Mechercharimyces mesophilus gen. nov., sp. nov.
and *Mechercharimyces asporophorigenens*
sp. nov., antitumour substance-producing marine
bacteria, and description of
Thermoactinomycetaceae fam. nov.

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A study was carried out to clarify the taxonomy of four Gram-positive, heterotrophic mesophiles isolated from marine lakes in the Republic of Palau. The strains, designated YM3-251^T, YM3-653, YM3-671 and YM11-542^T, formed aerial and substrate mycelia. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid, glutamic acid and alanine. The G + C content of their genomic DNA was approximately 45 mol%. The major fatty acid was iso-C_{15:0} and the major isoprenoid quinone was MK-9. The strains formed a distinct group in the 16S rRNA gene tree and shared a range of phenotypic properties that distinguished them from members of related genera in *Thermoactinomycetaceae* fam. nov. The name proposed to accommodate the new isolates is *Mechercharimyces* gen. nov., comprising two species based on genotypic and phenotypic criteria, including comparative *gyrB* and DNA–DNA relatedness data. The names proposed for these taxa are *Mechercharimyces mesophilus* sp. nov., the type species, and *Mechercharimyces asporophorigenens* sp. nov., with the type strains YM3-251^T (=MBIC06230^T = DSM 44894^T) and YM11-542^T (=MBIC06487^T = DSM 44955^T), respectively.

Lacey & Cross (1989) described the genus *Thermoactinomyces* as the only genus of thermoactinomycetes, which was placed in the family *Bacillaceae* (Stackebrandt & Woese, 1981). More recently, members of the genus *Thermoactinomyces* were divided into four genera, *Thermoactinomyces*, *Laceyella*, *Thermoflavimicrobium* and *Seinonella* (Yoon & Park, 2000; Yoon *et al.*, 2005). These four genera and the genus *Planifilum* form a coherent unit in phylogenetic trees based on 16S rRNA gene sequences (Yoon *et al.*, 2000; Hatayama *et al.*, 2005). They are aerobic, Gram-positive and thermophilic, with the exception of one mesophilic species, *Seinonella peptonophila* (Nonomura &

Ohara, 1971). Here, we report another mesophilic member of this phylogenetic group and *Thermoactinomycetaceae* fam. nov. is described.

As part of a study to characterize the bioactive compounds produced by marine micro-organisms, we isolated cytotoxic cyclopeptides from a mesophilic bacterium from our marine microbial library that showed filamentous growth (Kano *et al.*, 2005). All of the strains in our marine microbial library have been annotated with their 16S rRNA gene sequence, making it easy to find phylogenetically related strains by searching for 16S rRNA gene sequence similarity. Three other related strains were also identified as a result of a similarity search of our library. In this paper, we present the characteristics and relationships of isolates representing *Thermoactinomycetaceae* fam. nov., by using a polyphasic taxonomic approach.

Strains YM3-251^T, YM3-653 and YM3-671 were isolated from sediment samples collected from a marine lake (7° 9' 49" N 134° 22' 29" E) on Mecherchar Island, Republic of Palau, by using, respectively, 1/10 MYGS-AF medium, 1/10 PYGS-AF medium and skimmed milk medium. Strain YM11-542^T was isolated from a sediment sample collected

Abbreviation: *meso*-DAP, *meso*-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers determined in this study for the 16S rRNA gene sequences are AB239529–AB239532 and those for the *gyrB* gene sequences are AB242198–AB242206 and AB243408.

Details of media used for strain isolation, micrographs of spore-forming cells of strains YM3-251^T and YM11-542^T and a table detailing chemotaxonomic characteristics of strains YM3-251^T, YM3-653, YM3-671 and YM11-542^T are available as supplementary material in IJSEM Online.

from a marine lake (7° 17' 74" N 134° 26' 92" E) in the northern part of Urukthapel Island, Republic of Palau, by using 1/10 PYGS-AF medium. For isolation of strain YM3-251^T, the sediment sample was heated at 52 °C for 2 h prior to application to the medium. For isolation of strain YM3-653, the sediment sample was dried on filter paper for 24 h and on silica gel for 1 month. The other two strains were isolated from non-pretreated sediment samples. Details of the media used are available as supplementary material in IJSEM Online. Strains YM3-251^T, YM3-653 and YM3-671 grew at 15–37 °C (optimum 30 °C) and strain YM11-542^T at 20–37 °C (optimum 30 °C) on marine agar 2216 (Difco). All strains grew on marine agar 2216 containing 25 µg novobiocin ml⁻¹. Aerial and substrate mycelia were observed in the cultures of the four strains. Substrate mycelia were well-developed, branched and septate. Cultures of strains YM3-251^T, YM3-653 and YM3-671 exhibited endospores singly on short, unbranched sporophores. The YM11-542^T culture exhibited oval-shaped endospores in both substrate and aerial mycelia, but not sessile endospores or sporophores (phase-contrast micrographs are available as Supplementary Fig. S1 in IJSEM Online).

Genomic DNA was purified from the four strains by using a QIAGEN Genomic-tip and buffer set (Qiagen). The 16S rRNA gene fragment was amplified by using universal primers corresponding to positions 8–27 as the forward primer and 1492–1510 as the reverse primer (*Escherichia coli* numbering system; Weisburg *et al.*, 1991). Based on the 1439 bp-long 16S rRNA gene sequences, phylogenetically related bacteria were aligned by using the BLAST program (Altschul *et al.*, 1990) against the GenBank database and Classifier analysis in RDP-II release 9 (Cole *et al.*, 2005). The results suggested that the strains were affiliated with members of the order *Bacillales*. Phylogenetic trees were constructed to deduce the interspecies relationships in the order *Bacillales*. Members of the genera *Thermoactinomyces*, *Laceyella*, *Seinonella*, *Thermoflavimicrobium*, *Planifilum* and the four novel strains formed a coherent unit as shown in Fig. 1(a). In this coherent unit, the four strains formed a distinct monophyletic clade that was supported by a high bootstrap value (79 %). The 16S rRNA gene sequences of the four strains were highly conserved, as they had similarity values in the range 99.4–99.9 %, values that correspond to 2–9 nucleotide differences, respectively, at 1428 locations. Nevertheless, it is evident from Fig. 1(a) that the four strains could be assigned to two groups supported by high bootstrap values. In cases such as this, the nucleotide sequences of aligned stretches of DNA that are less conserved than those of the corresponding 16S rRNA gene sequences, such as the gene encoding the DNA gyrase B subunit (*gyrB*), can be used to highlight differences between strains (Yamamoto & Harayama, 1995). *gyrB* gene sequences were analysed to differentiate ten strains, including the type strains of members of the genera *Thermoactinomyces*, *Laceyella*, *Seinonella* and *Thermoflavimicrobium*. The *gyrB* gene fragment corresponding to positions 274–1525 in the *E. coli gyrB* gene was amplified by using the deoxyinosine

nucleotide-containing primers UP1Gi (5'-GAAGTCATCACCGTTCTGCAYGSIGGIGGIAARTTYGG-3') and GBgc-R (5'-GTGAGTAACAATGTACGDATRTGIGCICCRTCIAC-3'). The PCR products were purified by using Montage PCR₉₆ (Millipore). The nucleotide sequence was determined by using primers UP1s (5'-GAAGTCATCACCGTTCTGCA-3') and GBgc-Rs (5'-GTGAGTAACAATGTACG-3').

The 1167–1173 bp nucleotide sequences of *gyrB* were aligned using CLUSTAL_X software (Thompson *et al.*, 1997). Phylogenetic and molecular evolutionary analyses based on the 1167 bp nucleotide sequences of *gyrB* were conducted by using MEGA version 3.0 (Kumar *et al.*, 2004). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method were determined by using bootstrap values based on 1000 replications. It is clear from Fig. 1(b) that the *gyrB* sequence data provide a much better resolution of the interspecies relationship between the two novel species. For the four novel strains, the *gyrB* sequences had similarity values within the range 96.5–99.8 %, corresponding, respectively, to 2–40 nucleotide differences at 1167 locations. The taxonomic integrity of the two sharply delineated groups was supported by high bootstrap values. Alignment gaps were also helpful in confirming the branching pattern. The results were supported by data from DNA–DNA hybridization experiments, which were conducted by using the method of Ezaki *et al.* (1989). Strains YM3-653 and YM3-671 had DNA–DNA relatedness values of 95 and 81 %, respectively, with reference DNA from strain YM3-251^T; strains YM3-251^T and YM11-542^T had a DNA–DNA relatedness of 49 %. The DNA G + C contents of the strains were determined by using the method of Tamaoka & Komagata (1984); the values obtained were approximately 45 mol%, whereas the values for the type strains of other related genera were 40–60.3 mol%.

Chemotaxonomic characteristics of strains YM3-251^T and YM11-542^T were determined by using cells cultured in marine broth 2216 at the exponential phase of growth. Cell walls were prepared from approximately 100 mg (dry weight) bacterial cells, as described by Schleifer & Kandler (1972). Amino acids in an acid hydrolysate of the cell walls were identified by using two-dimensional descending chromatography on cellulose TLC plates (Tokyo Kasei), using the method of Harper & Davis (1979), and by HPLC, as phenylthiocarbamoyl derivatives, with a model LC-10AD HPLC apparatus (Shimadzu) equipped with a Wakopak WS-PTC column (Wako Pure Chemical Industries). Menaquinones and cellular fatty acids were determined as described by Nishijima *et al.* (1997) and Katsuta *et al.* (2005). The cell-wall peptidoglycan contained *meso*-diaminopimelic acid (*meso*-DAP), glutamic acid and alanine in the ratio 1.1 : 1 : 2.7 for strain YM3-251^T and 1.2 : 1 : 2.9 for strain YM11-542^T, but no characteristic sugars. MK-9 and MK-8 were observed in strains YM3-251^T (73 and 21.7 %, respectively) and YM11-542^T (76.1 and 23.9 %, respectively). This profile is similar to those of members of the genus *Laceyella* (Yoon *et al.*, 2005). The predominant

Fig. 1. Phylogenetic tree based on 16S rRNA (a) and *gyrB* (b) gene sequences of the four strains and members of the family *Thermoactinomycetaceae*. The tree was constructed by using the neighbour-joining method. Numbers at nodes are percentage bootstrap values based on 1000 replications (only values greater than 50% are shown). GenBank/EMBL/DBJ accession numbers are given in parentheses. Bar, 2 nucleotide substitutions (a) and 5 nucleotide substitutions (b) per 100 nucleotides.

cellular fatty acid was iso-C_{15:0}, similar to other members of the *Thermoactinomycetaceae*, except for two species of the genus *Planifilum* and *S. peptonophila* KCTC 9740^T. Details of menaquinone and cellular fatty acid compositions are available as Supplementary Table S1 in IJSEM Online.

Physiological and biochemical tests were performed by using GP2 MicroPlate (Biolog) and API ZYM (bioMérieux). No reproducible results were obtained from the GP2 MicroPlate because of weak growth of the strains. In API ZYM tests, alkaline phosphatase activity was observed in all

strains. Trypsin activity was observed for strains YM3-251^T, YM3-653 and YM3-671, but not for strain YM11-542^T. Degradation of substrates was tested by using marine agar 2216 supplemented with 2 % casein, 0.2 % starch, 0.5 % xanthine, 0.5 % hypoxanthine, 1 % gelatin, 0.1 % aesculin, 0.5 % L-tyrosine or 0.15 % chitin. The four strains all degraded gelatin and casein, but not the other substrates tested. All strains produced dark-brown pigment on L-tyrosine-containing marine agar.

Strains YM3-251^T, YM3-653, YM3-671 and YM11-542^T could be clearly distinguished from members of the family *Thermoactinomycetaceae*, based on optimal growth temperature and the predominant menaquinone (Table 1). Phylogenetic analyses based on 16S rRNA and *gyrB* gene sequences showed that the four strains constituted an independent clade within the family *Thermoactinomycetaceae*. Strain YM11-542^T could be distinguished from the other three strains based on 16S rRNA and *gyrB* gene sequence similarity, the level of DNA–DNA relatedness and the presence of endospores. The genotypic and phenotypic data acquired in the present study show that the four strains should be assigned as representing two novel species in a new genus in *Thermoactinomycetaceae* fam. nov., for which the names *Mechercharimyces mesophilus* gen. nov., sp. nov. (the type species) and *Mechercharimyces asporophorigenens* sp. nov. are proposed.

Description of *Thermoactinomycetaceae* fam. nov.

Thermoactinomycetaceae (Ther'mo.ac.ti.no.my.ce.ta'ce.ae. N.L. masc. n. *Thermoactinomyces* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Thermoactinomycetaceae* the *Thermoactinomyces* family).

Form aerial mycelia and substrate mycelia. Aerial mycelia are abundant and white or yellow. Form well-developed, branched and septate substrate mycelia. Form sessile spores, singly on aerial and substrate hyphae, or on simple or branched sporophores, with the structure and properties of bacterial endospores. Gram-positive, chemo-organotrophic and aerobic. The genera *Thermoactinomyces*, *Laceyella*, *Seinonella*, *Thermoflavimicrobium*, *Planifilum* and *Mechercharimyces* form a coherent phylogenetic unit on the basis of partial 16S rRNA gene sequences and comprise the family. Cell-wall peptidoglycan contains *meso*-DAP. Major menaquinones are unsaturated with seven or nine isoprene units. The G+C content of the DNA ranges from 40 to 60.3 mol%. The type genus is *Thermoactinomyces* Tsilinsky 1899.

Description of *Mechercharimyces* gen. nov.

Mechercharimyces (Me.ch.er.cha.ri'my.ces. N.L. n. *Mecherchar* a marine lake located on Mecherchar Island in the Republic of Palau, from where the organisms were isolated; Gr. masc. n. *mukes* fungus; N.L. masc. n. *Mechercharimyces* a fungus of Mecherchar).

Cells are aerobic, Gram-positive and mesophilic. Form aerial mycelia and substrate mycelia. Aerial mycelia are abundant and white. Form well-developed, branched and septate substrate mycelia on marine agar 2216. Do not produce soluble pigment. Cell-wall peptidoglycan contains *meso*-DAP, glutamic acid and alanine, but no characteristic sugars. The predominant menaquinone is MK-9. The major fatty acid is iso-C_{15:0}. The DNA G+C content is approximately 45 mol%. The type species is *Mechercharimyces mesophilus*.

Description of *Mechercharimyces mesophilus* sp. nov.

Mechercharimyces mesophilus [me.so.phi'lus. Gr. adj. *mesos* middle; Gr. adj. *philos* loving; N.L. masc. adj. *mesophilus* middle (temperature) -loving, mesophilic].

Exhibits the following properties in addition to those given in the genus description. Colonies are fast-growing, lightly ridged, with a moderate covering of white mycelia and a feathery margin on marine agar 2216 at 27 °C. Growth occurs at 15–37 °C, with optimum growth at 30 °C. Forms endospores singly on short, unbranched sporophores. Casein and gelatin are degraded, but not starch, hypoxanthine, xanthine or L-tyrosine. Produces dark-brown pigment on L-tyrosine-containing marine agar. Growth occurs in the presence of 25 µg novobiocin ml⁻¹. Major cellular fatty acids are iso-C_{15:0}, iso-C_{16:0}, iso-C_{17:1}ω11c and iso-C_{17:0}.

The type strain, YM3-251^T (=MBIC06230^T=DSM 44894^T), was isolated from a sediment sample collected from a marine lake on Mecherchar Island, Republic of Palau. The DNA G+C content of the type strain is 45.1 mol%.

Description of *Mechercharimyces asporophorigenens* sp. nov.

Mechercharimyces asporophorigenens (a.spo'ro.pho.ri.gen. ens. Gr. prep. *a* not; N.Gr. n. *sporophora* sporophore; L. part. adj. *genens* producing; N.L. part. adj. *asporophorigenens* sporophore non-producing).

Exhibits the following properties in addition to those given in the genus description. Colonies are fast-growing, lightly ridged, with a moderate covering of white mycelia and a feathery margin on marine agar 2216 at 27 °C. Growth occurs at 20–37 °C, with optimum growth at 30 °C. Forms oval-shaped endospores in substrate mycelia or aerial mycelia. Does not form sessile endospores or sporophores. Casein and gelatin are degraded, but not starch, hypoxanthine, xanthine or L-tyrosine. Produces dark-brown pigment on L-tyrosine-containing marine agar. Growth occurs in the presence of 25 µg novobiocin ml⁻¹. Major cellular fatty acids are iso-C_{15:0}, iso-C_{16:0}, iso-C_{17:1}ω11c and iso-C_{17:0}.

The type strain, YM11-542^T (=MBIC06487^T=DSM 44955^T), was isolated from a sediment sample collected

Table 1. Differential phenotypic characteristics of the new isolates and the five genera of the family *Thermoactinomycetaceae*

Taxa: 1, strain YM3-251^T; 2, strain YM3-653; 3, strain YM3-671; 4, strain YM11-542^T; 5, *Thermoactinomyces*; 6, *Laceyella*; 7, *Thermoflavimicrobium*; 8, *Planifilum*; 9, *Seinonella*. +, Positive reaction; –, negative reaction; +/–, variable reaction; ND, not detected; NO, not observed. Data from this study, Yoon *et al.* (2005) and Hatayama *et al.* (2005).

Characteristic	1	2	3	4	5	6	7	8	9
Colour of aerial mycelia	White	White	White	White	White	White	Yellow	NO*	White
Growth on 25 µg novobiocin ml ^{−1}	+	+	+	+	+	+	+	No data	–
Degradation of:									
Casein	+	+	+	+	+	+	+	+	–
Gelatin	+	+	+	+	+	+	+	No data	–
Starch	–	–	–	–	+	–	+	+	–
Hypoxanthine	–	–	–	–	–	–	+	–	–
Xanthine	–	–	–	–	–	–	+	–	–
L-Tyrosine	–	–	–	–	–	+/–	–	–	–
Optimal temperature for growth (°C)	30	30	30	30	50–55	48–55	55	55–63	35
Major menaquinone (peak area ratio; %)	MK-9 (73·0)	MK-9 (70·4)	MK-9 (64·3)	MK-9 (76·1)	MK-7	MK-9	MK-7	MK-7	MK-7
Detected menaquinone(s)†	MK-8 (21·7)	MK-8 (22·3)	MK-8 (36·6)	MK-8 (23·9)	MK-8 or MK-9	MK-7, MK-8 or MK-10	ND‡	ND‡	MK-8, MK-9, MK-10
Major fatty acids†	iso-C _{15:0} , iso-C _{17:1} ω11c	iso-C _{15:0} , iso-C _{17:1} ω11c	iso-C _{15:0} , iso-C _{16:0} , iso-C _{17:1} ω11c	iso-C _{15:0} , iso-C _{16:0} , iso-C _{17:1} ω11c, iso-C _{17:0}	iso-C _{15:0} , iso-C _{17:0} , anteiso-C _{15:0}	iso-C _{15:0} , anteiso-C _{15:0}	iso-C _{15:0} , anteiso-C _{15:0} , iso-C _{16:0}	iso-C _{17:0} , anteiso-C _{17:0} (iso-C _{15:0} or C _{16:0})	iso-C _{14:0} anteiso-C _{15:0} iso-C _{16:0}
DNA G+C content (mol%)	45·1	44·9	45·0	45·2	48	48–49	43	58·7–60·3	40

*Aerial mycelia were not observed on LB, CYC (Czapek–Dox-yeast-casein), SY (starch-yeast) or Bacto nutrient plates (Hatayama *et al.*, 2005).

†Other components making up >10% peak area ratio are shown.

‡MK-8 detected at a trace level (Hatayama *et al.*, 2005).

from a marine lake in the northern part of Urukthapel Island, Republic of Palau. The DNA G+C content of the type strain is 45.2 mol%.

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