Leadbetterella byssophila gen. nov., sp. nov., isolated from cotton-waste composts for the cultivation of oyster mushroom

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A bacterial strain, designated 4M15^T, was isolated from cotton-waste composts used as mushroom cultivation in South Korea. Properties of this isolate were studied on the basis of physiological and biochemical characteristics, fatty acid profile, isoprenoid quinone, DNA G + C content and phylogenetic position based on 16S rRNA gene sequence analysis. The strain was found to form a distinct phylogenetic lineage related to the family 'Flexibacteraceae' within the phylum 'Bacteroidetes'. No recognized species showed >85% 16S rRNA gene sequence similarity to strain 4M15^T. The fatty acid profile of strain 4M15^T included C_{16:1}ω7c/iso-C_{15:0} 2-OH (30.5%), iso-C_{15:0} (24.2%), iso-C_{15:0} 2-OH/C_{16:1}ω7c (15.9%), iso-C_{17:0} 3-OH (10.5%) and C_{16:0} (5.6%). The major isoprenoid quinone was menaquinone MK-7. The DNA G + C content was 33.0 mol%. Cells were Gram-negative, strictly aerobic, rod-shaped, non-motile, catalase-positive, oxidase-positive and flexirubin-positive. The strain hydrolysed aesculin, gelatin, starch and tyrosine. Several phenotypic tests could be used to differentiate strain 4M15^T from other members of the family 'Flexibacteraceae'. On the basis of the data presented, strain 4M15^T should be assigned to the phylum 'Bacteroidetes' as a novel genus and species, for which the name *Leadbetterella byssophila* gen. nov., sp. nov. is proposed. The type strain is 4M15^T (= KACC 11308^T = DSM 17132^T).

The *Cytophaga*–*Flavobacterium*–*Bacteroides* (CFB) group, which is also known as the phylum 'Bacteroidetes' (Ludwig & Klenk, 2001), is considered to comprise organisms associated with the degradation of organic compounds such as

complex polysaccharides (Höfle, 1982, 1992; Pinhassi *et al.*, 1999; Cottrell & Kirchman, 2000). The CFB group constitutes an important proportion of marine microbial communities (Glöckner *et al.*, 1999). Recently, many novel bacteria belonging to the CFB group have been isolated from marine environments and hypersaline lakes (Brettar *et al.*, 2004a, b; Donachie *et al.*, 2004; Frette *et al.*, 2004; Yoon *et al.*, 2004). However, a few CFB bacterial strains isolated from other habitats, such as soil, have also been described (Reichenbach, 1989).

Cotton wastes are mainly composed of cellulose, lignin and hemicellulose. In South Korea, cotton-waste composts are used as media for the cultivation of oyster mushroom (*Pleurotus ostreatus*). During the composting process,

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 4M15^T is AY854022.

A phase-contrast micrograph of cells of strain 4M15^T together with tables detailing its physiological characteristics based on several commercial systems and its fatty acid profile are available as supplementary material in IJSEM Online.

temperature in the material is gradually increased to 65 °C. We isolated one strain, designated 4M15^T, in a study of the bacterial diversity of these cotton-waste composts.

Strain 4T15^T was isolated by using the plating technique on trypticase soy agar (TSA, pH 7.0; Bacto) at 30 °C and then maintained on TSA medium. Gram staining was performed by using a Gram-stain kit (Difco) according to the manufacturer's recommended protocol. The KOH and amino-peptidase tests were also used in this regard (Gregersen, 1978). Gliding motility was observed by direct microscopic examination of the edge of colonies grown on 1:10 strength CASO (DSMZ medium no. 220; <http://www.dsmz.de/media/media.htm>)-supplemented 1% agar. After 16 h incubation at 30 °C, the inoculated area was examined by oil-immersion phase-contrast microscopy. Flexirubin pigments of the strain were identified by suspending cells in 20% KOH (Fautz & Reichenbach, 1980). CM-Cellulose (Sigma), hydroxyethylcellulose (Aldrich), Whatman powder CF11 and Whatman no. 1 filter paper were used to test cellulase activity. Hydrolyses of CM-cellulose, hydroxyethylcellulose and Whatman powder CF11 were tested by overlaying CASO agar with a thin layer of 0.1% of each of the components in tap-water agar and examining after 4 weeks. Hydrolysis of Whatman no. 1 filter paper was conducted by the method of Smibert & Krieg (1994). Oxidative or fermentative utilization of glucose was determined on Hugh–Leifson medium (Hugh & Leifson, 1953). Catalase activity was tested by using a 3% (v/v) H₂O₂ solution. Determinations of Gram staining, degradation of agar (1.5%, w/v), oxidase reactivity, hydrolysis of aesculin, starch, casein, gelatin, Tweens 20, 40 and 80 and DNA, and indole production were made according to the methods of Smibert & Krieg (1994). Hydrolysis of chitin from crab shells (1%, w/v; Sigma) and tyrosine (0.5%, w/v) was tested on CASO agar at 30 °C and observed by the appearance of a clear zone after 4 weeks. The urease test was conducted according to the method described by MacFaddin (2000). The pH range (pH 4.0–10.0 at intervals of 1.0 pH units) for growth was determined in CASO agar

that was buffered with citrate/phosphate buffer or Tris/hydrochloride buffer (Breznak & Costilow, 1994). Growth at 1, 3, 5, 7 and 10% NaCl (w/v) was investigated in CASO broth. Growth at various temperatures (5–50 °C) was measured on CASO. Growth on a 0.5% yeast extract medium (0.5% yeast extract, 0.5% NaCl; pH 7.0) was observed. Tests in the commercial systems API ZYM, API 20NE and API 50CH (bioMérieux) were generally performed according to the manufacturer's instructions. The API ZYM test strip was read after 4 h incubation at 30 °C, whilst the other API strips were examined after 48 h at 30 °C. For the Biolog system, strains were incubated on TSA at 30 °C for 24 h. GN2 microplates were inoculated according to the manufacturer's instructions and incubated at 30 °C for 48 h. Sensitivity to antibiotics was determined with the routine disc-diffusion plate method. The following antibiotics were tested: 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) and tetracycline (30 µg).

Fatty acid methyl esters were extracted and prepared by using the standard protocol of the Microbial Identification system (MIDI; Microbial ID) after cells were grown on TSA for 24 h at 30 °C. Isoprenoid quinones were analysed by HPLC as described by Groth *et al.* (1996). The DNA G+C content was determined by HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989) using a reversed-phase column (Supelcosil LC-18-S; Supelco).

The 16S rRNA gene sequence was determined by PCR amplification (Kwon *et al.*, 2003) and direct sequencing (Hiraishi, 1992). For the phylogenetic analyses, similar 16S rRNA gene sequences and sequences of representatives of different genera of the CFB were included in sequence alignments. The 16S rRNA gene sequences were aligned by using the MEGALIGN program of DNASTAR. An evolutionary-distance matrix was generated as described by Jukes & Cantor (1969). The evolutionary tree for the datasets was

Fig. 1. Phylogenetic position of strain 4M15^T in the *Cytophaga*–*Flavobacterium*–*Bacteroides* group on the basis of 16S rRNA gene sequence analysis. The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987), and the 16S rRNA gene sequence of *Rhodothermus marinus* DSM 4252^T was used as the outgroup. Numbers at nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. Bootstrap values below 50% are not indicated. Bar, 2 nucleotide substitutions per 100 nt.

inferred from the neighbour-joining method of Saitou & Nei (1987) by using the neighbour-joining program of MEGA version 2.1 (Kumar *et al.*, 2001). The stability of relationships was assessed by performing bootstrap analyses of the neighbour-joining data based on 1000 resamplings (see Fig. 1). The phylogenetic positions of strain 4M15^T were also assessed based on maximum-parsimony and maximum-likelihood analyses.

Cells of strain 4M15^T were Gram-negative and rod-shaped with a width of 0.6–0.9 µm and length of 2–7 µm (see Supplementary Figure in IJSEM Online). Colonies were circular, 1–2 mm in diameter, smooth, light orange, shiny and convex with entire margin when grown on TSA. With prolonged incubation, colonies became dark orange. Gliding motility was not observed by phase-contrast microscopy. The strain was positive for oxidase, catalase, KOH test, aminopeptidase, indole production and O/F test (glucose). Flexirubin pigmentation was positive, which was demonstrated by a fast shift of colony colour from light orange to deep orange after addition of 20 % KOH. Strain 4M15^T grew at temperatures of 15–45 °C and at a pH range of 6.0–8.0. The strain grew in the presence of 1 % (w/v) NaCl, but not at 3 % NaCl. Growth was observed in 0.5 % yeast extract broth. Strain 4M15^T degraded aesculin, gelatin, starch, tyrosine and Tween 20, but not casein, cellulose, chitin, DNA or Tweens 40 and 80. Phenotypic comparisons among the members of the family 'Flexibacteraceae' within the CFB group are illustrated in Table 1. Strain 4M15^T was unique in its isolation source from cotton-waste composts, and several properties differentiate the strain from related genera.

In commercial multitest systems, strain 4M15^T showed positive responses for eight tests, including tryptophan deaminase in the API 20NE test strip, production of acid from a total of 20 substrates, including D-arabinose, in the API 50CH test strip, and had 12 positive enzyme reactions, including phosphate, in the API ZYM test strip. In the Biolog GN2 test system, the strain assimilated a total of 47 carbohydrates, including α-cyclodextrin. Results of the commercial multitest systems are given in Supplementary Table S1, available in IJSEM Online.

The study of antibiotic susceptibility showed that strain 4M15^T was sensitive to ampicillin, carbenicillin, lincomycin, streptomycin and tetracycline. Antibiotic resistance was observed to benzylpenicillin, gentamicin, neomycin, oleanomycin and polymyxin B.

Predominant cellular fatty acids of strain 4M15^T were C_{16:1}ω7c/iso-C_{15:0} 2-OH (30.5 %), iso-C_{15:0} (24.2 %), iso-C_{15:0} 2-OH/C_{16:1}ω7c (15.9 %), iso-C_{17:0} 3-OH (10.5 %) and C_{16:0} (5.6 %) (the full results are given in Supplementary Table S2, available in IJSEM Online). The major isoprenoid quinone was menaquinone MK-7; a trace amount of menaquinone MK-8 was also detected. The DNA G+C content of strain 4M15^T was 33.0 mol% (Table 1).

To determine the phylogenetic position of strain 4M15^T, the 16S rRNA gene sequences corresponding to nt 235–1376 of the *Escherichia coli* 16S rRNA gene sequence (GenBank accession no. J01695) (Brosius *et al.*, 1978) were used. According to the neighbour-joining phylogenetic tree (Fig. 1), strain 4M15^T within the CFB group could be positioned in the family 'Flexibacteraceae'. In the other tree-building methods used in this study, strain 4M15^T was also shown to form a cluster with the members of the family 'Flexibacteraceae'.

According to a BLAST search result in GenBank, strain 4M15^T showed highest 16S rRNA gene sequence similarity (93 %) to uncultured bacterial clone Hot Creek 2 (GenBank accession no. AY168735). According to the CLUSTAL W alignment of the members of the CFB group, strain 4M15^T showed highest sequence similarity (85.4 %) to *Belliella baltica* BA134^T (GenBank no. AJ564643). The phenotypic comparisons were made with several genera that showed 16S rRNA gene sequence similarities of >82.0 % to strain 4M15^T. Our study demonstrated that strain 4M15^T has a distinct phylogenetic position in the CFB group and showed very low levels of sequence similarity to other members of the group. Thus, on the basis of the phenotypic and phylogenetic data presented, strain 4M15^T cannot be assigned to any of the recognized genera and should be placed in a novel genus and species within the CFB group, for which the name *Leadbetterella byssophila* gen. nov., sp. nov. is proposed.

Description of *Leadbetterella* gen. nov.

Leadbetterella (Lead.bet.te.rel'la. N.L. fem. n. *Leadbetterella* in honour of Dr Edward R. Leadbetter, who studied bacteria belonging to the CFB group).

Cells are strictly aerobic, Gram-negative, non-motile and non-gliding rods. Oxidase and catalase are positive. Flexirubin-type pigments are present. Several carbohydrates are used as sole carbon sources. Aesculin, gelatin, starch and tyrosine are degraded. Major fatty acids are C_{16:1}ω7c/iso-C_{15:0} 2-OH, iso-C_{15:0}, iso-C_{15:0} 2-OH/C_{16:1}ω7c, iso-C_{17:0} 3-OH and C_{16:0}. Respiratory quinone is menaquinone MK-7. The type species is *Leadbetterella byssophila*.

Description of *Leadbetterella byssophila* sp. nov.

Leadbetterella byssophila (bys.so'phi.la. Gr. n. *byssos* cotton; N.L. adj. *philus* friendly to; N.L. fem. adj. *byssophila* liking cotton).

Cells are rod-shaped (0.6–0.9 × 2–7 µm) and non-motile. Colonies on TSA are orange and convex with entire margin. Growth occurs at temperatures of 15–45 °C, pH 6.0–8.0 and in the presence of 1 % (w/v) NaCl, but not at 3 % NaCl. Grows on 0.5 % yeast extract medium. Aesculin, gelatin, starch, tyrosine and Tween 20 are degraded. Positive for O/F test (glucose) and indole production. Casein, cellulose, chitin, DNA, Tweens 40 and 80 and urea are not degraded. Growth on carbohydrates (API 20NE) is observed for glucose, arabinose, mannose, N-acetylglucosamine

Table 1. Characteristics useful in distinguishing strain 4M15^T from related species of the CFB group

Species/taxon: 1, strain 4M15^T; 2, *Algoriphagus antarcticus*; 3, *Algoriphagus aquimarinus*; 4, *Algoriphagus chordae*; 5, *Algoriphagus halophilus*; 6, *Algoriphagus ratkowskyi*; 7, *Algoriphagus winogradskyi*; 8, *Algoriphagus yeomjeoni*; 9, *Aquiflexum balticum*; 10, *Belliella baltica*; 11, *Cyclobacterium marinum*; 12, *Cytophaga arvensicola*; 13, *Cytophaga aurantiaca*; 14, *Cytophaga fermentans*; 15, *Cytophaga hutchinsonii*; 16, *Cytophaga xylanolytica*; 17, *Dyadobacter crusticola*; 18, *Dyadobacter fermentans*; 19, *Flexithrix dorotheae*; 20, *Hongiella mannitolivorans*; 21, *Hongiella marincola*; 22, *Hongiella ornithinivorans*; 23, *Persicobacter diffluens*; 24, *Reichenbachia agariperforans*. Data are from Bowman *et al.* (2003), Brettar *et al.* (2004a, b), Chelius & Triplett (2000), Haack & Breznak (1993), Nedashkovskaya *et al.* (2003, 2004), Raj & Maloy (1990), Reddy & Garcia-Pichel (2005), Reichenbach (1989), Van Trappen *et al.* (2004), Yi & Chun (2004), Yoon *et al.* (2004, 2005) and this study. ND, Not determined; +, positive; −, negative; V, variable; A, strict aerobic; F, facultative anaerobe.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
Habitat	Cotton compost	Marine	Marine	Marine	Marine	Marine	Marine	Marine	Marine	Marine	Marine	Marine	Soil	Soil	Marine	Soil	Freshwater	Soil	Plant	Marine	Marine	Marine	Marine	Marine	Marine
Reaction to oxygen	A	A	A	A	A	A	A	A	A	A	A	A	A	F	A	F	A	A	A	A	ND	A	A	A	
Oxidase/catalase	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	ND/+	+/-	-/+	+/+	+/+	-/-	+/+	+/+	+/+	+/+	+/-	+/+
Flexirubin	+	-	-	-	-	-	-	-	-	-	-	-	+	-	ND	+	-	+	V	ND	-	ND	-	-	+
Gliding motility	-	-	-	-	-	-	-	-	-	-	-	-	V	+	+	+	+	-	-	+	-	-	-	+	+
Hydrolysis of:																									
Agar	-	-	+	+	-	-	+	-	ND	ND	-	ND	-	+	-	ND	ND	-	+	-	-	-	+	+	
Gelatin	+	-	+	-	+	-	+	V	+	-	-	V	ND	ND	ND	ND	-	-	-	+	-	+	+	+	
Casein	-	-	+	-	-	V	-	+	-	-	-	ND	-	ND	ND	ND	-	ND	-	-	+	-	-	-	
Starch	+	-	-	-	+	V	+	-	+	+	-	V	-	+	-	+	-	-	-	+	ND	+	+	+	
Filter paper	-	ND	-	-	-	-	-	ND	-	-	-	ND	+	-	+	-	-	-	-	-	ND	-	-	-	
CM-Cellulose	-	-	-	-	-	-	-	ND	ND	ND	-	ND	+	ND	+	-	-	-	+	-	ND	-	+	-	
DNA G+C content (mol%)	33	40	41.3	37-40.5	37	35-37	39-42	41	38.4	35-36	38.4	43-46	42	39	39	45.5	ND	48-49	37	42	43	38	40-42	44.5	

and maltose. Positive for indole production and β -galactosidase (API 20NE). Negative for nitrate reduction and arginine dihydrolase (API 20NE). Enzymic activity is observed for alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase and α -fucosidase (API ZYM). Weak enzymic activity is detected for α -galactosidase and β -galactosidase (API ZYM). D-Arabinose, D-galactose, D-glucose, D-mannose, L-rhamnose, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, sucrose, D-trehalose, starch and gentiobiose are oxidized or weakly oxidized (API 50CH). *i*-Erythritol, D-melibiose, acetic acid, α -cyclodextrin, D-fructose, methyl β -D-glucoside, dextrin, L-fucose, uridine, glycogen, D-galactose, D-raffinose, L-alaninamide, L-ornithine, thymidine, gentiobiose, α -ketovaleic acid, α -D-glucose, D-galacturonic acid, DL-lactic acid, L-alanine, *N*-acetyl-D-glucosamine, sucrose, L-alanyl-glycine, *N*-acetyl-D-glucosamine, α -D-lactose, D-trehalose, L-asparagine, lactulose, turanose, L-aspartic acid, L-serine, glycerol, L-arabinose, maltose, L-glutamic acid, L-threonine, α -DL-glycerol phosphate, D-arabitol, methyl pyruvate, glycyl L-aspartic acid, glucose 1-phosphate, D-cellobiose, D-mannose, monomethyl succinate, glycyl L-glutamic acid and D-glucose 6-phosphate are assimilated or weakly assimilated (Biolog GN 20). The DNA G+C content of strain 4M15^T is 33.0 mol%.

The type strain, 4M15^T (=KACC 11308^T=DSM 17132^T), was isolated from cotton-waste composts in the Republic of Korea.

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