Branchiibius hedensis gen. nov., sp. nov., an actinobacterium isolated from a Japanese codling (*Physiculus japonicus*)

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A novel, Gram-stain-positive bacterial strain, Mer 29717^T, was isolated from the branchia (gills) of a Japanese codling, *Physiculus japonicus*, collected from bottom waters of Suruga Bay in Shizuoka, Japan. Phylogenetic analysis based on 16S rRNA gene sequences indicated that this strain represents a distinct lineage within the family *Dermacoccaceae* and was related most closely to members of the genera *Demetria* and *Yimella*. It shared highest 16S rRNA gene sequence similarity (95.1 %) with *Yimella lutea* YIM 45900^T. Strain Mer 29717^T contained MK-8(H₂) and MK-8(H₄) as menaquinones, and iso-C_{16:0}, C_{16:0}, C_{17:1} *cis*-9, C_{17:0}, C_{18:1} *cis*-9 and C_{19:1} *cis*-10 were the major cellular fatty acids. The cell-wall peptidoglycan of strain Mer 29717^T was composed of L-Lys, D-Ser, L-Ser, Gly, D-Glu and D-Ala. Polar lipids were phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and one unidentified phospholipid. Mycolic acids were not detected. The G+C content of the DNA of strain Mer 29717^T was 68 mol%. On the basis of differential chemotaxonomic, physiological and biochemical data, strain Mer 29717^T is considered to represent a novel species of a new genus, for which the name *Branchiibius hedensis* gen. nov., sp. nov. is proposed. The type strain of *Branchiibius hedensis* is Mer 29717^T (=NBRC 106121^T =DSM 22951^T).

The description of an increasing diversity of actinobacteria has led to the discovery of secondary metabolites that can be applied in the pharmaceutical industry. In this regard, we have used soil and plant material as sources and have isolated over 30 000 actinobacteria. Over the past decade, many novel bioactive secondary metabolites discovered from marine actinobacteria have been reported (Lam, 2006), and these metabolites may have pharmaceutical potential (Fiedler *et al.*, 2005; Jensen *et al.*, 2005; Fenical & Jensen, 2006). Due to the attractiveness of marine sources for biologically divergent micro-organisms, we have been seeking to expand the diversity of our library. Here we describe the taxonomic characterization and classification of a novel actinobacterial strain, Mer 29717^T, isolated from a marine resource. Phylogenetic analysis shows that this strain forms a monophyletic branch in the family *Dermacoccaceae* (Stackebrandt & Schumann, 2000).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Mer 29717^T is AB520822.

Two supplementary figures are available with the online version of this paper.

Strain Mer 29717^T was isolated from the branchia of a Japanese codling, *Physiculus japonicus*, which was collected from bottom waters of Suruga Bay in Shizuoka prefecture, Japan. The sample was treated with 70 % ethanol (v/v) for 1 min to sterilize the surface, cut into small pieces (approx. 3 × 3 mm²) and ground with a pestle in sterilized PBS. This suspension was diluted appropriately and spread onto isolation plates. The medium used for isolation of strain Mer 29717^T was HVA medium (Hayakawa & Nonomura, 1987). The isolation plates were incubated for 21 days at 28 °C. Colonies of actinomycetes on isolation plates were transferred to sterilized International *Streptomyces* Project (ISP) medium 2 (Difco) and maintained at 28 °C.

Colony morphology, size and colour were examined by using cultures grown for 2 weeks on ISP medium 2. Gram-staining was carried out by using a standard Gram reaction kit (BD). Salt tolerance was tested in ISP medium 2 supplemented with 0, 4, 7, 10 and 13 % (w/v) NaCl after 2 weeks of incubation at 28 °C. Growth at 5, 10, 15, 20, 25, 28, 30, 37, 42, 45 and 50 °C was examined on ISP medium 2. Cell motility and morphology were studied by using

phase-contrast and scanning electron microscopy (model JSM-5600; JEOL), respectively. Cells for morphological observation were cultured for 2 days at 28 °C on R2A medium (Difco). Enzyme profiling was performed with API ZYM microtubes (bioMérieux), according to the manufacturer's instructions. After 12 h of incubation at 37 °C, data were recorded both as positive/negative and on a scale of 0–5. Other cultural characteristics were determined according to the methods of the ISP (Shirling & Gottlieb, 1966). Chemotaxonomic characteristics were determined by using cells grown in trypticase soy broth (Difco) at 28 °C and 220 r.p.m. for 4 days. Respiratory quinones were extracted with chloroform/methanol (2:1, v/v), separated on preparative TLC plates and determined by LC-MS with an HP Agilent 6320 Ion Trap (Agilent Technologies). The G+C content of the DNA was determined as described by Katayama-Fujiwara *et al.* (1984) and Katoh *et al.* (1983). The profile of whole-cell fatty acid methyl esters was determined by using the MIDI Sherlock Microbial Identification system (Microbial ID) (Miller, 1982). Fatty acids unidentified by this system ($C_{16:1}$ *trans*-9 and $C_{19:1}$ *cis*-10) were analysed via GC-MS (PerkinElmer). Whole-cell sugars were analysed as described by Yang *et al.* (2005). Cell walls were isolated and purified according to the method of Schleifer & Kandler (1972). The amino acid composition of cell-wall hydrolysates (Becker *et al.*, 1964) was analysed by HPLC as described by Kawasaki *et al.* (2007). The *N*-acyl type of muramic acid was determined by the colorimetric method of Uchida & Aida (1977). The presence of mycolic acids was investigated by using the TLC method of Minnikin *et al.* (1975) and phospholipids were analysed as described by Minnikin *et al.* (1984).

DNA for 16S rRNA gene sequence analysis was prepared by using an InstaGene matrix (Bio-Rad). The 16S rRNA gene was amplified by PCR with a forward primer corresponding to positions 9–27 and a reverse primer corresponding to

positions 1541–1525 (*Escherichia coli* numbering system; Weisburg *et al.*, 1991) and was sequenced by using the BigDye Terminator version 3.1 cycle sequencing kit (Applied

Table 1. Cellular fatty acid compositions of strain Mer 29717^T and the type strains of related species

Strains: 1, Mer 29717^T; 2, *Demetria terrigena* HKI 0089^T (data from Groth *et al.*, 1997); 3, *Dermacoccus nishinomiyaensis* DSM 20448^T (Stackebrandt *et al.*, 1995); 4, *Kytococcus sedentarius* HO-9042^T (Stackebrandt *et al.*, 1995); 5, *Yimella lutea* YIM 45900^T (Tang *et al.*, 2010); 6, *Luteipulveratus mongoliensis* MN07-A370^T (Ara *et al.*, 2010). Values are percentages of the total fatty acids; –, <0.1%, not detected or not reported.

Fatty acid	1	2	3	4	5	6
Saturated straight-chain						
$C_{14:0}$	0.1	0.5	–	–	3.0	–
$C_{15:0}$	3.1	1.5	–	10.6	1.0	0.7
$C_{16:0}$	5.1	9.8	1.6	2.8	5.7	2.1
$C_{17:0}$	8.5	13.9	1.1	22.2	0.5	6.3*
$C_{18:0}$	1.1	14.2	–	–	–	1.6
$C_{19:0}$	0.3	2.2	–	–	–	–
Unsaturated straight chain						
$C_{15:1}$	–	–	1.6	2.6	–	0.4
$C_{16:1}$ <i>cis</i> -9	–	–	–	–	3.3	1.5
$C_{16:1}$ <i>trans</i> -9	0.3	3.4	7.2	3.1	–	–
$C_{17:1}$ <i>cis</i> -9	25.2	–	–	–	1.0	7.3
$C_{18:1}$ <i>cis</i> -9	15.8	25.2	4.6	–	2.0	1.2
$C_{18:1}$ 2-OH	–	–	–	–	1.6	–
$C_{19:1}$ <i>cis</i> -10	5.0	–	–	–	–	–
$C_{19:1}$ unknown 1†	1.4	–	–	–	–	–
Branched						
iso- $C_{13:0}$	–	–	–	–	4.2	–
anteiso- $C_{13:0}$	–	–	–	–	2.4	–
iso- $C_{14:0}$	0.3	–	–	–	0.5	0.8
iso- $C_{15:0}$	–	1.9	10.3	2.7	35.2	0.4
anteiso- $C_{15:0}$	–	–	–	1.2	12.1	0.4
iso- $C_{15:1}$	–	–	1.5	–	–	–
iso- $C_{16:0}$	22.3	7.4	6.0	2.0	4.8	45.8
iso- $C_{16:1}$	–	–	1.8	–	0.5	7.6
iso- $C_{17:1}$	–	–	18.0	10.6	–	–
anteiso- $C_{17:1}$	–	–	2.5	–	0.8	2.5
iso- $C_{17:0}$	–	6.2	17.8	8.1	5.5	1.1
anteiso- $C_{17:0}$	0.6	9.9	17.1	29.8	11.1	7.9
9-Methyl $C_{16:0}$	–	–	–	–	2.0	–
10-Methyl $C_{16:0}$	–	–	–	–	–	2.0
10-Methyl $C_{17:1}$	2.8	–	–	–	–	–
10-Methyl $C_{17:0}$	2.8	–	–	–	–	6.4
10-Methyl $C_{18:0}$	0.7	–	–	–	–	0.4
iso- $C_{18:0}$	–	–	2.4	–	–	2.4
anteiso- $C_{18:0}$	3.5	–	–	–	–	–
$t_{19}\ddagger$	–	1.8	–	–	–	–

*Ara *et al.* (2010) listed two components as $C_{17:0}$, representing 4.0 and 2.3% of the total. The identity of these components is not clear.

†The double bond position and *cis/trans* isomer were not identified.

‡Identified by Groth *et al.* (1997) as 10-methoxyl octadecanoic acid.

Fig. 1. Scanning electron micrograph of cells from a 2-day-old culture of strain Mer 29717^T grown on R2A medium at 28 °C. Bar, 1 μm.

Biosystems) on an automatic sequence analyser (3100 DNA analyser; Applied Biosystems). Strains related closely to strain Mer 29717^T were identified by performing sequence database searches with the BLAST program (Altschul *et al.*, 1990) and the EzTaxon server (Chun *et al.*, 2007). Sequence data for related strains were retrieved from GenBank and used for detailed phylogenetic analysis. The CLUSTAL X program (Thompson *et al.*, 1997) was used to align the 16S rRNA gene sequences. Phylogenetic analyses were performed by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. A phylogenetic tree was reconstructed with the neighbour-joining method of Saitou & Nei (1987) from K_{nuc} values (Kimura, 1980) by using MEGA version 4.0 (Tamura *et al.*, 2007). The topology of the phylogenetic tree was evaluated by the bootstrap method of Felsenstein (1985) based on 1000 replicates.

Cells of strain Mer 29717^T were Gram-stain-positive, non-motile, aerobic to microaerobic cocci, approximately 0.7–0.9 µm in diameter (Fig. 1). Colonies on ISP medium 2 after 2 weeks at 28 °C were circular, convex, pale yellow and usually 1.0 mm in diameter. The isolate grew on ISP medium 2 with up to 7% NaCl; optimum growth was observed in the absence of NaCl. Growth was observed at 15–37 °C, with an optimum between 25 and 30 °C. Other physiological properties are given in the genus and species descriptions.

The cell-wall peptidoglycan of strain Mer 29717^T contained L-Lys, D-Ser, L-Ser, Gly, D-Glu and D-Ala in a molar ratio of 1.6:1.4:1.0:0.4:2.0:0.9, corresponding to peptidoglycan type A4α of Schleifer & Kandler (1972). Strain Mer 29717^T contained MK-8(H₂) (52%) and MK-8(H₄) (48%) as menaquinones. The cell-wall muramic acid was of the acetyl type. Mycolic acids were not detected. The polar lipids consisted of phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and one unidentified phospholipid (see Supplementary Fig. S1, available in IJSEM Online). Whole-cell sugars were galactose, mannose, rhamnose, ribose, glucose and arabinose. The G+C content of the DNA of strain Mer 29717^T was 68 mol%. The cellular fatty acid profile of strain Mer 29717^T was

dominated by C_{17:1} *cis*-9, iso-C_{16:0}, C_{18:1} *cis*-9, C_{17:0}, C_{19:1} *cis*-10 and C_{16:0} (Table 1).

The almost-complete 16S rRNA gene sequence (1524 nt) of strain Mer 29717^T was determined. A BLAST search in GenBank and the EzTaxon server showed that strain Mer 29717^T was related most closely to *Yimella lutea* YIM 45900^T (95.1% 16S rRNA gene sequence similarity), *Demetria terragena* HKI 0089^T (94.6%), *Luteipulveratus mongoliensis* MN07-A0330^T (94.6%) and *Dermacoccus nishinomyaensis* DSM 20448^T (94.7%). These results suggest that the isolate belongs within the family *Dermacoccaceae*. However, levels of 16S rRNA gene sequence similarity to the type strains of species in the family were 92.2–95.1%. Furthermore, phylogenetic trees reconstructed by the three tree-making algorithms revealed that strain Mer 29717^T formed a distinct lineage within the family *Dermacoccaceae* (Fig. 2 and Supplementary Fig. S2).

At the time of writing, the family *Dermacoccaceae* comprises five genera, *Demetria* (Groth *et al.*, 1997), *Dermacoccus* (Stackebrandt *et al.*, 1995), *Kytococcus* (Stackebrandt *et al.*, 1995), *Luteipulveratus* (Ara *et al.*, 2010) and *Yimella* (Tang *et al.*, 2010). Strain Mer 29717^T could be differentiated from other genera of the family *Dermacoccaceae* based on a number of morphological and chemotaxonomic characteristics (Table 2). The cell-wall peptidoglycan of strain Mer 29717^T contained L-Lys, D-Ser, L-Ser, Gly, D-Glu and D-Ala in a molar ratio of 1.6:1.4:1.0:0.4:2.0:0.9. Similar to members of the genera *Demetria*, *Yimella* and *Luteipulveratus*, strain Mer 29717^T contains Ser in the peptidoglycan but it clearly differs from them by the absence of Asp. Strain Mer 29717^T can also be distinguished from *Dermacoccus* and *Kytococcus* in that it has a different amino acid composition of the cell wall. In addition, strain Mer 29717^T can be distinguished from the other genera in the family *Dermacoccaceae* by containing MK-8(H₂) and MK-8(H₄) as major menaquinones.

Other differential characteristics included phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and one unidentified phospholipid as total phospholipids. In addition, strain Mer 29717^T contained galactose, mannose, rhamnose, ribose, glucose and arabinose as whole-cell

Fig. 2. Neighbour-joining tree showing the phylogenetic positions of strain Mer 29717^T and related organisms, inferred from 16S rRNA gene sequence analyses. Bootstrap values >50% (from 1000 bootstrap resamplings) are indicated. Asterisks indicate phylogenetic lines that were also recovered in the maximum-parsimony tree. *Turicella otitidis* DSM 8821^T was used as an outgroup to define the root of the tree. Bar, 0.01 substitutions per nucleotide position. A maximum-likelihood tree is shown in Supplementary Fig. S2.

Table 2. Differential morphological and chemotaxonomic features between strain Mer 29717^T and members of the family *Dermacoccaceae*

Data for reference genera were taken from Groth *et al.* (1997) (*Demetria*), Stackebrandt *et al.* (1995) (unless indicated) (*Dermacoccus* and *Kytococcus*), Tang *et al.* (2010) (*Yimella*) and Ara *et al.* (2010) (*Luteipulveratus*). $\pm$, Weakly positive; v, variable; ND, no data available.

Characteristic	Mer 29717 ^T	<i>Demetria</i>	<i>Dermacoccus</i>	<i>Kytococcus</i>	<i>Yimella</i>	<i>Luteipulveratus</i>
Cell morphology	Coccoid	Coccoid or rods	Coccoid	Coccoid	Coccoid	Coccoid to short rods
Colony colour	Pale yellow	White to pale yellow	Bright orange	Deep buttercup yellow or cream-white	Orange	Cream to bright yellow
Cell size (μm)	0.7×0.9	0.8×1.2 or 0.8×3.0	0.9×1.6	0.8×1.1	$0.7\text{--}0.9 \times 1.3$ –1.8	0.5×1.0 to 0.8×1.2
Nitrate reduction	+	–	v	v	+	–
Hydrolysis of:						
Gelatin	+	–	+	+	+	+
Starch	–	+	–	–	–	$\pm$
Conditions for growth						
NaCl range (% w/v)	0–7	0–12	0–7	0–10	0–8	0–2
10 °C	–	ND	–	ND	–	+
37 °C	+	–	+	+	+	–
40 °C	+	–	ND	+	+	–
API ZYM results						
α -Fucosidase	–	–	+	ND	–	$\pm$
β -Glucosidase	+	–	+	ND	–	–
Lipase (C14)	–	$\pm$	+	ND	–	–
Trypsin	+	$\pm$	+	ND	+	+
Amino acid composition of peptidoglycan (ratio)	L-Lys (1.6), D-Ser (1.4), L-Ser (1.0), Gly (0.4), D-Glu (2.0), D-Ala (0.9)	L-Lys (0.8), D-Ala (0.8), L-Ala (1.0), D-Asp (1.2), D-Glu (1.0), L-Ser (0.8)	Ala (3.0), Ser (0.9), Glu (2.3), Lys (1.0)*	Ala (1.6), Gly (0.1), Glu (2.5), Lys (1.0)†	Ala (1.6), Gly (1.0), Ser (0.7), Asp (0.8), Glu (1.0), Lys (1.2)	L-Lys (0.8), Ala (2.7), Asp (0.6), Glu (1.0), Ser (0.9)
Major menaquinone(s)	8(H ₂), 8(H ₄)	8(H ₄)	7(H ₂), 8(H ₂), 9(H ₂)	8, 9, 10	8(H ₄)	8(H ₄), 8(H ₆)
Fatty acids‡	I, U, S, A	S, U, A, I	S, A, I	S, A, I	A, I	A, I, U
Polar lipids§	PI, PG, DPG, 1PL	PI, PG, DPG, PE, 2PL	DPG, PG, PI	DPG, PG, PI	DPG, PI, Glu-NU, PL	DPG, PG, PI
DNA G + C content (mol%)	68	66	66–71	68–69	65.8	68.2

*Data from Pathom-aree *et al.* (2006a, b).

†Data from Kämpfer *et al.* (2009).

‡I, Iso-branched; U, unsaturated; S, straight-chain saturated; A, anteiso-branched. Data from Kämpfer *et al.* (2009) and Stackebrandt *et al.* (1995).

§DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; Glu-NU, glucosamine-containing phospholipid; PL, unidentified phospholipid.

sugars. Mycolic acids were not present. The fatty acid profile was characterized by the occurrence of large amounts of unsaturated straight-chain components (C_{17:1} *cis*-9 and C_{18:1} *cis*-9) and one iso-branched-chain fatty acid (iso-C_{16:0}) (Table 1). The G + C content of the DNA of strain Mer 29717^T was 68 mol%. Therefore, on the basis of differential chemotaxonomic characteristics (Table 2), in particular the amino acid composition of the peptidoglycan, in combination with menaquinone components MK-8(H₄) and MK-8(H₆), strain Mer 29717^T can be

distinguished clearly from members of the family *Dermacoccaceae*.

The closest phylogenetic neighbour to strain 29717^T is *Demetria terrigena* HKI 0089^T (94.6% 16S rRNA gene sequence similarity), the latter differing in the amino acid composition of the peptidoglycan, predominant menaquinones and major fatty acids, as well as physiological, cultural and biochemical characteristics (Tables 1 and 2). Furthermore, morphological differences distinguish strain

Mer 29717^T from members of the genus *Demetria*. Obvious differences in chemotaxonomic, morphological and physiological properties were also found between strain Mer 29717^T and *Yimella lutea* (Table 2). Therefore, on the basis of the distinct phylogenetic position of the novel isolate within the family *Dermacoccaceae*, and differences in physiological, morphological and biochemical characteristics, we propose that strain Mer 29717^T represents a novel species of a new genus, for which the name *Branchiibius hedensis* gen. nov., sp. nov. is proposed.

Description of *Branchiibius* gen. nov.

Branchiibius [Bran.chi.i.bi'us. L. pl. n. *branchiae* the gills of fish; N.L. masc. n. *bius* (from Gr. masc. n. *bios*) life; N.L. masc. n. *Branchiibius* a life existing in gills of fish].

Cells are Gram-stain-positive, coccoid (0.7 × 0.9 µm), halotolerant, non-motile and aerobic to microaerobic. They do not form endospores. Colonies are circular, convex, entire, smooth and pale yellow. The cell-wall peptidoglycan contains L-Lys, D-Ser, L-Ser, Gly, D-Glu and D-Ala. The acyl type of the muramic acid in the peptidoglycan is acetyl. Menaquinones are MK-8(H₂) and MK-8(H₄). Phospholipids are phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and one unidentified phospholipid, and whole-cell sugars are galactose, mannose, rhamnose, ribose, glucose and arabinose. Mycolic acids are absent. The major fatty acids are C_{17:1} *cis*-9, C_{18:1} *cis*-9 and iso-C_{16:0}. Phylogenetically, the genus is a member of the family *Dermacoccaceae* and the type species is *Branchiibius hedensis*.

Description of *Branchiibius hedensis* sp. nov.

Branchiibius hedensis sp. nov. (he.den'sis. N.L. masc. adj. *hedensis* of or belonging to Heda, a town in Shizuoka prefecture, Japan, from where the codfish providing the source of the type strain was collected).

Morphological, chemotaxonomic and general characteristics are as given above for the genus. In addition, colonies grown on ISP medium 2 after 2 weeks at 28 °C are 1.0 mm in diameter. Good growth occurs on HVA medium and ISP media 2, 3 and 6. Optimal growth occurs at 20–37 °C; no growth occurs at 50 °C. Growth occurs in the presence of NaCl at concentrations up to 7.0%. Nitrates are reduced. Melanin is not produced. Gelatin is hydrolysed but coagulation of milk is not observed. Starch is hydrolysed weakly. Decomposes calcium malate and cellulose weakly. Hydrogen sulfide is not produced. According to API ZYM enzyme assays, positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase. Utilizes D-glucose, D-fructose, sucrose and D-mannitol as carbon sources, but not L-arabinose, D-xylose, L-rhamnose,

raffinose or inositol. In addition to the major fatty acids listed for the genus, minor amounts of C_{17:0}, C_{16:0}, C_{19:1} *cis*-10, anteiso-C_{18:0}, C_{15:0}, 10-methyl C_{17:1} and 10-methyl C_{17:0} are also present. The DNA G + C content of the type strain is 68 mol%.

The type strain, Mer 29717^T (=DSM 22951^T =NBRC 106121^T), was isolated from the branchia of a Japanese codling, *Physiculus japonicus*.

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