–8 days. Grows at 4–37 °C, but not at 42 °C, and at pH 6.0–10.0. Tolerates up to 8 % NaCl. Positive in methyl red, amylase, protease, lysine decarboxylase and ornithine decarboxylase tests but is negative for arginine dihydrolase. Hydrolyses starch and gelatin but not urea, Tween 20 or aesculin. Does not reduce nitrate to nitrite and is negative in the Voges–Proskauer test and for indole production, citrate utilization and H₂S production. Utilizes D-fructose, D-galactose, D-glucose, lactose, raffinose and sucrose but not acetate, adonitol, arabinose, citrate, fumarate, lactate, *myo*-inositol, salicin, sorbitol or succinate as sole sources of carbon. Assimilates histidine, L-isoleucine, L-lysine, ornithine, L-proline and glycine as sole nitrogen sources, but not L-arginine, DL-alanine or L-methionine. Produces acid from D-glucose but not from lactose or sucrose. Resistant to penicillin and tobramycin but sensitive to vancomycin, lincomycin, streptomycin, gentamicin and

chlortetracycline. The whole-cell fatty acids are listed in Table 2. The major respiratory quinone is MK-7. The phospholipids present are phosphatidylglycerol, diphosphatidylglycerol and an unknown phospholipid. The DNA G+C content of the type strain is 59.5 mol%.

The type strain, SRC-1^T (=MTCC 7260^T=DSM 17521^T), was isolated from muddy water from an occasional drainage system in Chandigarh, India.

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