Characterization and Description of *Hyphomicrobium denitrificans* sp. nov.

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The *Hyphomicrobium* strains which we examined in this study are gram negative and facultatively methylotrophic and utilize methanol and monomethylamine, but not methane, by the serine pathway with activated formaldehyde incorporation. The cellular fatty acids include a large amount of straight-chain unsaturated C_{18:1} acid, and the hydroxy fatty acids include large amounts of 3-OH C_{14:0} and 3-OH C_{16:0} hydroxy acids. The major ubiquinone is ubiquinone Q-9. However, the DNA base compositions of these *Hyphomicrobium* strains are heterogeneous, ranging from 58 to 66 mol% guanine plus cytosine. Strains TK 0415^T (T = type strain), TK 0414, TK 0416, and TK 0441 were distinguished from other *Hyphomicrobium* strains on the basis of physiological characteristics and DNA-DNA hybridization data. A new species, *Hyphomicrobium denitrificans*, is proposed for these organisms; the type strain of *H. denitrificans* is TK 0415 (= DSM 1869 = NCIB 11706 = Attwood and Harder strain X).

Previously (21), we described the phenotypic and chemotaxonomic characteristics of methanol-utilizing *Hyphomicrobium* species and other similar budding bacteria. *Hyphomicrobium* strains were clearly separated from other similar budding bacteria belonging to the genera *Hyphomonas*, *Pedomicrobium*, and *Rhodomicrobium* and from "*Hyphomicrobium indicum*." The strains of the genus *Hyphomicrobium* are facultatively methylotrophic, non-spore-forming, gram-negative, polarly flagellated, hyphal, budding, rod-shaped organisms that have a Q-9 ubiquinone system (17, 19, 21), cellular fatty acids that include large amounts of C_{18:1} acid (17, 20, 21), hydroxy fatty acids that include large amounts of 3-OH C_{14:0} and 3-OH C_{16:0} hydroxy acids (20, 21), and DNA base compositions ranging from 59 to 67 mol% guanine plus cytosine (G+C) (21, 22). "*H. indicum*" falls into a different taxon on the basis of its low DNA base composition, cellular fatty acids that include anteiso-C_{15:0} acid, and menaquinone system and should be separated from the genus *Hyphomicrobium*. In 1986, Gebers et al. (2) reported that *Hyphomicrobium* strains could be divided into seven groups (groups A to G) on the basis of DNA-DNA homology data. Furthermore, Hirsch (4) proposed that *Hyphomicrobium vulgare* IFAM MC-750 (= ATCC 27500) (7) should be the neo-type strain of the genus *Hyphomicrobium* and also described four new species, *Hyphomicrobium aestuarii*, *Hyphomicrobium hollandicum*, *Hyphomicrobium facilis*, and *Hyphomicrobium zavarzinii*. *Hyphomicrobium methylovorum* was described by Izumi et al. in 1982 (5), and this species was validated by the report of Hirsch in 1989 (4). "*Hyphomicrobium coagulans*" was described by Takada in 1975 (13), but this species has not been validated yet.

In this paper, we present a detailed emended description of *Hyphomicrobium* strains and propose the establishment of a new species, *Hyphomicrobium denitrificans*.

MATERIALS AND METHODS

Bacterial strains. The sources and reference collection numbers of the strains which we studied are shown in Table 1. All of these strains are methanol-utilizing bacteria that belong to the genus *Hyphomicrobium*. *Hyphomicrobium* sp. strains TK 0415^T (= X^T) (T = type strain), TK 0414 (= G), TK 416 (= 53-49), and TK 0441 (= B-4) were isolated by using methanol as a carbon source by Attwood and Harder (1), Uebayasi and Tonomura (16), and some of us (17). The morphological, physiological, and chemotaxonomic characteristics of these four strains were identical (21), but the taxonomic position of these strains was not determined.

Cellular fatty acid compositions. The cellular fatty acid compositions and hydroxy fatty acid compositions of *H. vulgare* ATCC 27500^T, *H. hollandicum* ATCC 27498^T and ATCC 27497, *H. facilis* ATCC 27484, and *H. facilis* subsp. *ureaphilum* ATCC 27492^T were determined as described previously (20).

Quinone systems and quinone homologs. The respiratory quinones of *H. vulgare* ATCC 27500^T, *H. hollandicum* ATCC 27498^T and ATCC 27497, *H. facilis* ATCC 27484, and *H. facilis* subsp. *ureaphilum* ATCC 27492^T were determined as described previously (19).

DNA base compositions. DNAs were extracted by the method of Saito and Miura (12), and G+C contents were determined by reversed-phase high-performance liquid chromatography (14).

DNA-DNA hybridization. DNA-DNA hybridization was carried out at 68°C by using the method of Kaneko et al. (6). DNAs from strains TK 0415^T, ATCC 27500^T, NCIB 11052^T, ATCC 27498^T, NCIB 10342^T, DSM 1566, and IFO 14180^T were labeled with [1',2',5-³H]dCTP by the nick translation method by using a type TK 700 kit (Amersham International plc, Amersham, United Kingdom).

RESULTS

Cellular fatty acid compositions. The cellular fatty acids of ATCC 27500^T, ATCC 27498^T, ATCC 27497, ATCC 27484, and ATCC 27492^T included large amounts of *n*-C_{18:1} acid and were identical to the cellular fatty acids of other *Hyphomicrobium* strains (20).

Hydroxy fatty acid compositions. Strains ATCC 27500^T, ATCC 27498^T, ATCC 27497, ATCC 27484, and ATCC 27492^T contained large amounts of 3-OH C_{14:0} and 3-OH C_{16:0} hydroxy acids, as has been reported previously for other *Hyphomicrobium* strains (20).

Quinone systems and quinone homologs. Strains ATCC 27500^T, ATCC 27498^T, ATCC 27497, ATCC 27484, and ATCC 27492^T contained large amounts of ubiquinone Q-9 and small amounts of Q-8 and Q-10, as was found for other *Hyphomicrobium* strains (19).

DNA base compositions. The DNA base compositions of the

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TABLE 1. Bacterial strains studied

Strain	Other designation(s)	Source ^a	Reference(s)
<i>Hyphomicrobium</i> sp. strain X ^T (= TK 0415 ^T)	DSM 1869 ^T , NCIB 11706 ^T	Attwood and Harder	1
<i>Hyphomicrobium</i> sp. strain G (= TK 0414)		Attwood and Harder	1
<i>Hyphomicrobium</i> sp. strain 53-49 (= TK 0416)		Uebayasi and Tonomura	16
<i>Hyphomicrobium</i> sp. strain B-4 (= TK 0441)		Urakami and Komagata	17
<i>H. vulgare</i> ATCC 27500 ^T		ATCC (MC 750 ^T)	4, 8
<i>H. aestuarii</i> NCIB 11052 ^T	ATCC 27483 ^T	NCIB (NQ 521Gr ^T)	4, 8, 9
<i>H. aestuarii</i> NCIB 9698	JCM 6889	NCIB (NQ 521)	4, 8, 9
<i>H. aestuarii</i> DSM 1564	ATCC 27488	DSM (MEV 533Gr)	4, 8, 9
<i>H. hollandicum</i> ATCC 27498 ^T		ATCC (KB 677 ^T)	4, 9
<i>H. hollandicum</i> ATCC 27497		ATCC (MC 651)	4, 9
<i>H. facilis</i> NCIB 10342 ^T	ATCC 27485 ^T , DSM 1565 ^T	NCIB (H 526 ^T)	4, 9
<i>H. facilis</i> NCIB 9699		NCIB (L 530)	4, 9
<i>H. facilis</i> NCIB 11053		NCIB (D 524)	4, 9
<i>H. facilis</i> ATCC 27484		ATCC (B 522)	4, 9
<i>H. facilis</i> subsp. <i>tolerans</i> NCIB 9697	ATCC 27491	NCIB (CO 558)	4, 9
<i>H. facilis</i> subsp. <i>ureaphilum</i> ATCC 27492 ^T		ATCC (CO 582 ^T)	4, 9
<i>H. zavarzinii</i> DSM 1566		DSM (ZV 620)	4, 9
<i>H. zavarzinii</i> NCIB 9696		NCIB (MC 630)	4, 9
<i>H. methylovorum</i> IFO 14180 ^T	JCM 6890 ^T	IFO (KM 146 ^T)	4, 5

^a Attwood and Harder, M. M. Attwood and W. Harder; Uebayasi and Tonomura, M. Uebayasi and K. Tonomura; Urakami and Komagata, T. Urakami and K. Komagata; ATCC, American Type Culture Collection; NCIB, National Collection of Industrial Bacteria; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; IFO, Institute of Fermentation, Osaka.

Hyphomicrobium strains studied were heterogeneous and ranged from 58 to 66 mol% G+C (Table 2). However, the DNA base compositions of the strains of each species were homogeneous; the G+C content of *H. vulgare* was 61.1 mol%, the G+C content of *H. aestuarii* strains was 65.5 mol%, the G+C content of *H. hollandicum* was 62.9 mol%, the G+C contents of *H. facilis* strains ranged from 59.7 to 60.5 mol%, the G+C content of *H. zavarzinii* was 63.3 mol%, the G+C content of *H. methylovorum* was 58.8 mol%, and the G+C

contents of *Hyphomicrobium* sp. strains TK 0415^T, TK 0416, and TK 0441 ranged from 60.4 to 60.6 mol%.

DNA-DNA hybridization. The levels of DNA-DNA reassociation between *Hyphomicrobium* strains are shown in Table 3. The bacteria were clearly divided into seven groups. *Hyphomicrobium* sp. strains TK 0415^T, TK 0414, TK 0416, and TK 0441 exhibited high levels of reassociation with each other (group 1), and these bacteria were different from the other bacteria studied. *H. hollandicum* ATCC 27497 was different from *H.*

TABLE 2. DNA base compositions of *Hyphomicrobium* strains

Strain	G+C content (mol%) as determined by:				Buoyant density method ^c
	HPLC method ^a		<i>T_m</i> method ^b		
	This study	Urakami and Komagata ^d	Urakami et al. ^e	Gebers et al. ^f	
<i>Hyphomicrobium</i> sp. strain TK 0415 ^T	60.4	60.5			
<i>Hyphomicrobium</i> sp. strain TK 0416		60.6			
<i>Hyphomicrobium</i> sp. strain TK 0441		60.5			
<i>H. vulgare</i> ATCC 27500 ^T	61.1			61.4	61.2
<i>H. aestuarii</i> NCIB 11052 ^T	65.5	66.1		64.1	66.8
<i>H. aestuarii</i> NCIB 9698	65.5	66.3	65.0		66.8
<i>H. aestuarii</i> DSM 1564			65.6	64.7	66.3
<i>H. hollandicum</i> ATCC 27497	65.5			62.9	64.3
<i>H. hollandicum</i> ATCC 27498 ^T	62.9			62.4	
<i>H. facilis</i> NCIB 10342 ^T	59.7		61.2	59.5	60.2
<i>H. facilis</i> NCIB 9699					59.2
<i>H. facilis</i> NCIB 11053					60.2
<i>H. facilis</i> ATCC 27484	59.6			59.3	60.2
<i>H. facilis</i> subsp. <i>tolerans</i> NCIB 9697		59.3	60.5	59.8	61.2
<i>H. facilis</i> subsp. <i>ureaphilum</i> ATCC 27492 ^T	60.5			60.5	62.2
<i>H. zavarzinii</i> DSM 1566	63.3	64.4			64.3
<i>H. zavarzinii</i> NCIB 9696		64.5			63.8
<i>H. methylovorum</i> IFO 14180 ^T	58.8		60.2		

^a Data obtained by the reversed-phase high-performance liquid chromatography (HPLC) method.

^b Data obtained by the thermal denaturation (*T_m*) method.

^c Data from reference 8.

^d Data from reference 21.

^e Data from reference 22.

^f Data from reference 2.

TABLE 3. Levels of DNA-DNA reassociation between *Hyphomicrobium* strains

Strain	% DNA-DNA reassociation with:							DNA-DNA homology group
	TK 0415 ^T	ATCC 27500 ^T	NCIB 11052 ^T	ATCC 27498 ^T	NCIB 10342	DSM 1566	IFO 14180 ^T	
<i>Hyphomicrobium</i> sp. strain TK 0415 ^T	100	10	13	13	14	7	23	1
<i>Hyphomicrobium</i> sp. strain TK 0414	87							1
<i>Hyphomicrobium</i> sp. strain TK 0416	89							1
<i>Hyphomicrobium</i> sp. strain TK 0441	83							1
<i>H. vulgare</i> ATCC 27500 ^T	9	100	28	23	6	18	12	2
<i>H. aestuarii</i> NCIB 11052 ^T	8	14	100	19	5	24	8	3
<i>H. aestuarii</i> NCIB 9698	10	11	83	21	4	30	6	3
<i>H. hollandicum</i> ATCC 27497	7	20	103	19	4	16	6	3
<i>H. hollandicum</i> ATCC 27498 ^T	8	15	28	100	5	13	6	4
<i>H. facilis</i> NCIB 10342 ^T	12	9	10	13	100	6	13	5
<i>H. facilis</i> ATCC 27484	10	7	8	7	47	4	12	(5)
<i>H. facilis</i> subsp. <i>ureaphilum</i> ATCC 27492 ^T	9	5	8	5	51	4	8	(5)
<i>H. zavarzinii</i> DSM 1566	10	21	35	23	7	100	12	6
<i>H. methylovorum</i> IFO 14180 ^T	23	6	7	23	6	18	100	7

hollandicum ATCC 27498^T (group 4) and was included in *H. aestuarii* (group 3). *H. facilis* ATCC 27484 and *H. facilis* subsp. *ureaphilum* ATCC 27492^T exhibited relatively low levels of reassociation (47 or 51%) with *H. facilis* NCIB 10342^T.

DISCUSSION

Hyphomicrobium strains are facultatively methylotrophic and utilize methanol and monomethylamine, but not methane, by the serine pathway with formaldehyde incorporation (3, 15, 18). Only "*H. coagulans*" was described as a methane utilizer (4, 13), but this finding was not confirmed by other researchers because the type strain was not deposited in a culture collection. Furthermore, *Hyphomicrobium* strains are non-spore-forming, gram-negative, rod-shaped, budding bacteria that have a Q-9 ubiquinone system, cellular fatty acids that include

a large amount of C_{18:1} acid, and hydroxy fatty acids that include large amounts of 3-OH C_{14:0} and 3-OH C_{16:0} hydroxy acids. However, the DNA base compositions of these bacteria are heterogeneous, ranging from 58 to 66 mol% G+C (Table 2) (2, 8, 21, 22). The DNA-DNA hybridization data obtained in this study are consistent with the results of Moore and Hirsch (10) and Gebers et al. (2). *Hyphomicrobium* sp. strains TK 0415^T, TK 0414, TK 0416, and TK 0441 can be clearly separated from other *Hyphomicrobium* strains. Furthermore, *H. vulgare*, *H. aestuarii*, *H. hollandicum*, *H. facilis*, *H. zavarzinii*, and *H. methylovorum* can be clearly distinguished from each other. On the other hand, *H. facilis* ATCC 27491 and *H. facilis* subsp. *ureaphilum* ATCC 27492^T exhibited relatively low levels of reassociation (47 or 51%) with *H. facilis* NCIB 10342^T. The heterogeneity of *H. facilis* strains was demonstrated by their serological relationships (Table 4) (11). *H. facilis* should be

TABLE 4. Taxonomy of the genus *Hyphomicrobium* based on DNA-DNA hybridization data, the electrophoretic types of enzymes, and serological relationships

Strain	DNA-DNA reassociation group as determined by:			Electrophoretic type ^c	Serological relationship group ^d
	This study	Gebers et al. ^a	Moore and Hirsch ^b		
<i>Hyphomicrobium</i> sp. strain TK 0415 ^T	1			1	
<i>Hyphomicrobium</i> sp. strain TK 0414	1			1	
<i>Hyphomicrobium</i> sp. strain TK 0416	1			1	
<i>Hyphomicrobium</i> sp. strain TK 0441	1			1	
<i>H. vulgare</i> ATCC 27500 ^T	2	A			II
<i>H. aestuarii</i> NCIB 11052 ^T	3	B	1		III
<i>H. aestuarii</i> NCIB 9698	3		1		
<i>H. aestuarii</i> DSM 1564		B	1		III
<i>H. hollandicum</i> ATCC 27497	3				
<i>H. hollandicum</i> ATCC 27498 ^T	4	D	2		
<i>H. facilis</i> NCIB 10342 ^T	5	E	3		IV
<i>H. facilis</i> NCIB 9699			3		IV
<i>H. facilis</i> NCIB 11053			3		
<i>H. facilis</i> ATCC 27484	(5)	E	3		IV
<i>H. facilis</i> subsp. <i>tolerans</i> NCIB 9697		E			Va
<i>H. facilis</i> subsp. <i>ureaphilum</i> ATCC 27492 ^T	(5)	E			V
<i>H. zavarzinii</i> DSM 1566	6	C			I
<i>H. zavarzinii</i> NCIB 9696					
<i>H. methylovorum</i> IFO 14180 ^T	7				

^a Data from reference 2.

^b Data from reference 10.

^c Data from reference 18.

^d Data from reference 11.

TABLE 5. Differential characteristics of *Hyphomicrobium* species

Strain	Nitrate reduction	Urease activity	Catalase activity	Nitrate respiration	Utilization of nitrate	Utilization of urea	Growth at:		Utilization of:						G+C content (mol%)
							37°C	42°C	Acetate	Formate	Succinate	Ethanol	Dimethylamine	Trimethylamine	
<i>H. methylovorum</i> IFO 14180 ^T	w ^a	—	+	—	—	w	—	—	—	w	—	—	+	+	58–59
<i>H. facilis</i> NCIB 10342 ^T	—	—	+	—	—	—	+	—	—	—	—	—	—	—	59–60
<i>H. denitrificans</i> TK 0415 ^T	+	+	+	+	+	+	—	—	+	w	—	w	+	+	60–61
TK 0414	+	+	+	+	+	+	—	—	+	w	—	w	+	+	60–61
TK 0416	+	+	+	+	+	+	—	—	+	—	—	—	+	+	60–61
TK 0441	w	+	+	+	+	+	+	—	+	w	—	—	+	+	60–61
<i>H. vulgare</i> ATCC 27500 ^T	+	—	+	+	+	—	+	—	+	+	+	—	+	+	61–62
<i>H. hollandicum</i> ATCC 27498 ^T	—	—	+	—	+	—	+	—	—	+	+	—	+	+	62–63
<i>H. zavarzinii</i> DSM 1566	w	—	—	+	+	—	+	—	w	w	—	w	+	+	63–65
<i>H. aestuarii</i> NCIB 11052 ^T	+	—	—	+	+	+	+	—	—	—	+	—	+	+	65–67
ATCC 27497	+	—	—	+	+	+	+	—	—	—	+	—	+	+	65–66

^a w, weak; —, negative; +, positive.

characterized more precisely in the future. On the other hand, *H. hollandicum* ATCC 27497 could be separated from *H. hollandicum* ATCC 27498^T and exhibited a high level of reassociation with *H. aestuarii* NCIB 11052^T. Strain ATCC 27497 (= MC 651) was included in *H. hollandicum* by Hirsch (4) on the basis of the findings of Gebers et al. (2). However, the relationship between strain ATCC 27497 (= MC 651) and *H. hollandicum* ATCC 27498^T (= KB 677^T) based on DNA-DNA homology data was not consistent with the findings of Gebers et al. (2). Moreover, the physical characteristics of strain ATCC 27497 were identical to those of *H. aestuarii* NCIB 11052^T (= TK 0401^T), NCIB 9698 (= TK 0401), and DSM 1564 (= TK 0403) (21). Therefore, strain ATCC 27497 was identified as *H. aestuarii*. Although "*H. coagulans*" was described by Takada in 1975 (13) and was included in *Bergey's Manual of Systematic Bacteriology* (4), this name has not been validated yet because the type strain of this species has not been available.

Hyphomicrobium sp. strains TK 0415^T, TK 0414, TK 0416, and TK 0441 exhibited high levels of reassociation with each other, contained the same electrophoretic types of various enzymes (Table 4) (18), had the same phenotypic characteristics (Table 5) (21), and had DNA G+C contents of 60 to 61 mol%. Furthermore, these four strains could be clearly separated from other *Hyphomicrobium* species on the basis of their phenotypic characteristics (Table 5) (21) and DNA-DNA homology data. Therefore, we believe that these four strains should be separated from previously described *Hyphomicrobium* species at the species level, and we propose a new species for these strains. The minimal characteristics for differentiating *Hyphomicrobium* species are shown in Table 5.

Description of *Hyphomicrobium denitrificans* Urakami, Sasaki, Suzuki, and Komagata sp. nov. *Hyphomicrobium denitrificans* (de.ni.tri'fi.cans. M. L. part. adj. *denitrificans*, denitrifying). Gram-negative, non-spore-forming, rod-shaped cells with pointed ends that are oval, egg-shaped, or bean shaped, 0.3 to 0.6 µm in diameter, and 1.0 to 3.0 µm long. Cells produce

mono- or bipolar filamentous outgrowths (hyphae) of varying lengths that are 0.3 to 0.4 µm in diameter. The hyphae are not septate. Cells multiply by budding at the tips of the hyphae. Mature buds become motile, break off, and often attach themselves to surfaces or to other cells to form clumps. Motility is lost soon after attachment. Cells do not grow in nutrient broth or peptone broth. Colonies on methanol-containing agar are shiny, smooth, raised, entire, white, and 1 to 2 mm in diameter after 3 to 6 days at 30°C. A water-soluble fluorescent pigment is not produced on King A or King B medium containing 5% (wt/wt) methanol. Granules of poly-β-hydroxybutyrate accumulate in the cells. Nitrate is reduced to nitrite. Nitrate respiration (anaerobic growth in the presence of nitrate) is strong. Methyl red and Voges-Proskauer tests are negative. Indole and hydrogen sulfide are not produced. Negative for gelatin and starch hydrolysis. Ammonia is not produced. Oxidase, urease, and catalase positive. Acid is not produced from sugars oxidatively or fermentatively. Methanol, monomethylamine, dimethylamine, trimethylamine, pectin, and acetate are utilized as sole sources of carbon, but methane is not utilized. Some strains utilize formate and ethanol. The following compounds are not utilized; L-arabinose, D-xylose, D-glucose, D-mannose, galactose, maltose, sucrose, lactose, trehalose, D-sorbitol, D-mannitol, inositol, glycerol, soluble starch, propionic acid, isobutyric acid, *n*-valeric acid, lactic acid, succinic acid, and oxalic acid. Vitamins and amino acids are not required for growth. Ammonia, nitrate, and urea are utilized as sole sources of nitrogen. Aerobic. Metabolism is strictly respiratory and not fermentative. Good growth occurs between pH 6.0 and 8.0 and at 30°C. Growth does not occur in media containing 3% sodium chloride. The DNA base composition ranges from 60 to 61 mol% G+C. The cellular fatty acids include large amounts of straight-chain unsaturated C_{18:1} acid. The hydroxy fatty acids include large amounts of 3-OH C_{14:0} and 3-OH C_{16:0} hydroxy acids. The major ubiquinone is ubiquinone Q-9. Methanol is utilized by the serine pathway via activated formaldehyde incorporation. The type strain of *H. denitrificans*

is TK 0415 (= DSM 1869 = NCIB 11706 = Attwood and Harder strain X).

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