Thermoflavifilum aggregans gen. nov., sp. nov., a thermophilic and slightly halophilic filamentous bacterium from the phylum Bacteroidetes

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A strictly aerobic, thermophilic, moderately acidophilic, non-spore-forming bacterium, strain P373T, was isolated from geothermally heated soil at Waikite, New Zealand. Cells were filamentous rods, 0.2–0.4 μm in diameter and grew in chains up to 80 μm in length. On the basis of 16S rRNA gene sequence similarity, strain P373T was shown to belong to the family Chitinophagaceae (class Sphingobacteriia) of the phylum Bacteroidetes, with the most closely related cultivated strain, Chitinophaga pinensis UQM 2034T, having 87.6 % sequence similarity. Cells stained Gram-negative, and were catalase- and oxidase-positive. The major fatty acids were i-15 : 0 (10.8 %), i-17 : 0 (24.5 %) and i-17 : 0 3-OH (35.2 %). Primary lipids were phosphatidylethanolamine, two unidentified aminolipids and three other unidentified polar lipids. The presence of sulfonolipids (N-acyl-capnines) was observed in the total lipid extract by mass spectrometry. The G+C content of the genomic DNA was 47.3 mol% and the primary respiratory quinone was MK-7. Strain P373T grew at 35–63 °C with an optimum temperature of 60 °C, and at pH 5.5–8.7 with an optimum growth pH of 7.3–7.4. NaCl tolerance was up to 5 % (w/v) with an optimum of 0.1–0.25 % (w/v). Cell colonies were non-translucent and pigmented vivid yellow–orange. Cells displayed an oxidative chemoheterotrophic metabolism. The distinct phylogenetic position and the phenotypic characteristics separate strain P373T from all other members of the phylum Bacteroidetes and indicate that it represents a novel species in a new genus, for which the name Thermoflavifilum aggregans gen. nov., sp. nov. is proposed. The type strain of the type species is P373T (= ICMP 20041T = DSM 27268T).

Members of the phylum Bacteroidetes are broadly distributed across a range of environments including soils, freshwater and marine environments, and the skin and gastrointestinal tracts of animals and humans. The majority of described members of the phylum Bacteroidetes are mesophilic or even slightly psychrophilic, particularly amongst marine representatives (Bowman et al., 1997; Schmidt et al., 2006). Indeed, thermophilic isolates belonging to this phylum are rare, with few true thermophilic representatives (optimum growth temperature >60 °C). In this study, we describe a novel thermophilic Bacteroidetes strain, isolated from geothermal soils in New Zealand.

There are only a few examples of thermophilic species of the phylum Bacteroidetes, three of which are members of the genus Rhodothermus isolated from submarine
et al. (1996) and are the most thermophilic species described to date. All three species have growth maxima at or just below 80 °C. Saliniibacter ruber (Antón et al., 2002) and Schleiferia thermophilica (Albuquerque et al., 2011) have an elevated growth optimum of 50 °C or just below, but are unable to grow at 60 or 62 °C, respectively. In addition, two species of the genus Thermoneauta isolated from hot springs in New Zealand and Italy have been reported to have growth optima at 60 °C (Hudson et al., 1989; Tenreiro et al., 1997). Previously, we have described the cultivation of novel thermophilic strains from geothermal soils using oligotrophic medium (Stott et al., 1997). Previously, we have described the cultivation of novel thermophilic strains from geothermal soils using oligotrophic medium (Stott et al., 2008). One of these isolates (strain P373T) was provisionally identified as a novel strain of the phylum Bacteroidetes, isolated from geothermally heated clay wall immediately adjacent to a 98 °C hot spring at Waikite, New Zealand. Here the phenotypic and phylogenetic characteristics of this novel isolate are described and a novel genus and novel species are proposed to accommodate this strain.

Soil samples from the clay wall directly above (15 cm above) the spring were aseptically collected with a sterile spatula and transferred into sterile 50 ml Falcon tubes. The soil temperature was 50.7 °C. Soil pH was measured at room temperature by suspending 1 g soil sample in double-distilled H2O. Soil crumbs were spread on half-strength R2A agar (Merck) (Reasoner & Geldreich, 1985) which was solidified by the addition of 1.5 % (w/v) gellan (Phytagel; Sigma) with 2 g MgCl2·6H2O l⁻¹. The pH was adjusted to 4.5 (H2SO4) to reflect the measured soil pH. Plates were incubated at 60 °C in Oxoid anaerobic jars with an aerobic atmosphere supplemented with 10 % (v/v) CO2. Individual bacterial colonies were picked and purified via the streak-plate method until axenic cultures were obtained (Stott et al., 2008). Unless otherwise stated, all physiological and metabolic determinations were conducted by growing P373T at 60 °C on R2A-P medium (3.0 g R2A broth l⁻¹ and 2.0 g NaCl l⁻¹ with no pH adjustment).

Genomic DNA was extracted from the novel isolates using a Nucleospin Tissue kit (Macherey-Nagel) as per the manufacturer’s instructions. Universal bacterial primers 9f and 1492r (Weisburg et al., 1991) were used to amplify the 16S rRNA gene sequence. A near full-length sequence was checked for chimeras (Huber et al., 2004) and subjected to a discontinuous MEGABLAST (Altschul et al., 1997) search against the NCBI database. The 16S rRNA gene sequence of strain P373T was 1396 bp long (Stott et al., 2008). Sequence similarity calculations (using Jukes–Canter correction) indicated that the most closely related strain(s) to P373T were as yet undescribed bacterial strains isolated from heated lumber mill waste (GenBank accession no. AB635381, 100 %) and from compost (JQ033715, 99.1 %), and an environmental clone from sugar cane bagasse (HM362488, 98.5 %) (Rattanachomsri et al., 2011). The most closely related formally described relatives were Chitinophaga pinensis UQM 2034T (Glavina Del Rio et al., 2010) (87.6 %) and Ferruginibacter lapsinanus HU1-HG42T (Lim et al., 2009) (87.4 %). A phylogenetic tree (Fig. 1) was reconstructed using the maximum-likelihood estimator (RAxML) using default settings within the ARB software environment (Ludwig et al., 2004). The 16S rRNA gene sequence of strain P373T was aligned with closely related phylotypes and well-characterized type strains of species of the phylum Bacteroidetes. Confidence estimations at the branching nodes were assessed via 200 bootstrap resamplings. Strain P373TT places within the family Chitinophagaceae within the class Sphingobacteria.

Strain P373T formed bright yellow-orange pigmented colonies on solid medium and similarly coloured pellicles in liquid medium (Fig. 2a, b). The colonies were circular, convex with an entire margin, ~0.2–0.5 cm in diameter, non-translucent and were fully developed 24 h after inoculation. In liquid culture, strain P373T grew in thick yellow-orange cell pellicles within 12 h (Fig. 2b). Cultivation required gentle shaking (120 r.p.m.) in an orbital incubator with no growth observed during static or rapid shaking incubation (>120 r.p.m.). Cells were unicellular, unbranched filaments, very thin with 0.2–0.4 μm diameter and variable length, but cell chains were up to 80 μm long (Fig. 2c). Cells of strain P373T stained Gram-negative. Cryogenic transmission electron microscopy (TEM, FEI Tecnai F30) imaging confirmed the typical Gram-negative tri-layered composition of the cell wall, consisting of the cytoplasmic membrane, a thin layer of peptidoglycan and the lipid outer membrane (Fig. 2d). Cells were non-spore-forming and non-motile, and no pili or flagella were detected via negative stain TEM (using uranyl acetate) analysis or via flagella staining techniques (Heimbrook et al., 1989). We were not able to detect exopolysaccharides using either TEM technique applied; however, exopolysaccharide-like layers were consistently observed when the flagella-stain was applied to cells during liquid medium-based cultivation and was congruent with the thick pellicle formation (O’Toole et al., 2000) and requires further investigation (Fig. S1, available in the online Supplementary Material).

The yellow pigmentation of strain P373T did not vary under any growth conditions tested. No colour change was observed following treatment with 20 % (w/v) aqueous Koh solution indicating that no flexirubin-type pigments were present (Fautz & Reichenbach, 1980). Acetone-, methanol- and hexane-extracted pigments were spectrophotometrically scanned (330–900 nm) with absorbance maxima at 454 nm (acetone), 448 nm (hexane) and 448–450 nm (methanol) with secondary peaks noted at 481 nm (acetone), 448 nm (hexane) and 475–477 nm (methanol). These initial analyses putatively place the primary P373T pigment in the xanthin-like family, possibly meso-zeaxanthin or alloxanthin, or even a version of β-carotene (Watanabe et al., 2000).

Fatty acid methyl esters (FAMEs) were prepared (Ferreira et al., 1999) and analysed without prior extraction as previously described (Lee et al., 2011). The fatty acid composition of strain P373T is presented in Table S1. The
The cell membrane of strain P373T was mainly composed of branched and often hydroxylated fatty acids with the primary fatty acids being i-15:0 (10.8%), i-17:0 (24.5%), and i-17:0 3-OH (35.2%). i-18:1 (1.2%) was also detected. Polar lipids were analysed via TLC and phospholipids were quantified by 31P-NMR (MacKenzie et al., 2009) (Supplementary Methods; Figs S2 and S3). TLC analysis of polar lipids revealed the presence of two phospholipids, two unidentified aminolipids and three other unidentified polar lipids. No glycolipids were observed. Phospholipids of strain P373T were phosphatidylethanolamine (92%) and a minor unidentified aminophospholipid (8%). The total phospholipid content was 22% (w/w) of total lipid.

Mass spectrometry (electrospray ionization time-of-flight negative mode) of the total lipid extract detected sulfonolipids (N-acyl-capnines) as the major peaks in the spectrum (Fig. S4). The quinone and DNA base composition of strain P373T were determined as previously described (Lee et al., 2011) by the Identification Service of the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. The primary quinones of strain P373T were MK-7 (92%) and MK-6 (8%), and the DNA base composition was 47.3 mol% G+C.

As detailed previously, strain P373T grows primarily as a pellicle mass, which made determination of positive or negative growth not practicable via standard optical outgroup.
Strain P373T was an obligate aerobe requiring homogenization to disrupt the cell pellicle prior to cultivation. Cell inoculum was obtained via centrifugation followed by cultivation of the resultant biomass on solid medium. As indicators of growth, and confirmed any positive growth, the MTT (Wang, 2010) and/or the phenol red assays were also tested using API 50 CH 300 strips (bioMérieux) with the substrates supporting positive growth listed in the species description and the full list of substrates tested listed in Table S3.

The preferred nitrogen source was determined by providing different nitrogen sources in otherwise nitrogen-free half-strength R2A medium. Nitrogen sources tested were KNO$_3$, KNO$_2$, (NH$_4$)$_2$SO$_4$, urea, Casamino acids and peptone. Strain P373T preferred peptone as its nitrogen source; Casamino acids provided weak growth. No growth was observed with any other of the tested nitrogen sources. Nitrogen fixation from air was not detectable. In addition, no improvement in biomass production was noted when additional CO$_2$ was supplemented to the oxic headspace indicating a lack of anapleurotic mechanisms such as pyruvate carboxylase or phosphoenolpyruvate carboxylase.

Strain P373T demonstrated positive reactions for catalase and oxidase. A selection of enzymic activities were also tested using the API ZYM 25-200 strips (bioMérieux; Table S4), the results of which are presented in the species description. Antibiotic sensitivity was assessed by growing strain P373T on R2A-P agar with either 10, 100 or 250 µg ml$^{-1}$ antibiotic. No colony growth was observed on solid medium containing 10 or 100 µg ampicillin, rifampicin or tetracycline. Strain P373T was resistant to chloramphenicol, kanamycin, erythromycin and neomycin at 250 µg ml$^{-1}$, and polymyxin B at 100 µg ml$^{-1}$. Metronidazole, monensin, streptomycin, trimethoprim and vancomycin (250 µg ml$^{-1}$) did not inhibit growth. Lasalocid A did not inhibit growth at 10 µg ml$^{-1}$, but was not tested at 100 or 250 µg ml$^{-1}$.

Strain P373T places within the family Chitinophagaceae with the closest described phylogenetic relatives being Chitinophaga pinensis and Ferruginibacter lapsinanis, both with ~87% 16S rRNA gene sequence similarity. Many of its metabolic and phenotypic characteristics are consistent with those of recognized members of the family Chitinophagaceae including a generally neutrophilic pH range and optima, density/turbidity measurements. Likewise, obtaining representative inoculum to give reliable and reproducible results was quite involved. The methodologies for phenotypic determinations have been described in detail in the Supplementary Methods. However, briefly, we employed the MTT (Wang et al., 2010) and/or the phenol red assays as indicators of growth, and confirmed any positive growth by cultivation of the resultant biomass on solid medium. Cell inoculum was obtained via centrifugation followed by homogenization to disrupt the cell pellicle prior to inoculation. Strain P373T was an obligate aerobe requiring a minimum of 5% (v/v) O$_2$ for growth, and was unable to grow anaerobically using nitrate or sulphate as terminal electron acceptors (Supplementary Methods). It had a thermophilic growth range between 35 and 63 °C (but did not at 29 °C or 67 °C) and had a growth optimum of ~60 °C. Strain P373T was able to grow at pH 5.5–8.7. No growth was observed at pH 4.9 and below or at pH 9.2. Optimum growth of strain P373T was observed between pH 7.3 and 7.4. NaCl was not required for growth, but promoted growth at concentrations between 1.0 and 2.5 g l$^{-1}$; strain P373T displayed poor growth with 50 g NaCl l$^{-1}$ and no growth with 60 g NaCl l$^{-1}$. Strain P373T was sensitive to ethanol concentrations >5% (v/v).

Substrate utilization tests were conducted in triplicate in 125 ml serum bottles (NRP-P medium; Supplementary Methods) and were incubated at 60 °C in an oxic atmosphere at 120 r.p.m. for 7 days. A list of substrates tested (0.5 g l$^{-1}$ unless otherwise stated) is given in Table S2 and a complete list of substrates that supported growth is listed in the species description. Strain P373T grew chemo-organotrophically and was able to grow on all mono-, di- and trisaccharides tested, but no growth was observed for sugar alcohols or sugar derivatives except for N-acetyl-D-glucosamine. It was able to degrade amorphous polymers, but was unable to hydrolyse more crystalline polysaccharides such as Avicel. Growth was also observed in medium containing yeast extract, peptone or tryptone, as well as standard complex medium such as nutrient broth (NB), R2A and Luria–Bertani broth (LB). Strain P373T showed no ability to utilize inorganic substrates as energy sources. Some organic salts like sodium fumarate and sodium lactate, but no organic acids, promoted growth. In addition, a selection of carbohydrates and their derivatives were also tested using API 50 CH 300 strips (bioMérieux) with the substrates supporting positive growth listed in the species description and the full list of substrates tested listed in Table S3.

**Fig. 2.** Cell and colony morphology of strain P373T. (a) Colony formation of P373T on solid 50% (w/v) R2A medium; ×10 magnification. (b) Growth of P373T in 50% (w/v) R2A medium. (c) Phase-contrast micrograph of P373T showing the filamentous structure and partial cell pellicle formation. (d) Cryogenic TEM micrograph of P373T showing the typical Gram-negative cell wall structure. CM, cytoplasmic membrane; P, peptidoglycan; OM, outer membrane; CG, copper mesh grid. Bars, 0.5 cm (a), 1 cm (b), 20 µm (c) and 200 nm (d).
yellow–orange–red pigmentation, a catalase-positive reaction, a modest halotolerance, low DNA G+C content, MK-7 as the primary menaquinone, and similar dominant iso-branching FAMEs (Tables 1 and S1). The presence of phosphatidylethanolamine as major phospholipid, together with various aminolipids and other polar lipids is a typical lipid pattern both for the phylum Bacteroidetes and family Chitinophagaceae. The sulfonolipids, N-acyl-capnines (a class of sphingolipids), have also been previously reported as major components of the cell envelope of various gliding bacteria, all of the phylum Bacteroidetes. However, strain P373T differs notably from other described strains by its temperature optima and the relative proportion of its dominant FAMEs (Table 1). Strain P373T represents the most thermostable strain (\(T_{\text{opt}} 60^\circ\text{C}\)) of the class Sphingobacteria and to our knowledge, only the sixth true thermostable isolate, with the type strains of three species of the genus Rhodothermus (Kristjansson et al., 1986; Marteinsson et al., 2010; Sako et al., 1996) and two species of the genus Thermonema (Hudson et al., 1989; Tenreiro et al., 1997), in the phylum Bacteroidetes (Table S5). The FAME content of strain P373T is dominated by i-15:0 (10.8%), i-17:0 3-OH (35.2%) and i-17:0 (24.5%), of which i-17:0 is uncommon as a dominant fatty acid amongst members of the family Chitinophagaceae, and i-15:0, which only represents ~10% of the P373T FAME content compared to >25% for all other described strains (Kämpfer et al., 2011). The differences in FAME content may represent modifications in membrane stability to facilitate the increased temperature optima. Based on these data, it can be concluded that strain P373T is phylogenetically and physiologically dissimilar from other described isolates of species in the family Chitinophagaceae. These findings demonstrate that strain P373T is a novel and distinctive bacterium within the phylum Bacteroidetes and we therefore propose a novel species in a new genus, Thermoflavifilum aggregans gen. nov., sp. nov., to accommodate this isolate.

**Description of Thermoflavifilum gen. nov.**

*Thermoflavifilum* (Ther’mo.flav.if.illum. Gr. n. thérme heat; L. adj. flavus yellow; L. n. filum thread, filament; N.L. neut. n. *Thermoflavifilum* a thermophilic yellow-pigmented filament).

Cells are strictly aerobic, Gram-negative, catalase- and oxidase-positive, non-motile and filamentous. Spores are not formed. Cell division is by binary fission. Thermophilic and neutrophilic. Heterotrophic. Typical growth substrates include simple oligo- and monosaccharides, and proteinaceous substrates. The major respiratory quinone is MK-7. The primary fatty acids are i-15:0, i-17:0 and i-17:0 3-OH. The type species is *Thermoflavifilum aggregans*.

**Description of Thermoflavifilum aggregans sp. nov.**

*Thermoflavifilum aggregans* (ag’gre.gans. L. v. aggregate to flock or band together; L. part. adj. *aggregans* pellicle-forming, aggregating).

Cells are unicellular, unbranched, filamentous rods, varying in size with 0.2–0.4 \(\mu\text{m}\) diameter and forming chains up to 80 \(\mu\text{m}\) long. Devoid of flagella or gliding motility, and forms exopolysaccharides. Colonies are circular, convex with an entire margin, about 0.2–0.5 cm in diameter, not translucent, and brightly yellow–orange pigmented. Pigments have an absorbance maximum at 454 nm (acetone), 448 nm (hexane) and 448–450 nm (methanol). Growth occurs at 35–63 °C (optimum 60 °C), at pH 5.5–8.7 (optimum 7.3–7.4), with 0–5% NaCl (w/v; optimum 0.1–0.25%) and with up to 5% (v/v) ethanol. Exhibits an oxidative heterotrophic metabolism. Growth occurs on simple sugars [DL-arabinose, (+)-D-ribose, (+)-D-xylitol, L-ribose, (+)-D-galactose, D-glucose, (+)-D-mannose, (-)-D-fructose, (+)-celllobiose, lactose, (+)-maltose, sucrose, (+)-trehalose, (+)-raffinose] and N-acetyl-D-glucosamine. Dextrin, pullulan, starch, glucomannan, xylan, glycogen and xanthan support growth, but Avicel, agarose, alginic acid sodium salt, CM-cellulose, galactomannan (Locust bean gum), pectin, chitin, gellan and Phytage do not. Grows on short chain alcohols (methanol, ethanol, 1-propanol, 2-propanol), sodium fumarate, sodium lactate, sodium pyruvate, yeast extract, peptone, tryptone, NB, R2A and LB. Peptone is the preferred nitrogen source. No heterotrophic CO\(_2\) fixation observed. API 50 CH 300 strips (bioMérieux) exhibited positive reactions for D-arabinose, L-arabinose, D-ribose, D-xylitol, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, methyl \(\alpha\)-D-mannopyranoside, methyl \(\alpha\)-D-glucopyranoside, N-acetylgalactosamine, amydalin, arbutin, aesculin ferric citrate, salicin, cellobiose, maltose, lactose (bovine origin), melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose and L-fucose. API ZYM strips (bioMérieux) show enzymic activity for alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, acetylamidase, cysteine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, \(\beta\)-galactosidase, \(\alpha\)-glucosidase, \(\beta\)-glucosidase, N-acetyl-\(\beta\)-glucosaminidase and \(\alpha\)-mannosidase. Resistant to chloramphenicol, erythromycin, kanamycin, kanamycin, metronidazole, monensin, neomycin, streptomycin, trimethoprim and vancomycin, but susceptible to ampicillin, rifampicin, polymyxin B and tetracycline. The predominant isoprenoid quinone was MK-7. The fatty acids detected were primarily methyl-branched (iso- or anteiso-) with the major FAMEs being i-15:0, i-17:0 and i-17:0 3-OH. A monounsaturated FAME, i-18:1, was also detected. The primary lipids were phosphatidylethanolamine, two unidentified aminolipids and three other unidentified polar lipids. The sulfonolipid, N-acetyl-capnine, was also observed.

The type strain, P373T (=ICMP 20041\(^T\)=DSM 27268\(^T\)), was isolated from geothermally heated soils at Waikite hot springs, New Zealand. The DNA G+C content of the type strain is 47.3 mol%.
Table 1. Differential characteristics of strain P373<sup>T</sup> and selected type strains of species of the family Chitinophagaceae

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Acknowledgements
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References


