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ORIGINAL PAPER

Rubidimonas crustatorum gen. nov., sp. nov., a novel member of the family *Saprospiraceae* isolated from a marine crustacean

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Abstract A strictly aerobic, Gram-negative, reddish-orange pigmented, non-motile and rod-shaped bacterium, designated AK17-053^T was isolated from a marine crustacean (*Squillidae*) living on tidal flats on the coast of the Ariake Sea, Nagasaki, Japan. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that the novel isolate could be affiliated with the family *Saprospiraceae* of the phylum *Bacteroidetes* and that it showed highest sequence similarity (84%) with *Lewinella marina* MKG-38^T. The strain could be differentiated phenotypically from recognized members of the family *Saprospiraceae*. The G+C content of DNA was 55.3 mol%, MK-7 was the major menaquinone and iso-$C_{15:0}$ and $C_{16:1}\omega7c$ were the major fatty acids. On the basis of polyphasic taxonomic studies, it was concluded that strain AK17-053^T represents a new genus of the family *Saprospiraceae*. We propose the name *Rubidimonas crustatorum* gen. nov., sp. nov. for this strain; its type strain is AK17-053^T (= MBIC08356^T = NBRC 107717^T).

Keywords *Rubidimonas crustatorum* gen. nov. · sp. nov. · 16S rRNA gene · Marine crustacean · Polyphasic taxonomy

Introduction

A number of molecular phylogenetic studies based on 16S rRNA gene sequence analysis have revealed that members of the phylum *Bacteroidetes* (Ludwig and Klenk 2001) previously known as the *Cytophaga–Flavobacterium–Bacteroides* group, are omnipresent in a wide range of global ecosystems (DeLong et al. 1993; Bowman et al. 1997; Glöckner et al. 1999; O'Sullivan et al. 2002). Species of the phylum *Bacteroidetes* are generally associated with the degradation of complex organic materials (Cottrell and Kirchman 2000) but there have been relatively few studies of their detailed physiology and ecological niches.

The family *Saprospiraceae* within the phylum *Bacteroidetes* was circumscribed on the basis of 16S

The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain AK17-053^T is AB602438.

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rRNA gene sequences (Sly et al. 1998). In the second edition (Vol 4) of *Bergey's Manual of Systematic Bacteriology* (Family III. Saprospiraceae fam. nov.), the family *Saprospiraceae* incorporates the genera *Saprospira* (type genus), *Haliscomenobacter*, *Lewinella* and *Aureispira*. The genera *Saprospira* and *Haliscomenobacter* each accommodate one species, *Saprospira grandis* (Reichenbach 1989) and *Haliscomenobacter hydrossis* (van Veen et al. 1973) and the genus *Lewinella* accommodates seven species, *Lewinella agarilytica* (Lee 2007), *Lewinella antarctica* (Oh et al. 2009), *Lewinella cohaerens* (Khan et al. 2007), *Lewinella lutea* (Khan et al. 2007), *Lewinella marina* (Khan et al. 2007), *Lewinella nigricans* (Khan et al. 2007) and *Lewinella persica* (Khan et al. 2007). The genus *Aureispira* (Hosoya et al. 2006) comprised two species, *Aureispira marina* (Hosoya et al. 2006) and *Aureispira maritima* (Hosoya et al. 2007). The two latter species were found to be arachidonic acid-producing bacteria. In this study, we carried out a polyphasic taxonomic characterization of a novel marine bacterium (strain AK17-053^T) isolated from a marine crustacean in Japan.

Materials and methods

Isolation of bacterial strain and culture conditions

Strain AK17-053^T was isolated from an undetermined species of marine crustacean (family *Squillidae*) collected at a tideland (N32°56′42.7″ E130°12′04.3″) on the coast of the Sea of Ariake, Nagasaki Prefecture, Japan. The specimen (approximately 1 cm^3) was homogenized with a glass rod in 5 ml of sterile artificial seawater. A 50 µl sample of the homogenate was applied to the surface of a 1/10 strength R2A agar medium sprayed with dibenzothiophene vapour after gelification. The composition of the medium was as follows (per liter): R2A broth (DAIGO), 0.32 g; agar, 15 g; aged seawater, 1,000 ml. Colonies appeared after incubation for a week at 25°C. A reddish-orange-colored colony was subcultured to purify on 1/10 strength marine agar 2216 (Difco).

The strain was routinely cultured on full-strength marine agar 2216 (MA) at 25°C and maintained in marine broth 2216 supplemented with 20% (v/v) glycerol at –70°C.

Morphological, physiological and biochemical analysis

Cell morphology was observed using light microscopy (BX60; Olympus) and flagella staining was carried out according to Blenden and Goldberg (1965). Gliding motility was determined by using a semisolid medium as described by Perry (1973). The temperature (4–45°C) and pH (5–10) ranges for growth were determined by incubating the isolates on MA. The NaCl concentration for growth was determined on MA containing 0–10% (w/v) NaCl (Atlas 1993). Gram-staining was performed as described by Murray et al. (1994). Spore formation was determined by staining with malachite green. Anaerobic growth was tested for up to 2 weeks on MA in a jar containing AnaeroPack-Anaero (Mitsubishi Gas Chemical Co., Inc.), which works as an O_2 absorber and CO_2 generator. Catalase activity was determined by bubble formation in a 3% H_2O_2 solution. Oxidase activity was determined using cytochrome oxidase paper (Nissui Pharmaceutical Co., Inc.). Degradation of DNA was tested using DNase agar (Scharlau), with DNase activity detected by flooding plates with 1 M HCl. Starch hydrolysis were tested as described by Choi et al. (2007). The ability to hydrolyse casein, Tween 20, Tween 80 and tyrosine were determined according to Hansen and Sørheim (1991). API 20E, API 50 CH and API ZYM strips (bioMérieux) were used to determine the biochemical characteristics. All suspension media for the API test strips were supplemented with 0.85% (w/v) NaCl solution (final concentration). API 20E and API 50CH test strips were read after 72 h of incubation at 30°C and API ZYM test strips were read after 4 h incubation at 37°C. Flexirubin-type pigments were investigated by using the bathochromatic shift test with a 20% (w/v) KOH solution (Bernardet et al. 2002).

Determination of G+C content of DNA, 16S rRNA gene sequencing and phylogenetic analysis

The genomic DNA was prepared according to the method of Marmur (1961) from cells grown on MA and the DNA base composition was determined by using the HPLC method of Mesbah et al. (1989). An approximately 1,500 bp fragment of the 16S rRNA gene was amplified from the extracted DNA by using the bacterial universal primers: 27F and 1492R (*Escherichia coli* numbering system; Weisburg et al.

1991). To ascertain the phylogenetic position of the novel isolate, the 16S rRNA gene sequence of strain AK17-053^T (GenBank/EMBL/DDBJ Accession Number AB602438) was compared with sequences obtained from GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). Multiple alignments of the sequences were performed using CLUSTAL_X (version 1.83) (Thompson et al. 1997). Alignment gaps and ambiguous bases were not taken into consideration when 1,319 bases of the 16S rRNA gene were compared. Aligned sequences were analyzed by the MEGA 4 software (Tamura et al. 2007). The evolutionary distances [distance options according to the Kimura two-parameter model (Kimura 1983)] and clustering with the neighbor-joining (Saitou and Nei 1987) and maximum-parsimony (Fitch 1971) methods were determined by using bootstrap values based on 1,000 replications (Felsenstein 1985). The similarity values were calculated using the same software.

Chemotaxonomic analysis

Gas chromatography analysis of the cellular fatty acid methyl esters was performed using a culture grown on MA at 37°C for 4 days. Fatty acid methyl esters were extracted and prepared according to standard protocols provided by the MIDI/Hewlett Packard Microbial Identification system (Sasser 1990; MIDI Inc. 1999). Determination of the respiratory quinone system was carried out as described previously (Xie and Yokota 2003).

Results and discussion

Molecular phylogenetic analysis

Comparative phylogenetic analysis of the 16S rRNA gene sequence revealed that strain AK17-053^T was affiliated with the family *Saprospiraceae* within the phylum *Bacteroidetes* (Fig. 1). Analysis of the 16S rRNA gene sequence also indicated that strain AK17-053^T showed the highest sequence similarity to *L. marina* MKG-38^T (84%), followed by *L. agarilytica* SST-19^T (82.5%), *L. antarctica* IMCC3223^T (82.5%), *L. cohaerens* II-2^T (82.5%) and *L. lutea* FYK2402M69^T (82.2%). Sequence similarity was less than 82% with all other members of the family *Saprospiraceae* with validly published names. This suggests that strain AK17-053^T may represent a novel genus and species of the family *Saprospiraceae*.

Morphological, physiological and biochemical characteristics

Cells were mostly straight and short- rod-shaped. Cells varied between 0.3 and 0.5 μm in diameter and 2.0–3.0 μm in length. Gliding motility was not observed and flexirubin-type pigments were not produced (Table 1). Strain AK17-053^T also showed distinct phenotypic, physiological and biochemical features that discriminated it from the closest described members in the family *Saprospiraceae* as shown in Table 1.

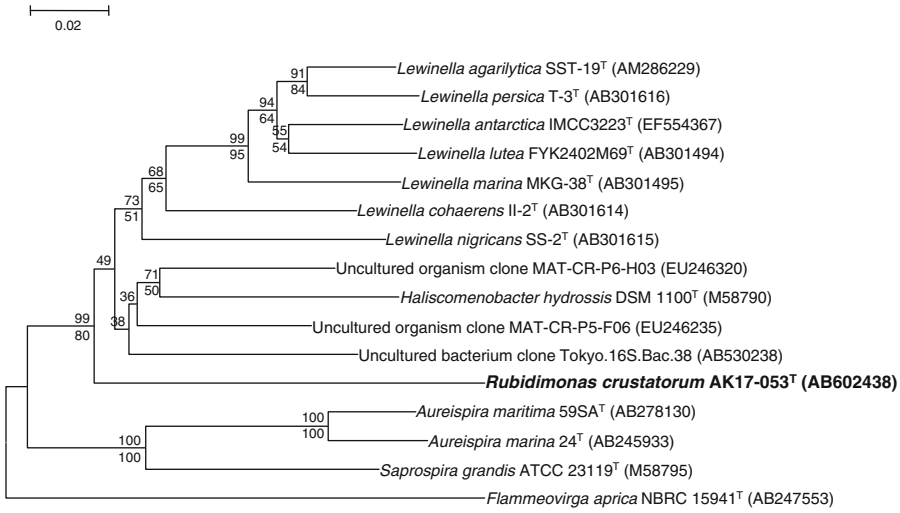

Fig. 1 Neighbour-joining tree of 16S rRNA gene sequence similarity, showing the phylogenetic position of strain AK17-053^T and representatives of the family *Saprospiraceae*. The sequence of *Flammeovirga aprica* NBRC 15941^T was used as an outgroup. The sequence determined in this study is shown in *bold*. Bootstrap values both neighbor-joining (above nodes) and maximum-parsimony (below nodes) are shown. *Bar* 2% sequence divergence

Chemotaxonomic characteristics

As shown in Table 2, the predominant cellular fatty acids of strain AK17-053^T were iso-$C_{15:0}$ (37.1%) and $C_{16:1}\omega7c$ (18.7%). On the basis of the fatty acid composition, strain AK17-053^T can be differentiated from recognized species of the genera *Saprospira*, *Haliscomenobacter*, *Lewinella* and *Aureispira* as shown in Table 2. These results also suggest that strain AK17-053^T represents an independent genus of the family *Saprospiraceae*.

Polyphasic taxonomic conclusion

From the distinct phylogenetic position and combinations of genotypic and phenotypic characteristics, strain AK17-053^T cannot be assigned to any previously recognized bacterial genus and thus can be described as a novel species within a new genus, *Rubidimonas crustatorum* gen. nov., sp. nov.

Description of *Rubidimonas* gen. nov

Rubidimonas (Ru.bi.di.mo'nas. L. adj. *rubidus*, red, reddish; L. fem. n. *monas*, a monad, unit; N.L. fem. n. *Rubidimonas*, a reddish-colored unit (bacterium)).

Cells are short-rod-shaped, Gram-negative and strictly aerobic. Do not form endospores. Catalase-positive, but oxidase-negative. Flexirubin-type pigments are absent. The major respiratory quinone is menaquinone 7 (MK-7). The G+C content of the genomic DNA of the type strain is 55.3 mol%. The predominant cellular fatty acids are iso-$C_{15:0}$ and $C_{16:1}\omega7c$. A member of the family *Saprospiraceae*, phylum *Bacteroidetes*, according to 16S rRNA gene sequence analyses.

The type species is *Rubidimonas crustatorum*.

Description of *Rubidimonas crustatorum* sp. nov

Rubidimonas crustatorum (crus.ta.to'rum. L. pl. n. crustata -orum, crustaceans; L. gen. pl. n. *crustatorum*, of crustaceans).

The main characteristics are the same as those given for the genus. In addition, cells are short-rods 0.3–0.5 μm in diameter and 2–3 μm in length. Cells lack flagella and are non-motile. Gliding motility is not observed. Colonies grown on MA are 1–2 mm in diameter, circular, shiny with entire edges and orange-pigmented, becoming reddish-orange after one week of incubation. The temperature range for growth is 10–40°C, the optimal temperature is between 25 and 37°C but no growth was occurs at 4 or 45°C. The pH range for growth is pH 6–9 (optimum, pH 7), no growth is observed below pH 6 or above pH 10. NaCl is required for growth and can be tolerated at a concentration of up to 7% (w/v). No growth occurs in the presence of 8% (w/v) NaCl. Nitrate and nitrite reduction are negative. Agar, casein, DNA, tyrosine, Tween 20 and 80 and urea are not hydrolyzed but gelatin and starch are hydrolyzed. The *o*-nitrophenyl-*β*-D-galactosidase (ONPG) test is positive but the reactions for the Voges–Proskauer test, citrate utilization, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities and hydrogen sulfide and indole production are negative (API 20E). In the API 50 CH strip, acid is produced from D-arabinose, L-arabinose, D-xylose, L-xylose, methyl-*β*-D-xylopyranoside, galactose, glucose, fructose, mannose and rhamnose but not from methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetyl-glucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, inulin, ribose, raffinose, gentiobiose, glycerol, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, sorbitol, starch, glycogen, xylitol, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate or 5-keto-gluconate. In the API ZYM strip, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, α-chymotrypsin and acid phosphatase activities are present but naphthol-AS-BI-phosphohydrolase, *N*-acetyl-*β*-glucosaminidase, α-galactosidase, *β*-galactosidase, α-glucosidase, *β*-glucosidase, trypsin, *β*-glucuronidase, α-mannosidase and α-fucosidase activities are absent. The major (> 5.0%) fatty acids are C18:0, iso-C15:0, $C15:1\omega6c$, $C16:1\omega7c$ and iso-C17:0 3-OH. The G+C of the genomic DNA is 55.3 mol%.

The type strain is AK17-053^T (= MBIC08356^T = NBRC 107717^T), which was isolated from an undetermined species of marine crustacean (Family *Squillidae*) living on tidal flats on the coast of the Ariake Sea, Nagasaki, Japan.

Table 1 Differential characteristics of strain AK17-053^T and other closely related taxa

Characteristics	1	2	3	4	5	6
Cell shape	Short rods	Short to elongated rods	Short to elongated rods	Short to elongated rods	Flexible rods or filaments	Short to elongated rods
Colony color	Reddish orange	Light orange	Black	Dark orange	Light orange	Orange
Gliding motility	−	−	−	−	+	−
Oxidase	−	+	+	+	+	−
Flexirubin-type pigments	−	−	−	−	−	−
NaCl range for growth (%)	1–7	2–4	2–4	2–4	0.5–1	0.5–5
Temperature range for growth (°C)	10–40	10–30	15–37	4–30	4–37	3–25
Hydrolysis of:						
Agar	−	−	−	−	+	−
Casein	−	+	+	+	+	−
DNA	−	+	+	+	+	ND
Starch	+	+	+	+	+	+
DNA G+C content (mol%)	55.3	44.9	53.1	52.6	51.3	50.3
Characteristics	7	8	9	10	11	12
Cell shape	Short to elongated rods	Short to elongated rods	Thin rods	Flexible helical filaments	Flexible helical filaments	Flexible helical filaments
Colony color	Dark orange	Dull orange	Pink	Yellowish orange	Yellow	Orange-red
Gliding motility	−	−	+	+	+	+
Oxidase	+	+	ND	*v*	*v*	ND
Flexirubin-type pigments	−	−	ND	−	−	−
NaCl range for growth (%)	2–4	1–7	ND	ND	ND	ND
Temperature range for growth (°C)	10–35	4–42	8–30	25–30	25–30	30–37
Hydrolysis of:						
Agar	−	−	ND	−	−	−
Casein	+	+	ND	+	+	+
DNA	+	+	ND	−	+	ND
Starch	+	+	+	−	−	−
DNA G+C content (mol%)	56	61	49	38–39	38.7	46–48

Strains 1 AK17-053^T (*Rubidimonas crustatorum* gen. nov., sp. nov.; present study); *2 Lewinella cohaerens* II-2^T (Khan et al. 2007); *3 Lewinella nigricans* SS-2^T (Khan et al. 2007); *4 Lewinella persica* T-3^T (Khan et al. 2007); *5 Lewinella agarilytica* SST-19^T (Lee 2007); *6 Lewinella antarctica* IMCC3223^T (Oh et al. 2009); *7 Lewinella lutea* FYK2402M69^T (Khan et al. 2007); *8 Lewinella marina* MKG-38^T (Khan et al. 2007); *9 Haliscomenobacter hydrossis* ATCC 27775^T (van Veen et al. 1973; Mulder 1989); *10 Aureispira marina* 24^T (Hosoya et al. 2006); *11 Aureispira maritima* 59SA^T (Hosoya et al. 2007); *12 Saprospira grandis* ATCC 23119^T (Reichenbach 1989). *Symbols* + positive; − negative; *v* variable; *ND* no data. The data presented for reference strains are taken from the literature sources indicated and have been obtained using different growth conditions and methods, in part reflecting the specific culture methods needed for different members of the family *Saprospira*. Typically for biochemical and physiological tests, API 20E, API 50 CH and API ZYM strips (bioMérieux) were used to determine the characteristics of the reference strains, except for the representatives of the genera *Haliscomenobacter* and *Saprospira*

Table 2 Comparison of cellular fatty acids for strain AK17-053^T and other closely related taxa

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12
$C_{14:0}$	1.3	–	–	–	1.3	tr	–	–	tr	–	–	–
$C_{15:0}$	1.1	13	6	tr	tr	tr	tr	tr	tr	–	–	–
$C_{16:0}$	4.3	tr	7	2	10.9	2.3	3	4	5.7	16.9	25.6	–
$C_{16:1}$	–	–	–	–	–	–	–	–	17.3	–	–	–
$C_{18:0}$	6.2	–	–	–	13.5	–	–	–	6.9	–	–	–
iso-$C_{13:0}$	tr	–	–	–	–	–	–	–	tr	–	–	1.4
iso-$C_{15:0}$	37.1	30	32	28	11.1	15.9	16	22	21.1	12.2	3	43.3
iso-$C_{15:1}$	1.7	29	19	4	–	–	2	3	–	–	–	–
iso-$C_{16:0}$	–	3	3	tr	3.2	–	–	–	2.8	8.8	3.7	–
iso-$C_{16:1}$	–	1	–	tr	–	–	1	–	–	–	–	–
iso-$C_{17:0}$	–	2	tr	tr	–	–	1	4	1	25.8	7.1	–
anteiso-$C_{15:0}$	3.5	–	–	–	tr	–	–	–	–	–	–	–
$C_{15:1}\omega 6c$	7.7	–	–	2	2.4	8.9	2	1	–	–	–	–
$C_{15:1}\omega 8c$	–	–	–	2	–	2	–	–	–	–	–	–
$C_{16:1}\omega 5c$	tr	2	–	tr	–	–	–	–	–	–	–	–
$C_{16:1}\omega 7c$/iso-$C_{15:0}$ 2-OH	18.7	tr	9	30	24.1	39.4	53	–	15.5	–	–	4.1
$C_{17:1}\omega 6c$	–	tr	tr	5	2.6	–	2	3	–	–	–	–
$C_{17:1}\omega 7c$	–	–	–	–	–	10.5	–	–	–	–	–	–
$C_{20:4}\omega 6c$	–	–	–	–	–	–	–	–	–	46.3	43.6	–
iso-$C_{17:1}\omega 9c$	–	tr	2	10	tr	2.6	9	23	–	–	–	–
$C_{16:0}$ 3-OH	2.1	–	–	–	1.3	tr	–	–	–	–	tr	3.8
iso-$C_{11:0}$ 3-OH	2.7	3	–	–	–	–	–	–	–	–	–	–
iso-$C_{15:0}$ 3-OH	tr	3	2	2	–	–	4	2	22.8	–	–	–
iso-$C_{16:0}$ 3-OH	–	–	–	–	–	–	–	–	–	1.5	–	–
iso-$C_{17:0}$ 3-OH	7.1	3	6	5	–	–	4	6	tr	4.3	1.5	24.8

Strains 1 AK17-053^T (*Rubidimonas crustatorum* gen. nov., sp. nov.; present study); *2 Lewinella cohaerens* II-2^T (Khan et al. 2007); *3 Lewinella nigricans* SS-2^T (Khan et al. 2007); *4 Lewinella persica* T-3^T (Khan et al. 2007); *5 Lewinella agarilytica* SST-19^T (Lee 2007); *6 Lewinella antarctica* IMCC3223^T (Oh et al. 2009); *7 Lewinella lutea* FYK2402M69^T (Khan et al. 2007); *8 Lewinella marina* MKG-38^T (Khan et al. 2007); *9 Haliscomenobacter hydrossis* ATCC 27775^T (Kämpfer 1995); *10 Aureispira marina* 24^T (Hosoya et al. 2006); *11 Aureispira maritima* 59SA^T (Hosoya et al. 2007); *12 Saprospira grandis* ATCC 23119^T (Reichenbach 1989). Values are percentages of total fatty acids; – not detected; *tr* traces (< 1%). The data presented for reference strains are taken from the literature sources indicated. The data were typically obtained by MIDI analyses, although the data for the genera *Haliscomenobacter* and *Saprospira* were analyzed by the FAME system

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