

Note

Reichenbachia agariperforans gen. nov., sp. nov., a novel marine bacterium in the phylum *Cytophaga–Flavobacterium–Bacteroides*Olga I. Nedashkovskaya,¹ Makoto Suzuki,² Mikhail V. Vysotskii³ and Valery V. Mikhailov¹

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A heterotrophic, pigmented, agarolytic, gliding bacterium was isolated from a seawater sample collected from the Gulf of Peter the Great, Sea of Japan, during June 2000. 16S rDNA sequence analysis indicated that the novel bacterium, strain KMM 3525^T, was a member of the phylum *Cytophaga–Flavobacterium–Bacteroides*. On the basis of phenotypic, chemotaxonomic, genotypic and phylogenetic data, it is proposed that the marine bacterium represents the sole species of a novel genus, *Reichenbachia*, the type species of which is *Reichenbachia agariperforans* (KMM 3525^T = IFO 16625^T = JCM 11238^T).

Gliding, Gram-negative, heterotrophic bacteria are widely distributed in marine coastal ecosystems. Historically, bacteria belonging to the phylum *Cytophaga–Flavobacterium–Bacteroides* (CFB) have been poorly investigated in terms of their phylogeny. During the last decade, many novel taxa belonging to the phylum CFB have been described, and some bacterial species that previously had unclear taxonomic positions within this phylum have been reclassified due to the use of a polyphasic taxonomic approach. Descriptions of genera such as *Polaribacter*, *Gelidibacter*, *Psychroserpens*, *Psychroflexus* and *Salegentibacter* have resulted from intensive studies into the microbial communities of Antarctic seas and hypersaline lakes (Gosink *et al.*, 1998; Bowman *et al.*, 1997, 1998; McCammon & Bowman, 2000). Novel bacteria have also been found in shore marine waters in temperate latitudes, and the novel genera *Cellulophaga*, *Tenacibaculum* and *Arenibacter* have been created for these organisms (Johansen *et al.*, 1999; Suzuki *et al.*, 2001; Ivanova *et al.*, 2001). However, many other taxa belonging to the phylum CFB remain to be described. In this study, we have characterized a novel bacterium, strain KMM 3525^T, which was isolated from a seawater sample. On the basis of the results of a polyphasic taxonomic study (i.e. phylogenetic, phenotypic and genotypic analyses, and analyses of menaquinone and cellular fatty acid compositions) on this novel

bacterium, we describe a novel genus of the phylum CFB, *Reichenbachia*, the type species of which is *Reichenbachia agariperforans*.

Agarolytic strain KMM 3525^T was isolated from a seawater sample collected in the Amursky Bay of the Gulf of Peter the Great, Sea of Japan, during June 2000. The strain was cultured on Marine Agar 2216E (Difco). Flexirubin pigments of the strain were determined by the method of Fautz & Reichenbach (1980). Determinations for the degradation of alginic acids (1%, w/v) and agar (1.5%, w/v), growth at different temperatures, NaCl concentrations or pH, production of acid from carbohydrates, and hydrolysis of starch, casein, gelatin, cellulose [carboxymethylcellulose (CM-cellulose) and filter paper], DNA and urea by strain KMM 3525^T were carried out according to the methods of Smibert & Krieg (1994). Hydrolysis of chitin (1%, w/v) by strain KMM 3525^T was determined by the appearance of clear zones around colonies on chitin agar. Susceptibility of the novel strain to antibacterials was examined by the routine disc-diffusion plate method. Discs were impregnated with the following antibacterials: ampicillin (10 µg); benzylpenicillin (10 µg); carbenicillin (100 µg); gentamicin (10 µg); kanamycin (30 µg); lincomycin (15 µg); neomycin (30 µg); oleandomycin (15 µg); polymyxin B (300 U); streptomycin (10 µg); tetracycline (30 µg).

The analysis of fatty acid methyl esters of the novel strain was performed by GLC (30 m × 0.25 mm Supelcowax 10 column, 205 °C) as described by Svetashev *et al.* (1995).

Abbreviation: CFB, *Cytophaga–Flavobacterium–Bacteroides*.

The GenBank accession number for the 16S rDNA sequence of *Reichenbachia agariperforans* KMM 3525^T is AB058919.

Isoprenoid quinones were extracted and analysed by the method of Nakagawa & Yamasato (1993). DNA was isolated from strain KMM 3525^T following the method of Marmur (1961) and the G+C content (mol%) of the DNA was determined by the thermal denaturation method (Marmur & Doty, 1962). The 16S rRNA gene sequence of strain KMM 3525^T was determined by PCR amplification and direct sequencing (Hiraishi, 1992). Conditions and reagents used for PCR amplification and sequencing of the 16S rDNA have been described previously (Suzuki *et al.*, 2001). The 16S rDNA sequence of strain KMM 3525^T was aligned with sequences retrieved from the databases by using CLUSTAL W (Thompson *et al.*, 1994). The aligned sequences were modified manually according to the secondary structure of the *Escherichia coli* 16S rRNA gene sequence (Gutell *et al.*, 1994). The regions in which the secondary structure of the rRNA of the analysed strains varied (positions 194–220, 450–487, 1284–1289, *E. coli* numbering system) and the sequences before position 111 and after position 1376, for which sequences of some reference organisms had not been determined, were excluded from the analysis. Evolutionary distances were then computed by using DNADIST contained within the PHYLIP package (version 3.572; Felsenstein, 1995) with the Kimura two-parameter correction (Kimura, 1980), and the phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987). To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed with the SEQBOOT and CONSENSE programs contained within the PHYLIP package (version 3.572).

Strain KMM 3525^T was a Gram-negative, chemo-organotrophic bacterium with a respiratory-type metabolism. It was motile by gliding and appeared as single, flexible rods of 0.5–0.7 µm in diameter and 5–15 µm in length. On marine agar, colonies were round, 3–5 mm in diameter, orange-pigmented and formed deep hollows in agar plates. The organism had no known resting stages. Strain KMM 3525^T was oxidase-, catalase- and alkaline-phosphatase-positive, and required Na⁺ ions for growth. Growth of the organism occurred in media containing 1–6% NaCl. The temperature range for growth was 4–35 °C, with optimum growth occurring at 28 °C. The pH range for growth was 5.5–10.0, with optimum growth occurring between pH 7.5 and 8.5. Flexirubin pigments were formed by the novel bacterium. Agar, starch, alginate, gelatin, Tween 20, DNA and urea were degraded by strain KMM 3525^T, but cellulose (CM-cellulose and filter paper), chitin, casein, Tween 40, Tween 60 and Tween 80 were not hydrolysed by the strain. H₂S and indole were not produced by the bacterium and it formed no acid from arabinose, galactose, glucose, lactose, maltose, melibiose, rhamnose, sucrose, xylose, adonitol, dulcitol, inositol or mannitol. Utilization of citrate, fumarate and malate as sole carbon sources was not observed. Nitrate reduction was negative. Strain KMM 3525^T was susceptible to carbenicillin, oleandomycin, lincomycin and tetracycline, but it was not susceptible to ampicillin, benzylpenicillin, streptomycin, gentamicin, neomycin or

polymyxin B. Predominant cellular fatty acids were straight-chain unsaturated and branched-chain saturated, namely C_{15:0} (55.6%) and C_{16:1(n-7)} (23.7%) fatty acids. The DNA G+C content of the novel strain was 44.5 mol%, as determined by the thermal denaturation method.

A phylogenetic analysis of the 16S rDNA sequence data revealed that strain KMM 3525^T formed a distinct lineage within the phylum CFB and belonged to a cluster containing *Persicobacter diffluens* and marine flexibacteria (Fig. 1), but this was without significant bootstrap support. The members of this cluster share many phenotypic traits. Features useful for distinguishing strain KMM 3525^T from related members of the phylum CFB are listed in Table 1. The marine flexibacteria were separated from the genus *Flexibacter* and placed into the genus *Microscilla* by Pringsheim (1951); the taxonomy of this polyphyletic group was studied by Lewin (1969). More recently, on the basis of 16S rRNA gene sequence data, Nakagawa *et al.* (1997) have suggested that the genus *Microscilla* be restricted to a single species, *Microscilla marina*, the type species and only member of this taxon, which occupies a separate phylogenetic lineage within the phylum CFB. The taxonomic positions of other species of marine flexibacteria belonging to different rDNA branches are unclear and need further investigation (Bernardet *et al.*, 1996; Nakagawa *et al.*, 1997; Sly *et al.*, 1998). The 16S rDNA sequence of strain KMM 3525^T was most similar to that of '*Microscilla sericea*' (89.5%). Its level of 16S rDNA sequence similarity with other members of the phylum CFB ranged from 79.1 to 89.1%. Such low levels of sequence similarity of strain KMM 3525^T with other marine flexibacteria clearly demonstrate that the novel bacterium described in this study represents a novel genus within the phylum CFB. This conclusion, supported by the polyphasic data presented in this study, demonstrates that strain KMM 3525^T could not be assigned to any of the currently recognized genera included in the phylum CFB. Thus, we propose that strain KMM 3525^T be placed into a novel genus, *Reichenbachia*, as *Reichenbachia agariperforans*.

Description of *Reichenbachia* gen. nov.

Reichenbachia (Rei.chen.bach'i.a. N.L. fem. n. *Reichenbachia* after Hans Reichenbach, a German microbiologist, who has made a great contribution to the taxonomy of bacteria belonging to the phylum CFB).

Rod-shaped cells with gliding motility. Gram-negative. Does not form endospores or resting stages. Requires Na⁺ ions for growth. Strictly aerobic. Produces non-diffusible orange pigment. Flexirubins are synthesized. Chemo-organotroph. Cytochrome-oxidase-, catalase- and alkaline-phosphatase-positive. Major respiratory quinone is MK-7. The main cellular fatty acids are straight-chain unsaturated and branched-chain saturated fatty acids, C_{15:0} and C_{16:1(n-7)}. As determined by 16S rDNA sequence analysis, the genus *Reichenbachia* is a member of the phylum CFB. The type species is *Reichenbachia agariperforans*.

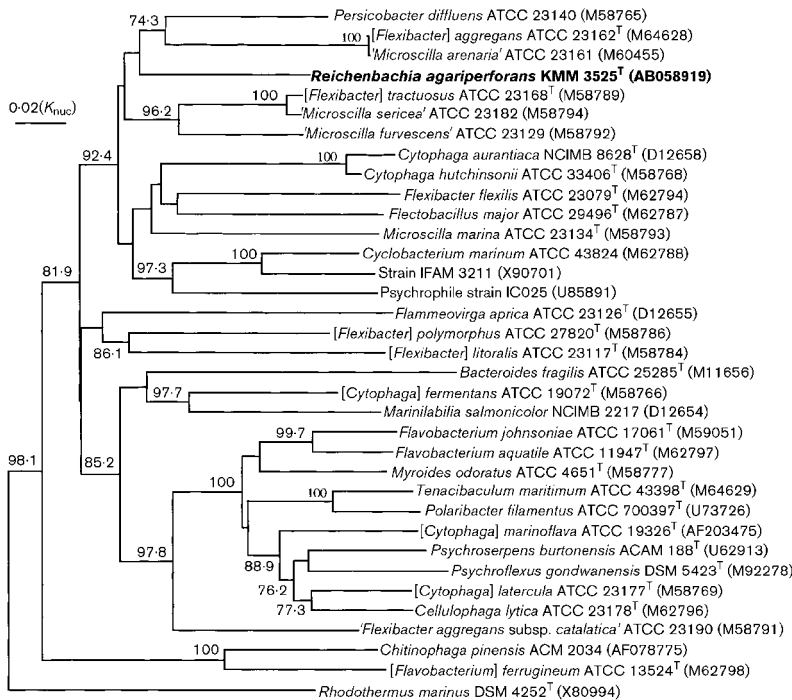


Fig. 1. Phylogenetic relationship of *Reichenbachia agariperforans* KMM 3525^T with other species of the phylum CFB on the basis of 16S rDNA sequence comparisons. The phylogenetic tree was generated by the neighbour-joining method (Saitou & Nei, 1987), and the 16S rDNA sequence of *Rhodothermus marinus* was used as the outgroup. The numbers shown at the nodes indicate the percentage bootstrap values (based on 1000 replications; only values of $\geq 70\%$ are shown). Bar, genetic distance of 0.02 (K_{nuc}).

Description of *Reichenbachia agariperforans* sp. nov.

Reichenbachia agariperforans [a.ga.ri.per.fo'rans. Malayan n. agar agar; N.L. n. *agarum* agar (algal polysaccharide); L. part. adj. *perforans* perforating (making holes); N.L. part.

adj. *agariperforans* making holes in agar, bacterium making deep hollows in agar].

Main characteristics are those given for the genus. In addition to the above data, cells are 0.5–0.7 μm in width and 5–15 μm in length. Colonies are 3–5 mm in diameter,

Table 1. Characteristics useful in distinguishing strain KMM 3525^T from related marine genera of the phylum CFB

Species/taxon: 1, strain KMM 3525^T; 2, *Cytophaga hutchinsonii*; 3, [*Microscilla furvescens*]; 4, [*Microscilla sericea*]; 5, [*Flexibacter*] *tractuosus*; 6, [*Marinilabilia salmonicolor*]; 7, [*Cytophaga*] *fermentans*; 8, *Persicobacter diffluens*; 9, [*Flammeovirga aprica*]; 10, [*Microscilla marina*]; 11, [*Cyclobacterium marinum*]; 12, [*Flexibacter*] *aggregans*; 13, [*Flexibacter*] *polymorphus*; 14, [*Flexibacter*] *litoralis*. Data are from Reichenbach (1989), Lewin & Lounsbury (1969), Nakagawa *et al.* (1997), Suzuki *et al.* (1999), Raj & Maloy (1990) and this study. ND, Not determined; +, positive; -, negative; v, variable; A, strict aerobic; F, facultative anaerobe.

Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Reaction to oxygen	A	A	A	A	A	F	F	A	A	A	A	A	A	A
Oxidase/catalase	+/+	+/-	ND	ND	ND	+/+	-/-	+/-	+/-	+/+	+/+	+/+	ND/-	ND/-
Flexirubin	+	+	ND	ND	ND	-	-	-	-	-	-	-	ND	ND
Gliding motility	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Requirement for Na ⁺ or seawater for growth	+	-	ND	+	-	+	+	+	+	+	+	+	+	+
Carbohydrate utilization	-	+	+	+	+	+	+	+	+	+	-	+	ND	-
Hydrolysis of:														
Agar	+	-	+	-	-	V	+	+	+	-	-	+	-	-
Gelatin	+	ND	+	+	V	+	ND	+	+	+	-	-	+	+
Casein	-	ND	ND	ND	ND	+	ND	-	-	ND	-	-	ND	ND
Starch	+	-	+	+	V	+	+	+	+	-	-	-	-	+
Filter paper	-	+	ND	ND	ND	-	-	-	-	ND	-	-	ND	ND
CM-cellulose	-	+	+	-	-	-	-	-	-	-	-	±	-	-
DNA G+C content (mol%)	44.5	39–42	44	38–39	37	41.5	40–42	40–42	35–37	42	38.4	37–42	29	31

circular, sunken into agar and shiny with entire edges on solid media containing high-nutrient components. Growth occurs at 4–35 °C; optimal temperature range for growth is 25–28 °C. Growth occurs in presence of 1–6% NaCl. Decomposes agar, starch, alginate, gelatin, DNA, urea and Tween 20. Does not hydrolyse cellulose (CM-cellulose and filter paper), chitin, casein, Tween 40, Tween 60 or Tween 80. Forms no acid from arabinose, galactose, glucose, lactose, maltose, melibiose, rhamnose, sucrose, xylose, adonitol, dulcitol, inositol or mannitol. Does not utilize citrate, fumarate or malate. Nitrate is not reduced. Does not produce H₂S or indole. DNA G + C content is 44.5 mol%. Type strain is KMM 3525^T (= IFO 16625^T = JCM 11238^T). Isolated from a seawater sample collected in the Amursky Bay of the Gulf of Peter the Great, Sea of Japan.

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