

Hyphomonas adhaerens sp. nov., *Hyphomonas johnsonii* sp. nov. and *Hyphomonas rosenbergii* sp. nov., marine budding and prosthecate bacteria

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Three strains of prosthecate, budding bacteria, MHS-2^T, MHS-3^T and VP6^T, were isolated from marine habitats including the open ocean (the pelagic zone), the offshore region (the neritic zone) and the hydrothermal vent region. A polyphasic approach including 16S rDNA sequencing, phenotypic analyses, serology, fatty acid analyses, membrane protein profiles and DNA-DNA hybridizations was used to place these strains in the genus *Hyphomonas*, a taxon of the α -Proteobacteria. The results of these analyses also showed that strains MHS-3^T, MHS-2^T and VP6^T each represent a new species of *Hyphomonas*. The names *Hyphomonas adhaerens* (type strain MHS-3^T, ATCC 43965^T), *Hyphomonas johnsonii* (type strain MHS-2^T, ATCC 43964^T) and *Hyphomonas rosenbergii* (type strain VP6^T, ATCC 43869^T) are proposed for the new species. With these additions, *Hyphomonas* now contains eight species.

Keywords: *Hyphomonas*, prosthecate, budding bacteria, biofilm, marine bacteria

INTRODUCTION

Hyphomonas, along with *Pedomicrobium*, *Hyphomicrobium*, *Rhodomicrobium* (Urakami & Komaga, 1987) and *Hirschia* (Schlesner *et al.*, 1990), belongs to the budding/prosthecate bacteria (Fig. 1), a heterogeneous group in the α -Proteobacteria (Stackebrandt *et al.*, 1988) which includes more than 50 genera, including *Agrobacterium*, *Rhodobacter*, *Nitrobacter* and *Rhizobium*. This report adds three species to *Hyphomonas* and amends the description of the genus.

Pongratz (1957) first described the genus *Hyphomonas* upon isolating a budding, prosthecate bacterium from the nasal mucus of a diver with infectious sinusitis. It was subsequently named *Hyphomonas polymorpha*. It was given a new genus designation (Liefson, 1964)

because, although morphologically similar to *Hyphomicrobium* (Stutzer & Hartleb, 1988) and *Rhodomicrobium* (Duchow & Douglas, 1949), it was physiologically different. Since 1957, four additional species have been added to the genus. *Hyphomonas neptunium* was moved from *Hyphomicrobium* to *Hyphomonas* on the basis of DNA homology (Moore *et al.*, 1984) and physiology (Havenner *et al.*, 1979). *Hyphomonas oceanitis*, *Hyphomonas jannaschiana* and *Hyphomonas hirschiana* were added in 1985 (Weiner *et al.*, 1985). All of the species thus far classified as *Hyphomonas* have been isolated from diverse marine or estuarine niches.

On the basis of nearly identical biphasic, morphogenic life cycles, it was believed that *Hyphomonas* shared a very close relationship with *Hyphomicrobium*. Differentiation of *Hyphomicrobium* and *Hyphomonas* by means of morphology, DNA base composition (57–65 mol% G+C; Gebers *et al.*, 1984, 1985; Mandel *et al.*, 1972) and genome size (Kolbel-Boelke *et al.*, 1985; Moore & Hirsch, 1973) did not dispel this notion. The only obvious phenotypic differences were that *Hyphomicrobium* species utilized one-carbon compounds for carbon and energy (Hirsch, 1974; Harder *et al.*, 1975), whereas *Hyphomonas* species catabolized

Abbreviation: MPPI, membrane protein profile index.

The GenBank accession numbers for the *Hyphomonas* sequences reported in this paper are as follows: *H. adhaerens* MHS-3^T, AF082790; *H. hirschiana* VP5^T, AF082794; *H. jannaschiana* VP1, AF082799; *H. jannaschiana* VP2^T, AF082789; *H. jannaschiana* VP3, AF082792; *H. jannaschiana* VP4, AF082793; *H. johnsonii* MHS-2^T, AF082791; *H. neptunium* LE670^T, AF082798; *H. oceanitis* SCH89^T, AF082797; *H. polymorpha* PR727^T, AF082796; *H. rosenbergii* VP6^T, AF082795.

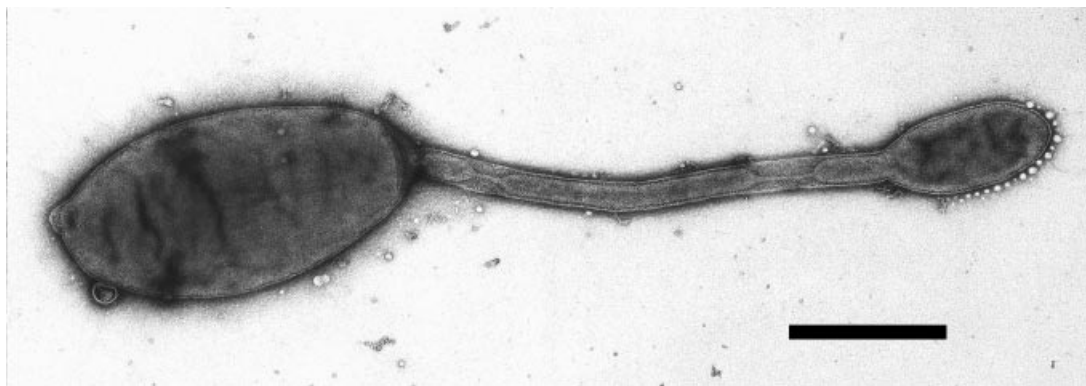


Fig. 1. *Hyphomonas adhaerens* sp. nov. strain MHS-3^T negatively stained with uranyl acetate and viewed by transmission electron microscopy. The main body of the reproductive cell, the prosthecum and the progeny (forming) bud are visible. Bar, 1 μ m. Reprinted with the permission of the American Society for Microbiology.

proteins, peptides and amino acids and had an absolute requirement for > 1% marine salt formulations (Pongratz, 1957; Havenner *et al.*, 1979; Moore *et al.*, 1984).

Molecular taxonomic approaches have supported the phenotypically based placements (Moore & Weiner, 1984) of many prosthecate bacteria into one of three genera, i.e. *Hyphomicrobium*, *Hyphomonas* or *Caulobacter*. The initial confirmation came when the G+C content of the DNA (mol%) was plotted against genome size and nucleotide distribution (Kolbel-Boelke *et al.*, 1985; Gebers *et al.*, 1985). rRNA-cistron similarities (Moore, 1977; Roggentin & Hirsch, 1989), DNA-DNA hybridization data (Moore & Hirsch, 1972; Gebers *et al.*, 1986) and quinone, fatty acid, hydroxy fatty acid and phospholipid profiles (Sittig & Hirsch, 1992; Sittig & Schlesner, 1993) also justified (or did not contradict) the placements.

Recent taxonomic studies based on 16S rDNA analyses have revealed that the marine 'evolutionary line of descent' (Stahl *et al.*, 1992) of *Caulobacter* species demonstrates a closer relationship with *Hyphomonas* species than that between *Hyphomicrobium* species and *Hyphomonas*. Also, saltwater caulobacters are, remarkably, more closely related to *Hyphomonas* than they are to freshwater caulobacters (Stahl *et al.*, 1992). Thus, marine physiology (i.e. the ability to pass macromolecules through a prosthecum to progeny buds) may be a more unifying criterion than cell biology.

Prosthecate- and budding bacteria are widespread in natural ecosystems (Moore, 1981) and may represent up to one-third of the total microbial biomass of some ecosystems (Hirsch, 1974; Nikitin & Nikitina, 1978). They are found in environments as diverse as Antarctic sea ice (Bowman *et al.*, 1997), fresh water (Staley *et al.*, 1980), ocean hydrothermal vents (Jannasch & Wirson, 1981) and soils (Hirsch, 1974). Many prosthecate species are oligotrophic (Poindexter, 1981) and evo-

lutionarily remote from eutrophic bacteria (Nikitin *et al.*, 1990).

Some of the prosthecate bacteria, in particular, *Hyphomonas* and *Caulobacter*, have been implicated as primary colonizers on submerged surfaces in the marine environment (Baier *et al.*, 1983). They are not readily isolated though they can be well represented (Weidner *et al.*, 1996). They have been at the forefront of several recent and ongoing studies involving their role in biofilm formation mediated by production of localized-capsule, extracellular, polymeric substances (holdfasts; see Quintero & Weiner, 1995; Frolund *et al.*, 1996; Baty *et al.*, 1996; Stahl *et al.*, 1992). Some of these biofilms have been shown to mediate invertebrate set (macrofouling; see Chang *et al.*, 1996). The mechanism of DNA segregation and the cell biology of capsule formation have also been elucidated recently (Langille & Weiner, 1998; Quintero *et al.*, 1998; Zerfas *et al.*, 1997).

Hyphomonas species have a stereotypical biphasic life cycle (Wali *et al.*, 1980), a G+C DNA content of 57–64 mol%, an optimum temperature range of 22–37 °C, a requirement for sea salts, a characteristic fatty acid fingerprint with octadecenoic acid (18:1) as the major fatty acid. Most species grow only with proteins, peptides and/or amino acids and synthesize Q₁₁ as the major quinone (Urakami & Komagata, 1987). Here we add the DNA-DNA overall similarity values and 16S rDNA sequence matrices of all reported species of *Hyphomonas* to clarify the taxonomy of *Hyphomonas*; these and other criteria show that three additional isolates, MHS-2^T, MHS-3^T and VP6^T, each represent a new species.

METHODS

Strain and culture conditions. The strains used in this study are listed in Table 1. *Hyphomonas neptunium*, *Hyphomonas jannaschiana*, *Hyphomonas hirschiana*, *Hyphomonas oceanitis* and *Hyphomonas polymorpha* were maintained at

Table 1. Source of *Hyphomonas* type strains used in this study

ATCC, American Type Culture Collection, Manassas, VA, USA. The temperatures shown are those used for cultivation.

<i>Hyphomonas</i> species	Strain designation	ATCC no.	Temp. (°C)	Isolation source
<i>H. polymorpha</i>	PS728 ^T	33881	30	Nasal mucosa of diver
<i>H. jannaschiana</i>	VP2 ^T	33882	30	Galapagos vent area
<i>H. hirschiana</i>	VP5 ^T	33886	25	Galapagos vent area
<i>H. oceanitis</i>	SCH89 ^T	33879	25	Baltic Sea
<i>H. neptunium</i>	LE670 ^T	15444	30	Barcelona Harbour
<i>H. johnsonii</i> sp. nov.	MHS-2 ^T	43964	30	Mud slough
<i>H. adhaerens</i> sp. nov.	MHS-3 ^T	43965	30	Mud slough
<i>H. rosenbergii</i> sp. nov.	VP6 ^T	43869	30	Guayamas vent region

–70 °C in our laboratory. Two of the three strains, belonging to the newly proposed species, were originally isolated and donated by J. Smit. Strain VP6^T was obtained from H. Jannasch. These strains were deposited in the American Type Culture Collection and also maintained in our laboratory at –70 °C.

Bacto Marine Broth 2216 (MB; Difco) was used for the routine culture of all strains. Solid marine media (MA) contained 2% Bacto agar (Difco). Working stock cultures were maintained on slants at 4 °C. For characterizations, all strains were grown with aeration at 25, 30 or 37 °C and harvested during the exponential phase of growth. Growth was measured turbidimetrically. It was verified microscopically that the culture consisted of pure prosthecate/budding and swarm cells. Species purity was confirmed serologically.

Biochemical tests. The pH and temperature requirements were examined using MB. Salt requirements were determined using variable amounts of Instant Ocean (IO; Aquarium Systems), Bacto-peptone at 0.5% and yeast extract at 0.1% (Difco). The NaCl concentration requirements were determined by using 30% MB and adding increments of NaCl. The biochemical tests were done as described by Smibert & Krieg (1981), except that IO was used instead of sodium chloride at a final concentration of 2.5%. Phenotypic tests included those for oxidase, urease, catalase, DNase, coagulase, gelatin hydrolysis, starch hydrolysis, citrate utilization, indole production, hydrogen sulfide production, gas from glucose, acid from mannose, sucrose, fructose, glucose, galactose and triple iron sugar, haemolysis on sheep-blood agar and nitrate reduction. The reagents were obtained from Sigma.

Inhibitors. The methods used for susceptibility testing were as described previously (Weiner *et al.*, 1985). Tester strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as standard controls. Streptomycin and penicillin Sensi-discs were obtained from BBL. Novobiocin, ampicillin and other inhibitory substances were purchased from Sigma and used to impregnate sterile blank discs at known concentrations. Inhibition by 1.0% Tween 80 was determined by incorporating it into the MA.

Fatty acid methyl ester analysis. All strains (Table 1) were grown at 27 °C and harvested during the late-exponential phase of growth. Fatty acids were processed and analysed according to the methods of Lechevalier & Lechevalier (1988).

Electrophoretic pattern of cell envelope protein. Cell envelope purification, membrane protein purification, SDS-PAGE and membrane protein profile index (MPPI) calculations were done as described previously (Dagasan & Weiner, 1986).

Serology. Rabbit antisera were produced against all strains, using formalin-killed cells harvested during the late-exponential phase of growth in MB as the antigens. The antisera were processed as described previously (Langille & Weiner, 1998). The antigenic similarities of all *Hyphomonas* strains were tested against each antiserum using the ELISA with low-binding, easy-wash assay plates (Corning Glass Works). The secondary antibody was peroxidase-conjugated goat anti-rabbit (Sigma). Serological similarities (%) among strains of *Hyphomonas* were calculated as (log of heterologous titre/log of homologous titre) × 100. The ELISA readings were taken at an optical density of 1.5 (at an absorbance wavelength of 490 nm) using a Lambda Reader (Perkin-Elmer). *Alteromonas macleodii* strain RF34650^T (= ATCC 27126^T) and antisera from rabbit prebleeds were used as negative controls.

Monoclonal antibodies to strain *Hyphomonas* MHS-3^T lipopolysaccharide were prepared as described previously (Quintero *et al.*, 1998) and analysed by Western blotting of SDS-PAGE-separated lipopolysaccharide as described previously (Sledjeski & Weiner, 1991).

DNA isolation, preparation and hybridization. DNA was isolated by using a variation of the Marmur (1961) procedure involving an extra phenol purification step. The purity of the DNA was determined spectrophotometrically and was considered acceptable with ratios of 1.75–1.85, 260/280 nm. The G + C content (mol%) was calculated from the thermal melting point (T_m) according to the methods of Johnson (1994). For the DNA–DNA hybridization studies, DNA was prepared, iodinated and analysed for overall similarity using the S₁ Homology DNA free-solution hybridization technique described by Johnson (1994) and incorporating ¹²⁵I according to the procedures of Werman *et al.* (1996).

PCR amplification and cloning. rRNA genes (rDNA) were amplified by the PCR, using the universal 16S rRNA (rRNA) primer 1392R (5'-ACGGGCGGTGTGTRC-3') and the bacterial primer 27F (5'-AGAGTTTGTATCMTG-GCTCAG-3') (the numbers of the primers correspond to *E. coli* positions; R = purine, M = A, C). The PCR was performed using 40 pmol of each primer, 10 mM Tris/HCl

(pH 8.8), 50 mM KCl, 0.1% (w/v) gelatin, 2.5 mM MgCl₂ and 10 mM dNTP. Target rDNA was amplified (ABI 9600 thermocycler; Perkin-Elmer) by using the following PCR protocol: denaturing at 92 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min for 30 cycles with a final extension at 72 °C for 15 min. Amplification of the target rDNA was confirmed by agarose gel electrophoresis and a 377 automated sequencer (Perkin-Elmer) was used to obtain rDNA sequences from the PCR products.

Phylogenetic analysis. Sequences were analysed for chimera formation, after which phylogenetic analysis was performed using maximum-likelihood (Larsen *et al.*, 1993) and a similarity matrix (Felsenstein, 1993). Sequences used for comparisons were obtained from the RNA Database Project (Larsen *et al.*, 1993).

RESULTS AND DISCUSSION

Phenotypic characteristics

The phenotypic characteristics of *Hyphomonas* strains MHS-2^T, MHS-3^T and VP6^T are summarized in Table 2. Each shared genus specific characteristics, e.g. prosthecate-budding morphology and a requirement for sea salts, yet were clearly different from one another. The fine structure, the colonial morphology

and the mechanism of rosette formation (or lack thereof) of *Hyphomonas* MHS-3^T and VP6^T have been reported (Langille & Weiner, 1998; Quintero & Weiner, 1995).

VP6^T had a broader optimum growth temperature (25–45 °C) than the other two new strains (25–37 °C). It also produced a reddish brown pigment (a pyomelanin; Kotob *et al.*, 1995). Of all the strains of *Hyphomonas*, only MHS-2^T used sugars as the sole carbon source for growth. Each of the three new isolates was oxidase-positive, catalase-positive and urease-negative. None hydrolysed starch or gelatin or utilized citrate. None produced indole or hydrogen sulfide.

None of the more recently acquired strains phenotypically matched closely enough with any of the extant *Hyphomonas* spp. to be considered an additional strain in an existing species. However, it would have been extremely difficult to identify properly the new strains to species using only phenotypic methods. The phenotypes, particularly the biphasic life cycle, the requirement for marine salts and the preferential utilization of peptone, were valuable in placing these strains in *Hyphomonas*.

Table 2. Phenotypic characteristics and G+C content of the DNA of newly proposed species of *Hyphomonas*

The following characteristics applied to all three species: growth in broth was granular/ropy; cells grew in peptone as the sole carbon source; and cells were susceptible to streptomycin (10 µg) and SDS (0.2 mg) but resistant to neutral red (80 µg) and Tween 80 (1.0%). Abbreviations: VS; very susceptible (diameter of inhibition zone, > 20 mm); S, susceptible (diameter of inhibition zone, 10–20 mm); R, resistant (diameter of inhibition zone, < 10 mm).

Characteristic	<i>H. adhaerens</i> MHS-3 ^T	<i>H. johnsonii</i> MHS-2 ^T	<i>H. rosenbergii</i> VP6 ^T
Optimal temp. for growth (°C)	25–37	25–37	25–45
Ocean salts growth range	1.5–12.0	1.5–6.0	1.0–12.0
Film formation on growth vessel	+++	-/+	++
Optimal pH range	5.7–8.7	5.7–8.1	5.7–8.9
Haemolysis on sheep-blood agar	Gamma	Alpha	Gamma
Rosette formation	–	+	+
Pyomelanin production	+/-	+/-	+
Susceptibility to:			
Novobiocin (30 µg)	S	S	VS
Penicillin (10 U)	R	S	S
Ampicillin (10 µg)	R	S	VS
Tellurite (0.1 mg)	VS	VS	S
Crystal violet (40 µg)	S	VS	S
Brilliant green (40 µg)	S	VS	S
Methylene blue (2.0 mg)	VS	R	S
Neutral red (160 µg)	S	R	R
Methyl violet (16 µg)	R	S	S
Growth in glucose*	–	+	–
DNA G+C content (mol%)†	60	64	61

* Sole carbon source.

† This study and personal communication (R. Gherna).

Table 3. Fatty acid profiles of *Hyphomonas*

Total fatty acid values are rounded up to the nearest one per cent of total detected fatty acids. Abbreviations: SH, Sittig & Hirsch (1992) (cells harvested in the late-exponential phase at 25 °C); TS, this study (cells harvested in the late-exponential phase at 27 °C); UK, Urakami & Komagata (1987) (cells harvested during stationary phase at 30 °C); ND, no data.

Species/strain	Reference	Total fatty acids (%)						
		n15:0	n16:0	n16:1	n17:0	cyc17:0	n17:1	n18:1
<i>H. neptunium</i> LE670 ^T	SH	10	5	1	19	ND	18	19
	TS	8	8	4	10	ND	14	37
	UK	8	2	2	16	55	ND	10
<i>H. polymorpha</i> PS728 ^T	SH	9	0	1	25	ND	14	12
	TS	6	2	1	23	ND	24	15
	UK	10	2	2	19	44	ND	10
<i>H. oceanitis</i> SCH89 ^T	SH	0	34	6	0	ND	0	53
	TS	0	27	6	0	ND	0	49
	UK	0	22	13	1	1	ND	60
<i>H. jannaschiana</i> VP2 ^T	SH	5	4	1	18	ND	14	22
	TS	5	2	0	31	ND	22	11
	UK	1	7.2	3	6	10	ND	65
<i>H. hirschiana</i> VP5 ^T	SH	0	30	2	0	ND	0	54
	TS	7	9	3	11	ND	13	39
	UK	1	9	7	2	11	ND	67
<i>H. johnsonii</i> sp. nov. MHS-2 ^T	TS	0	21	3	0	ND	0	64
<i>H. adhaerens</i> sp. nov. MHS-3 ^T	TS	0	10	2	0	ND	0	80
<i>H. rosenbergii</i> sp. nov. VP6 ^T	TS	2	32	6	0	ND	3	43

G + C content

Table 2 shows the G + C content of MHS-2^T, MHS-3^T and VP6^T to be between 60 and 64 mol %, strains MHS-3^T and VP6^T falling within the existing range of *Hyphomonas* species. The G + C content of strain MHS-2^T was 2 mol % higher than that of any other strain of *Hyphomonas*.

Fatty acid profiles

There are three separate studies on the fatty acid profiles of all but the newly proposed species of *Hyphomonas* and the results are included for purposes of comparison (Table 3). There was a consensus on most, but not all, profiles among the three studies. Variance was attributed to different conditions of cultivation, especially temperature and the phase of growth at harvesting. This study and that of Sittig & Hirsch (1992) harvested cells under similar conditions and the results are generally in good agreement.

Sittig & Hirsch (1992) and Urakami & Komagata (1987) examined several genera of budding/prosthecate bacteria, including *Hyphomonas*. *Hyphomicrobium* spp. were found to contain, on average, approximately 75 % octadecenoic acid, while all the other fatty acids constituted only 0.2–13.2 % of the total. *Hyphomonas* was the only genus, among the budding/prosthecate bacteria studied, in which some species, e.g. *Hyphomonas neptunium* and *Hyphomonas polymorpha*, contained higher amounts of hepta-

decanoic (17:0) and heptadecenoic acids (17:1) than octadecenoic acid (18:1; Sittig & Hirsch, 1992). In fact, species of *Hyphomonas* contained a mean of 50 % octadecenoic acid as opposed to approximately 75 % for species of *Hyphomicrobium*.

Like the analyses of phenotype and G + C content, the fatty acid profiles did not preclude placement of the new isolates into *Hyphomonas*. Indeed, they supported such placement. All *Hyphomonas* species synthesized high percentages of octadecenoic acid (18:1; Table 3), albeit in varying amounts. These ranged from 11 % in *Hyphomonas jannaschiana* to 80 % in MHS-3^T, a value exceeding those of all extant *Hyphomonas* species but correlating well with those of most other budding/prosthecate bacteria (i.e. *Rhodomicrobium*, *Pedomicrobium* and *Hyphomicrobium*; Urakami & Komagata, 1987; Sittig & Hirsch, 1992). *Hyphomonas* was also found to contain novel lipids (Batrakov *et al.*, 1996).

Membrane protein

Under defined growth parameters, the membrane protein content of bacteria is characteristic for each species (Dagasan & Weiner, 1986) and therefore serves as a useful taxonomic tool (Vauterin *et al.*, 1993). *Hyphomonas* species synthesized at least four outer-membrane proteins with high apparent molecular masses (> 100 kDa), which is a distinguishing characteristic of this genus (Shen *et al.*, 1989; Dagasan &

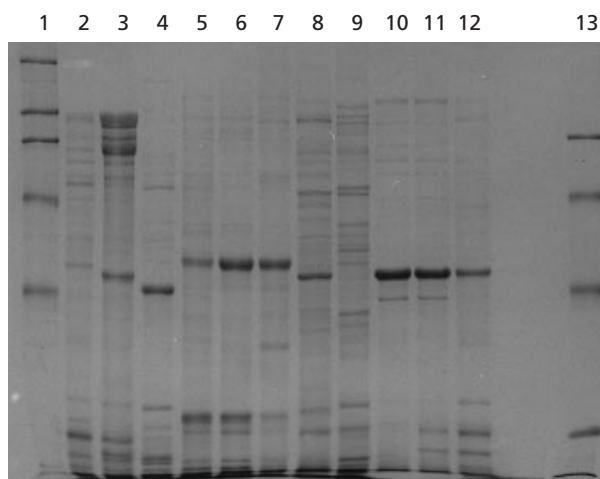


Fig. 2. SDS-PAGE of membrane proteins of seven strains of *Hyphomonas*. The gels were stained with Coomassie brilliant blue R-250. Lanes: 1, high-molecular-mass standards (170, 116, 92, 66 and 45 kDa); 2, strain VP1 (*H. jannaschiana*); 3, strain VP2^T (*H. jannaschiana*); 4, strain VP5^T (*H. hirschiana*); 5–7, strain VP6^T (proposed as *H. rosenbergii*); 8, unclassified strain MHS-1; 9, strain MHS-2^T (proposed as *H. johnsonii*); 10–12, strain MHS-3^T (proposed as *H. adhaerens*); 13, low-molecular-mass standards (92, 66, 45, 31 and 14.4 kDa).

Table 4. Serological similarities (%) among strains of *Hyphomonas*

Strains: MHS-2^T, *H. johnsonii* sp. nov.; MHS-3^T, *H. adhaerens* sp. nov.; VP1–VP4, *H. jannaschiana*; VP5^T, *H. hirschiana*; VP6^T, *H. rosenbergii* sp. nov.; LE670^T, *H. neptunium*; SCH89^T, *H. oceanitis*; PS728^T and PR727, *H. polymorpha*.

Antiserum to strain	Whole-cell strain:			
	MHS-2 ^T	MHS-3 ^T	VP1	VP6 ^T
MHS-2 ^T	100	47	52	50
MHS-3 ^T	48	100	45	< 38
VP1	72	72	100	66
VP2 ^T	62	68	76	68
VP3	71	74	97	68
VP4	48	70	88	51
VP5 ^T	61	61	47	59
VP6 ^T	54	56	63	100
LE670 ^T	62	49	39	38
SCH89 ^T	69	58	50	38
PS728 ^T	38	39	42	38
PR727	50	39	49	45
<i>Aleromonas macleodii</i>	38	39	39	44

Weiner, 1986). The results for the MPPI supported the division of extant members of this genus into five species (Dagasan & Weiner, 1986).

The MPPIs of strains MHS-2^T, MHS-3^T and VP6^T were also revealing (Fig. 2). They ranged from 30 to

80% MPPI similarity with the other species of *Hyphomonas*, suggesting their placement in *Hyphomonas* as unique species. MHS-3^T (lanes 10–12) and VP6^T (lanes 5–7) synthesized substantial amounts of a *Hyphomonas* signature protein (approx. 46–48 kDa; Dagasan & Weiner, 1986). MHS-2^T (lane 9) had the most unique protein profile and, unlike all the other strains, did not synthesize a very high-copy-number protein. It did, however, synthesize genus-typical (Dagasan & Weiner, 1986) 31 and 36 kDa proteins.

Serology

Table 4 shows the serological relatedness of the more recently isolated strains of *Hyphomonas* to one another and to the extant strains. The homologous reaction was set to 100% similarity and negative controls had less than 45% similarity. Different strains of the same species normally had > 75% similarity. Like the MPPI and phenotypes, the results must be considered in the larger framework of the molecular analyses. Only exposed antigens of whole cells would react with the strain-specific polyclonal antiserum and even these can be masked by the synthesis of surface structures such as capsules.

MHS-2^T, MHS-3^T and VP6^T were related to the other species, averaging $55 \pm 10\%$, $58 \pm 12\%$ and $52 \pm 12\%$ similarity, respectively. *Hyphomonas polymorpha* and *Hyphomonas oceanitis* had the least serological identity with the new isolates, while *Hyphomonas jannaschiana* had the most. *Hyphomonas jannaschiana* and *Hyphomonas rosenbergii*, having higher growth temperature capabilities, generally had more serological similarity to one another than to other strains. As with the MPPI, this measure of serological relatedness was useful in identifying isolates as members of the genus *Hyphomonas* (Bowden, 1993) and, in some cases, in matching the most closely related strains (same species); however, it was not useful in determining the relatedness of different species of *Hyphomonas* to one another.

Monoclonal antibodies against the lipopolysaccharide of *Hyphomonas* strain MHS-3^T did not cross-react with that of any of the other 12 strains of *Hyphomonas*. This is consistent with the considerable antigenic variability that is characteristic of LPS (DeWeger *et al.*, 1987).

DNA–DNA hybridization

Table 5 shows the results of hybridization between each extant *Hyphomonas* species and each of the newly proposed species. These results were in > 90% agreement with those of previous studies (only *Hyphomonas polymorpha* and *Hyphomonas neptunium* were previously tested; Moore & Hirsch, 1972; Gebers *et al.*, 1984). Of the new isolates, MHS-3^T had the highest overall DNA–DNA similarity (66% with another species, namely *Hyphomonas neptunium*). A guideline for the inclusion of different strains within the same

Table 5. DNA homologies of the eight *Hyphomonas* species

Results were calculated as the relationship shown by at least three separate experiments, averaging six values including the reciprocal. All the values listed are combined means from the reciprocal hybridizations, which are within 10% of one another.

Strain	MHS-3 ^T	LE670 ^T	MHS-2 ^T	VP5 ^T	SCH89 ^T	VP1	PS728 ^T	VP6 ^T
<i>H. adhaerens</i> sp. nov. MHS-3 ^T	100							
<i>H. neptunium</i> LE670 ^T	66	100						
<i>H. johnsonii</i> sp. nov. MHS-2 ^T	25	25	100					
<i>H. hirschiana</i> VP5 ^T	19	54	12	100				
<i>H. oceanitis</i> SCH89 ^T	11	12	23	20	100			
<i>H. jannaschiana</i> VP1	41	32	18	23	26	100		
<i>H. polymorpha</i> PS728 ^T	32	27	13	34	28	8	100	
<i>H. rosenbergii</i> sp. nov. VP6 ^T	30	11	21	19	2	4	5	100

species has been proposed to be $\geq 70\%$ homology (Johnson, 1984). In comparison, *Hyphomonas hirschiana* averaged 26% homology and *Hyphomonas jannaschiana* averaged 22% homology with the other species of *Hyphomonas*.

MHS-2^T was the most distantly related new strain, yet falling, in most cases, within 20% overall DNA–DNA homology. The hybridization data were consistent with other criteria, such as the membrane protein profile and phenotypic characteristics, in placing MHS-2^T as a new, more outlying, species of *Hyphomonas*.

Phylogenetic relationships

The 16S rDNA nucleotide sequences of strains MHS-2^T (1301 nucleotides), MHS-3^T (1334 nucleotides), VP6^T (1354 nucleotides), as well as the extant species PS728 (1309 nucleotides), VP1 (1352 nucleotides), VP2 (1368 nucleotides), VP3 (1351 nucleotides), VP4 (1354 nucleotides), VP5 (1354 nucleotides), SCH89 (1309 nucleotides) and LE670 (1316 nucleotides), were determined and deposited in the GenBank database.

Fig. 3 shows the similarity levels for the three newly proposed species and the five extant species. For *Hyphomonas jannaschiana* all four strains were included. *Hyphomonas jannaschiana* VP1 and MHS-3^T had been previously sequenced (Stahl *et al.*, 1992), in each case having $> 99.4\%$ similarity with the same strain in this study.

The five existing and three proposed species had signature sequences that placed them in the α -*Proteobacteria* (Stackebrandt *et al.*, 1988). Each was related to the other above the 96% similarity level. A member of the most closely related genus, *Hirschia* (Schlesner *et al.*, 1990), was related to each of the strains of *Hyphomonas* at the $90 \pm 1\%$ similarity level. The 16S rDNA sequence data supported previous findings that *Hyphomonas*, a genus of marine bacteria, is more closely related to the marine line of *Caulobacter* than to terrestrial, budding, prosthecate genera (e.g. *Hyphomicrobium*; Fig. 3).

Clearly, each of the new isolates (strains MHS-2^T, MHS-3^T and VP6^T) belong in the genus *Hyphomonas*. In addition to 16S rDNA sequences and phenotypic characteristics, the DNA reassociation values, the MPPI, the serological identities and the fatty acid analysis all support the placements of these isolates. The DNA–DNA reassociation values provided the best (but not the only) criteria by which the strains were shown to represent three distinct and new species. None of these strains had reassociation values $> 66\%$ with any extant species, while all showed some DNA–DNA similarity. Thus, it is proposed that *Hyphomonas* should be revised by the addition of three new species, bringing the total to eight species comprising 13 strains.

Interrelationship of *Hyphomonas* species based on genetic similarity

The phylogenetic tree (Fig. 3) shows the relationships between each of the species of *Hyphomonas* according to 16S rDNA sequence analysis. *Hyphomonas rosenbergii* sp. nov., *Hyphomonas hirschiana*, *Hyphomonas polymorpha* and *Hyphomonas neptunium* clustered at the 99.4% similarity level. *Hyphomonas adhaerens* sp. nov. and *Hyphomonas jannaschiana* (99.3%) and, as expected, each of the strains of *Hyphomonas jannaschiana* were related at a high level (99.3%) as well. *Hyphomonas johnsonii* sp. nov. was most closely related to *Hyphomonas oceanitis* (a 98.7% similarity level) but otherwise neither of these species was closely related to other species of *Hyphomonas*. The bootstrapping analyses support these placements (Fig. 3).

Thus, the 16S rDNA sequence analyses divide the eight species of *Hyphomonas* into: (i) a tight group of four species, i.e. *Hyphomonas rosenbergii* sp. nov., *Hyphomonas hirschiana*, *Hyphomonas polymorpha* and *Hyphomonas neptunium*; (ii) a group with two species, i.e. *Hyphomonas adhaerens* sp. nov. and *Hyphomonas jannaschiana*; and (iii) a less tight group of two species, i.e. *Hyphomonas johnsonii* sp. nov. and *Hyphomonas oceanitis*. Likewise, the DNA–DNA hybridization data show *Hyphomonas neptunium* to be closely related

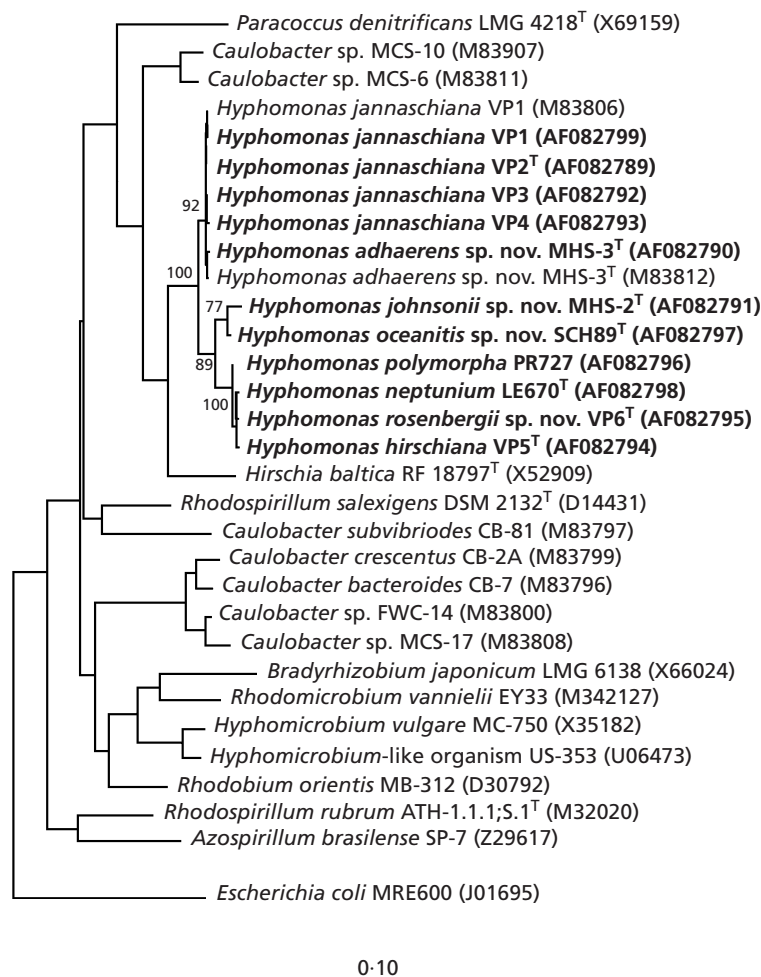


Fig. 3. Phylogenetic analysis of *Hyphomonas*. The tree was constructed using the fast DNA maximum-likelihood program from the RNA Database Project (Larsen *et al.*, 1993). Sequences obtained in this study are in bold type. Sequences used for comparison were obtained from the RNA Database Project (Larsen *et al.*, 1993). Bootstrap values (expressed as percentages of 100 replications), pertinent to *Hyphomonas* species, are shown at the appropriate branch points. *Escherichia coli* MRE600 was used as an outgroup to establish the root of the tree.

to *Hyphomonas hirschiana* and support both of the two species groupings, i.e. *Hyphomonas adhaerens* sp. nov. and *Hyphomonas jannaschiana* plus *Hyphomonas johnsonii* sp. nov. and *Hyphomonas oceanitis*. On the other hand, the DNA–DNA hybridization data suggest that *Hyphomonas neptunium* forms a tighter relationship with *Hyphomonas adhaerens* sp. nov. on the basis of DNA–DNA homology values than on the basis of 16S rDNA sequence comparisons. However, DNA–DNA homology values can be inflated by shared plasmids and other forms of horizontal genetic exchange.

The possibility was considered, and the serological identities hinted, that the vent prosthecate strains (VP1–VP6^T) could form a sub-group within *Hyphomonas* since each was isolated from the deep sea, each was barophilic and each came from dynamic niches with respect to temperature and ion concentration. Indeed, the VP strains, especially VP1–VP4, were phenotypically more ‘plastic’ (i.e. able to flourish in a wide variety of environmental conditions). However, genotypically, *Hyphomonas jannaschiana*, *Hyphomonas hirschiana* and *Hyphomonas rosenbergii* sp. nov. grouped as strongly, or more strongly, with species isolated from non-vent ocean niches as with one

another. For example, *Hyphomonas adhaerens* sp. nov. MHS-3^T and *Hyphomonas jannaschiana* VP1 had a higher 16S rDNA similarity matrix with one another than with *Hyphomonas hirschiana* VP5^T or *Hyphomonas rosenbergii* sp. nov. VP6^T. Thus, it appears that *Hyphomonas* is a group of pelagic ‘wanderers’, able to colonize (and possibly adapt to) different solid, submerged substrata, including those near hydrothermal vents.

Amended description of genus *Hyphomonas*

Hyphomonas species are of marine origin, share a biphasic life cycle and normally generate only a single polar prosthecum. They catabolize amino acids for energy and growth (with the exception of strain MHS-2^T, which can utilize sugars). They are isolated from many ocean niches, including the open ocean (the pelagic zone), mud sloughs and two different hydrothermal vent sites (surface waters and depths of 2600 m). Members of the genus *Hyphomonas*, like other budding, prosthecate bacteria, undergo a rather complex life cycle termed a ‘biphasic life cycle’. It requires 265 min for *Hyphomonas neptunium* to complete a full cycle at 36 °C. The life cycle consists of

a swarm-cell stage that eventually metamorphoses into a benthic, reproductive cell during the biphasic developmental cycle. Members of *Hyphomonas*, especially the vent strains, grow in a broad range of environmental conditions, including those involving considerable temperature, pressure and salinity variations. They are primary colonizers of submerged marine surfaces, some species producing dense biofilms.

Briefly, the morphological characteristics of *Hyphomonas* cells are as follows: rod-shaped to oval mature cells measuring 0.5–1.0 × 1.0–3.0 µm; buds are produced at the tips of polar prosthecae, which measure 0.2–0.3 µm in diameter and are 1–5 times the length of the cell body; and swarm cells are motile by means of a single polar to lateral flagellum located on developing buds of younger daughter cells.

Gram-negative, non-acid-fast, aerobic, non-spore-forming and chemo-organotrophic. All strains investigated so far are catalase-positive, oxidase-positive, urease-negative, indole-negative, hydrogen sulfide-negative, non-saccharolytic and non-pathogenic for mammals. Peptone is normally required for growth. With one exception, all strains denitrify. The optimum growth temperature range is 22–37 °C at 101 kPa. *Hyphomonas neptunium*, *Hyphomonas polymorpha*, *Hyphomonas oceanitis* and *Hyphomonas hirschiana* have a Q-11 ubiquinone type along with a significant amount of Q-10 (approx. 10% of total ubiquinones) and minor amounts of Q-9 and Q-12. *Hyphomonas jannaschiana* has Q-10 as its major quinone, with trace amounts of Q-9 and Q-11. Synthesize many apparent high-molecular-mass proteins (≥ 64 kDa). The dominant fatty acid, common to all *Hyphomonas* spp., is octadecanoic acid (18:1). The G+C content of the DNA is 57–64 mol%.

Description of *Hyphomonas adhaerens* sp. nov.

Hyphomonas adhaerens (ad.hae'rens. L. part. adj. *adhaerens* hanging on/sticking to).

The main body of the mother cell is prolate spheroid, approximately 1–2 µm in diameter and has one prosthecum 0.2 µm wide × 1–5 µm long. Buds are motile by a single flagellum. The main body of the reproductive cell, but not the prosthecum, is surrounded by capsular polysaccharide. Gram-negative. Not acid-fast. No endospores. Aerobic. Colonies are round, undulate and about 1.5 mm in diameter after 3 d at 30 °C on marine agar. In liquid media, there is granular turbidity due to adhering cell masses. A thick biofilm forms on the surface of the growth vessel. Do not form rosettes. The optimum temperature range is 25–37 °C. The ocean salts growth range is 1.5–12%. The optimum pH growth range is 5.7–8.7. Nitrate is reduced. Sheep erythrocytes are not haemolysed. Susceptible to novobiocin, streptomycin, tellurite, crystal violet, brilliant green and methylene blue but resistant to 1.0% Tween 80, penicillin and ampicillin.

The major fatty acid is octadecanoic acid (18:1), constituting 80% of the total fatty acids. The G+C content of the DNA is 60 mol%. The type strain is MHS-3^T (= ATCC 43965^T).

Description of *Hyphomonas johnsonii* sp. nov.

Hyphomonas johnsonii (john.so'nii. M.L. gen. n. *johnsonii* of Johnson, named after the American molecular taxonomist John Johnson).

The main body of the mother cell is prolate spheroid, approximately 1 µm in diameter and has one prosthecum 0.2 µm wide × 1 µm long. Buds are motile by a single flagellum. Gram-negative. Not acid-fast. No endospores. Aerobic. Colonies are round, convex and approximately 1.5 µm in diameter after 3 d at 30 °C on marine agar. In liquid medium, growth is granular and/or ropey. A thin film may form on the growth vessel. Can utilize sugars, in the presence of amino acids for carbon and energy. The optimum temperature range is 25–37 °C. The ocean salts growth range is 1.5–6.0%. The pH growth range is 5.7–8.0. Nitrate is reduced. Sheep erythrocytes are α-haemolysed. Susceptible to novobiocin, penicillin, ampicillin, streptomycin, tellurite, crystal violet and brilliant green but resistant to 1.0% Tween 80, methylene blue and neutral red. The major fatty acid is octadecanoic acid (18:1), constituting 64% of the total fatty acids. The outer-membrane-protein profiles, DNA homology and serology have low but significant identity with other strains of *Hyphomonas*. The G+C content of the DNA is 64 mol%. The type strain is MHS-2^T (= ATCC 43964^T).

Description of *Hyphomonas rosenbergii* sp. nov.

Hyphomonas rosenbergii (ro.sen.ber'gi.i. M.L. gen. n. *rosenbergii* of Rosenberg, named after the Israeli microbial ecologist Eugene Rosenberg).

The main body of the mother cell is prolate spheroid, approximately 1 µm in diameter and has one prosthecum 0.2 µm wide × 1 µm long. Buds are motile by a single flagellum. Synthesizes a capsule that surrounds the entire cell at all growth stages and a polar holdfast that is temporally synthesized. Gram-negative. Not acid-fast. No endospores. Aerobic. Colonies are round, convex and approximately 1.5 µm in diameter after 3 d at 37 °C on marine agar. In liquid medium, growth is granular and/or ropey. A biofilm forms on the walls of the growth vessel. Cells form rosettes. The optimum temperature range is 25–45 °C. The ocean salts growth range is 1.0–2.0%. The pH growth range is 5.7–8.9. Nitrate is reduced. Sheep erythrocytes are not haemolysed. Susceptible to novobiocin, penicillin, ampicillin, streptomycin, tellurite, crystal violet, brilliant green and methylene blue but resistant to 1.0% Tween 80 and neutral red. The major fatty acid is octadecanoic acid (18:1), constituting 43% of the total fatty acids. The outer-membrane-protein profiles, DNA homology and serology have

low but significant identity with other strains of *Hyphomonas*. The G+C content of the DNA is 61 mol%. The type strain is VP6^T (= ATCC 43869^T).

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