Hongiella mannitolivorans gen. nov., sp. nov., *Hongiella halophila* sp. nov. and *Hongiella* *ornithinivorans* sp. nov., isolated from tidal flat sediment

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Three marine strains of the *Cytophaga*–*Flavobacterium*–*Bacteroides* group, designated JC2050^T, JC2051^T and JC2052^T, were obtained from a single sediment sample of getbol, the Korean tidal flat. Comparative 16S rDNA sequence studies revealed that the test strains were not closely related to any validly published genera and that these strains were only distantly related to the genus *Cyclobacterium* (88.7–91.2%). Phylogenetic analyses demonstrated that the three getbol isolates formed a distinct monophyletic clade within the family *Cytophagaceae*. Physiological, biochemical and chemotaxonomic data also indicated that these three getbol isolates differed significantly from members of other genera and were sufficiently different from each other to be recognized as separate species. On the basis of polyphasic evidence, a new genus, *Hongiella* gen. nov., is proposed, with three novel species, *Hongiella mannitolivorans* sp. nov. (type strain JC2050^T=IMSNU 14012^T=DSM 15301^T), *Hongiella halophila* sp. nov. (type strain JC2051^T=IMSNU 14013^T=DSM 15292^T) and *Hongiella ornithinivorans* sp. nov. (type strain JC2052^T=IMSNU 14014^T=DSM 15282^T). *Hongiella mannitolivorans* is the type species of the genus.

INTRODUCTION

During a course of study on the culturable aerobic bacterial community in getbol (Korean tidal flat), a large number of novel bacterial strains was isolated (Yi & Chun, 2002; Yi *et al.*, 2003). Three of these isolates were members of the *Cytophaga*–*Flavobacterium*–*Bacteroides* (CFB) lineage and were the subject of a taxonomic investigation. On the basis of polyphasic evidence, these test strains formed a new genus in the CFB group, for which the name *Hongiella* gen. nov. is proposed.

METHODS

Bacterial strains. Test strains were isolated from a single sample of tidal flat sediment from Ganghwa Island, Korea (37°35'31.9"N; 126°27'24.5"E). Strains JC2050^T and JC2052^T were isolated on marine agar 2216 (MA; Difco) and strain JC2051^T was isolated on

MR2A [R2A (Difco) supplemented with artificial sea salts (Sigma)]. The isolates were routinely cultured on MA at 30 °C and maintained as a glycerol suspension (20 %, w/v) at –80 °C.

Molecular systematics. Bacterial DNA preparation, PCR amplification and 16S rDNA sequencing were carried out as described previously (Chun & Goodfellow, 1995). The resultant sequences were aligned manually against sequences obtained from GenBank. Phylogenetic trees were inferred using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1972) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices were generated according to Jukes & Cantor (1969). The resultant tree topologies were evaluated in bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out using the programs PHYDIT (available at <http://plaza.snu.ac.kr/~jchun/phydit/>) and PAUP 4.0 (Swofford, 1998) as described previously (Chun *et al.*, 2000).

Cultural, morphological and physiological properties. Cultural characteristics were studied using several bacteriological growth media: CY (3 g casitone, 1 g yeast extract, 1 g CaCl₂·2H₂O, 40 g sea salts and 15 g agar in 1000 ml distilled water), CSY-3 (Sawabe *et al.*, 1998); 1/5 LBM (Suzuki *et al.*, 2001), MA, SMM (Shioi's marine medium; Shiba, 1992) and YTSS (Gonzalez *et al.*, 1997). Growth at various temperatures was tested over the range 5–50 °C at intervals of 5 °C using MA. Growth at different pH values (4–14) was examined on MA. Growth in NaCl (0–12 % at 0.5 % intervals) or sea salts (0–15 % at 0.5 % intervals) was tested on CSY-3.

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

The GenBank accession numbers for 16S rDNA sequences of strains JC2050^T, JC2051^T and JC2052^T are respectively AY264838, AY264839 and AY264840.

Cellular morphology was examined by phase-contrast microscopy and SEM after growth on MA at 30 °C. Gliding motility was observed by direct microscopic examination of the edge of colonies and motility was observed by the hanging-drop technique for cells in exponential phase in CY broth.

Carbon-source utilization was tested on 96-well tissue-culture microplates as described by Gosink *et al.* (1998). Strain JC2051^T was able to grow on Baumann's basal medium (BM; Baumann *et al.*, 1971) without added growth factors, but strains JC2050^T and JC2052^T were unable to grow on BM without trace elements and vitamins. Thus, BM, supplemented with 2% (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957) and 1% (v/v) vitamin solution (Staley, 1968), was used for carbon-source testing. β -Galactosidase activity was determined by streaking cultures onto MA agar plates supplemented with 0.1 mM IPTG and 20 μ g X-Gal ml⁻¹ (Gosink *et al.*, 1998). Other physiological and biochemical properties were tested using standard procedures as described previously (Yi *et al.*, 2003).

Chemotaxonomy. Chemotaxonomic characteristics were determined in cells grown at 30 °C for 2 days on MA or in MB (marine broth 2216; Difco). The presence of flexirubin-like pigments was tested by measuring the absorbance spectrum of an ethanol and alkaline-ethanol extract of lysed cells (Weeks, 1981). Fatty acid methyl ester analysis was performed by GLC according to the instructions of the Microbial Identification system (MIDI). Isoprenoid quinones were isolated according to Minnikin *et al.* (1984) and analysed using HPLC as described by Collins (1985). G + C content (mol%) was determined by HPLC analysis of deoxy-ribonucleosides as described by Mesbah *et al.* (1989) using a reverse-phase column (Supelcosil LC-18-S; Supelco).

RESULTS AND DISCUSSION

Phylogenetic analysis

Nearly complete 16S rDNA sequences of strains JC2050^T (1376 bp), JC2051^T (1427 bp) and JC2052^T (1425 bp) were obtained and used for an initial BLAST search against GenBank. The result clearly indicated that the getbol isolates belonged to the family *Cytophagaceae* within the CFB group. The newly determined sequences were then aligned manually against representatives of the CFB group based on secondary structure of bacterial 16S rRNA. Sequence similarities among the three isolates were 94–96%, all below the cut-off value of 97% for bacterial species definition (Stackebrandt & Goebel, 1994). The highest similarity values obtained between our isolates and species with validly published names were observed with *Cyclobacterium marinum* ATCC 43824 (88.7% for JC2050^T, 90.1% for JC2051^T and 91.2% for JC2052^T). No other validly named bacterial species showed more than 90% 16S rDNA sequence similarity to the test strains. The distinctiveness of our isolates was also evident in the phylogenetic tree (Fig. 1). Strains JC2050^T and JC2052^T formed a monophyletic clade with 94% bootstrap support. Strain JC2051^T formed a sister group to this clade, and all three isolates formed a monophyletic clade supported by a bootstrap value of 100%. *Cyclobacterium marinum* ATCC 43824 was recovered as a further sister group with a bootstrap value of 100%. Tree topology was supported by all tree-making methods used in this study. However, the branching

Fig. 1. Phylogenetic relationship of strains JC2050^T, JC2051^T and JC2052^T and related taxa within the CFB group based on 16S rDNA sequences. The tree was created using the neighbour-joining method and numbers at nodes are levels of bootstrap support (%) from 1000 resampled datasets. Solid circles indicate that the corresponding nodes (groupings) are also recovered in Fitch–Margoliash, maximum-likelihood and maximum-parsimony trees. *Helicobacter pylori* ATCC 43504^T (GenBank accession number U01330) was used as an out-group (not shown). Bar, 0.1 nt substitution per position.

patterns of the other genera varied depending on the tree-building method used and the relationships were supported by relatively low bootstrap values. On the basis of low 16S rDNA similarity values (<92%) between our isolates and known genera and their significantly monophyletic grouping, it is fair to conclude that the test strains should be placed in a new genus of the CFB group.

Culture and growth conditions

Abundant growth was observed on CSY-3, MA and SMM. When grown on CY, 1/5 LBM and YTSS agar, smaller colonies with smaller amounts of pigment were observed. Strains grew at temperatures of 10–40 °C (optimum 35–40 °C) for JC2050^T, 5–40 °C (optimum 35 °C) for JC2051^T and 10–45 °C (optimum 35–40 °C) for JC2052^T. Although all of the test strains were isolated from a marine habitat, only strain JC2051^T showed slight halophilicity, as it grew on media containing 1–10% (w/v) NaCl (optimum 5% NaCl) or 0.5–11% (w/v) artificial sea salts-based media (optimum 1–2% sea salts). Strains JC2050^T and JC2052^T were able to grow without NaCl or sea salts; strain JC2050^T grew at 0–7% NaCl (optimum 1% NaCl) or 0–7% sea salts (optimum 0.5–1.5% sea salts) and strain JC2052^T grew at 0–10% NaCl (optimum 1% NaCl) or 0–13% sea salts (optimum 1.0–2.5% sea salts). Optimum growth occurred at pH 7; the pH range for growth was pH 6–11 for strain JC2050^T and pH 6–13 for strains JC2051^T and JC2052^T. No growth was detected under anaerobic conditions generated by a GasPack system (BBL).

Morphological properties

Colonies of strains JC2050^T and JC2052^T were circular, convex, glistening, non-luminescent, butyraceous, opaque

Fig. 2. SEM of strain JC2050^T. Cells were grown on MA at 30 °C for 2 days. Bar, 1 µm.

and orange with entire margins on agar plates. Colonies of strain JC2051^T were slightly different, i.e. flat, translucent and reddish-orange. Cells were rod-shaped with rounded ends (Fig. 2), non-flagellated and non-motile. Gliding motility was not observed on agar plates, nor was motility observed in broth media. Spore formation was not observed.

Physiological and biochemical properties

The physiological and biochemical properties of the test strains are given in Table 1. A summary of the major differences between the test strains and phylogenetically related species is given in Table 3.

Chemotaxonomic characteristics

Absorption maxima of crude extracts of strains JC2050^T and JC2052^T were approximately 480 nm; that of strain JC2051^T was 475 nm. Bathochromatic shift, a characteristic of flexirubins, was not observed in any strains. The predominant cellular fatty acids of the three strains were 15:0 iso, iso 17:1ω9c, 17:0 iso 3-OH and mixture of 16:1ω7c and 15:0 iso 2-OH (Table 2). The overall fatty acid profiles of our isolates differed significantly from those in phylogenetically related taxa, except for the genus *Runella* (Table 3). However, the latter showed a much higher DNA G+C content (49–50 mol%) than our isolates (37–42 mol%). MK-7 was the predominant isoprenoid quinone in all test strains. The DNA G+C contents of strains JC2050^T, JC2051^T and JC2052^T were respectively 42, 37 and 38 mol%.

Taxonomic conclusions

Polyphasic analyses demonstrated that the three getbol isolates belong to a coherent group that corresponds to a novel genus within the CFB group. Phenotypic data clearly demonstrated that test strains are not closely affiliated with any previously described genera (Table 3)

Table 1. Phenotypic features of strains JC2050^T, JC2051^T and JC2052^T

+, Positive; –, negative; W, weakly positive. All three strains were positive for catalase, oxidase, hydrolysis of aesculin and X-Gal, alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase and N-acetyl-β-glucosaminidase. All three strains were negative for arginine dihydrolase, fluorescein, fermentation of glucose, polyhydroxybutyrate accumulation, urease, production of acetoin, H₂S and indole, hydrolysis of agar, alginic acid, casein, cellulose, chitin and egg yolk, esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, α-mannosidase and α-fucosidase. The following compounds were utilized as sole carbon sources by all test strains: D-cellobiose, D-glucose, D-mannose, D-raffinose, D-salicin, D-trehalose, D-xylose, lactose, N-acetylglucosamine and sucrose. The following compounds were not utilized by any of the three strains: acetamide, acetate, benzoate, citrate, D-ribose, D-sorbitol, ethanol, glycerol, glycine, inositol, inulin, 2-propanol, L-arabinose, L-arginine, L-ascorbate, L-asparagine, L-lysine, polyethylene glycol, salicylate, succinate, tartrate and thiamin.

Characteristic	JC2050 ^T	JC2052 ^T	JC2051 ^T
Pigment-absorption max. (nm)	480	480	475
NaCl requirement	–	–	+
Nitrate reduction	+	–	–
Amylase	+	+	–
DNase	+	+	–
Gelatinase	+	W	+
Tweenase	–	+	+
Utilization of:			
D-Fructose	–	+	+
D-Galactose	–	–	+
D-Mannitol	+	–	–
L-Ornithine	–	+	–
API ZYM result:			
Valine arylamidase	+	+	–
Trypsin	+	+	–
α-Chymotrypsin	+	+	W
β-Galactosidase	+	W	+
β-Glucuronidase	–	–	+
α-Glucosidase	–	+	+
β-Glucosidase	–	+	+

and are sufficiently different from each other to be recognized as separate species (Table 1). In many aspects, strain JC2051^T was shown to be rather distantly related to the other two strains, namely colonial morphology, NaCl requirement, growth capability on BM and the maximum absorption of pigment. The 16S rDNA sequence similarity and the phylogenetic tree also reflected this relationship.

Based on the polyphasic data presented in this study, the name *Hongiella* gen. nov. is proposed for the getbol isolates, with three novel species, *Hongiella mannitolivorans* sp. nov. for strain JC2050^T, *Hongiella halophila* sp. nov. for strain

Table 2. Cellular fatty acid content of strains JC2050^T, JC2051^T and JC2052^T

Values are percentages of total fatty acid content. Only fatty acids representing at least 1% of the total fatty acids of at least one of the strains are shown.

Fatty acid	JC2050 ^T	JC2052 ^T	JC2051 ^T
11:0 anteiso	1.7	0.0	1.7
14:0 iso	1.2	0.5	0.4
15:1 iso G	1.0	1.1	0.0
15:0 iso	28.7	29.2	22.7
15:0 anteiso	4.6	4.9	2.6
15:1ω6c	2.5	1.1	1.0
15:0	1.7	0.7	0.8
16:0 iso G	0.0	1.9	0.0
16:1 iso H	2.8	1.6	2.5
16:0 iso	3.7	3.6	5.2
Summed feature 3*	13.7	14.0	20.7
16:1ω5c	1.3	1.7	3.6
15:0 iso 3-OH	3.6	3.4	2.5
iso 17:1ω9c	5.6	7.8	8.7
Summed feature 4*	4.8	4.9	4.5
17:1ω6c	3.1	2.6	3.7
16:0 iso 3-OH	4.0	2.3	2.0
16:0 3-OH	0.6	0.5	1.4
17:0 iso 3-OH	7.9	11.1	9.2
17:0 2-OH	0.7	1.6	0.8

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains 16:1ω7c and/or 15:0 iso 2-OH; summed feature 4 contains 17:1 iso I and/or anteiso B.

JC2051^T and *Hongiella ornithinivorans* sp. nov for strain JC2052^T.

Description of *Hongiella* gen. nov.

Hongiella (Hong.i.el'la. N.L. dim. fem. n. *Hongiella* named after Soon-Woo Hong, a Korean microbiologist who devoted his life to the study of soil micro-organisms).

Gram-negative, oxidase- and catalase-positive. Strictly aerobic, chemoheterotrophic and mesophilic. Grows optimally at neutral pH. Cells are rod-shaped with rounded ends, non-flagellated and non-motile. Colonies are circular, entirely margined, glistening, butyraceous and orange on MA and do not glide. Abundant growth occurs on CSY-3, MA and SMM media. Spores are not formed. Flexirubin-type pigment is absent. The major isoprenoid quinone is MK-7. The predominant cellular fatty acids are 15:0 iso (23–29%), iso 17:1ω9c (6–9%), 17:0 iso 3-OH (8–11%) and a mixture of 16:1ω7c and 15:0 iso 2-OH (14–21%). DNA G+C content is 37–42 mol%. Many phenotypic characters differentiate this genus from related taxa (Table 3).

Phylogenetically, the genus belongs to the family *Cytophagaceae*. The type species is *Hongiella mannitolivorans*.

Description of *Hongiella mannitolivorans* sp. nov.

Hongiella mannitolivorans (man.ni.to.li.vo'rans. N.L. n. *mannitolium* mannitol; L. v. *vorare* to devour; N.L. part. adj. *mannitolivorans* utilizing mannitol).

Cells are approximately 1.1–1.7 × 0.4–0.5 μm. Colonies are convex, opaque and orange on MA. Optimal growth is observed at 35–40 °C, pH 7 and 1% NaCl or 0.5–1.5% artificial sea salts. Grows without NaCl or sea salts. Reduces nitrate to nitrite. Produces amylase, DNase and gelatinase, but not Tweenase. Detailed physiological and biochemical characteristics are given in Table 1. Maximum absorption of pigment occurs at 480 nm. Cellular fatty acid composition is given in Table 2. The type strain is JC2050^T (=IMSNU 14012^T=KCTC 12050^T=DSM 15301^T); the DNA G+C content of the type strain is 42 mol%. Isolated from sediment of getbol, the Korean tidal flat.

Description of *Hongiella halophila* sp. nov.

Hongiella halophila (ha.lo'phi.la. Gr. n. *hals*, *halos* salt; Gr. adj. *philos* loving; N.L. fem. adj. *halophila* salt-loving).

Cells are approximately 1.0–1.8 × 0.3–0.5 μm. Colonies are flat, translucent and reddish-orange on MA. Optimal growth is observed at 35 °C, pH 7 and 5% NaCl or 1–2% artificial sea salts. Does not grow without NaCl or sea salts. Nitrate is not reduced to nitrite. Produces gelatinase and Tweenase, but not amylase or DNase. Detailed physiological and biochemical characteristics are given in Table 1. Maximum absorption of pigment occurs at 475 nm. Cellular fatty acid composition is given in Table 2. The type strain is JC2051^T (=IMSNU 14013^T=KCTC 12051^T=DSM 15292^T); the DNA G+C content of the type strain is 37 mol%. Isolated from sediment of getbol, the Korean tidal flat.

Description of *Hongiella ornithinivorans* sp. nov.

Hongiella ornithinivorans (or'ni.thi.ni.vo'rans. N.L. n. *ornithinum* ornithine; L. v. *vorare* to devour; N.L. part. adj. *ornithinivorans* utilizing ornithine).

Cells are approximately 0.8–2.6 × 0.3–0.4 μm. Colonies are convex, opaque and orange on MA. Optimal growth is observed at 35–40 °C, pH 7 and 1% NaCl or 1.0–2.5% artificial sea salts. Grows without NaCl or sea salts. Nitrate is not reduced to nitrite. Produces amylase, DNase, gelatinase (weakly) and Tweenase. Detailed physiological and biochemical characteristics are given in Table 1. Maximum absorption of pigment occurs at 480 nm. Cellular fatty acid composition is given in Table 2. The type strain is JC2052^T (=IMSNU 14014^T=KCTC 12052^T=DSM 15282^T); the DNA G+C content of the type strain is 38 mol%. Isolated from sediment of getbol, the Korean tidal flat.

Table 3. Characteristics that can be used to differentiate strains JC2050^T, JC2051^T and JC2052^T from phylogenetically related species

Genera: 1, *Hongiella* (strains JC2050^T, JC2051^T and JC2052^T); 2, *Cyclobacterium*; 3, *Cytophaga*; 4, *Dyadobacter*; 5, *Flectobacillus*; 6, *Flexibacter*; 7, *Runella*; 8, *Spirosoma*; 9, *Sporocystophaga*. Data are from this study and others (Chelius & Triplett, 2000; Fujita *et al.*, 1996; Gosink *et al.*, 1998; Raj & Maloy, 1990; Reichenbach, 1989; Urakami & Komagata, 1986). +, Positive; –, negative; w, weakly positive; v, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Pigment	Orange	Pink	Yellow	Yellow	Pink	Orange	Salmon	Yellow	ND
Flexirubin reaction	–	–	+	+	–	v	–	+	ND
Cell shape	Rod	Ring-like	Rod	Rod	Ring-like	Rod	Ring-like	Ring-like	Rod
Gliding	–	–	+	–	–	+	–	–	+
NaCl requirement	v	+	ND	ND	–	ND	–	–	ND
Catalase	+	+	v	+	w	v	w	+	+
Oxidase	+	–	+	+	+	+	+	+	ND
Urease	–	–	ND	ND	+	ND	–	–	ND
Nitrate reduction	v	–	+	ND	–	v	–	–	ND
Fermentation of glucose	–	–	ND	+	–	ND	–	–	ND
Amylase	v	–	v	–	+	v	w	w	ND
Caseinase	–	–	–	ND	–	v	–	w	ND
Aesculinase	+	ND	–	ND	ND	ND	ND	ND	ND
Gelatinase	+	–	ND	–	+	+	–	+	ND
Tweenase	v	–	–	ND	+	ND	ND	ND	ND
DNA G+C content (mol%)	37–42	34–39	37–42	48	40	37–47	49–50	51–53	36
Major fatty acids (% of total):									
15:0 iso	23–29	45	ND	17	33	ND	20	11	ND
15:0 anteiso	3–5	32	ND	0	0	ND	4	2	ND
16:1 ω 7/15:0 iso 2-OH	14–21	0	ND	44	0	ND	32	0	ND
16:1	0	14	ND	0	1	ND	0	43	ND
17:0 iso	0	0	ND	0	48	ND	0	20	ND

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