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Biology of Budding Bacteria

I. Enrichment, Isolation and Morphology of Hyphomicrobium spp.

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With 28 Figures in the Text

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Hyphomicrobium vulgare (Stutzer and Hartleb 1898) and *Rhodomicrobium vannielii* (Duchow and Douglas 1949) are the sole representatives of the genera which comprise the family *Hyphomicrobiaceae* (order *Hyphomicrobiales*, BERGEY's Manual 1957). They are characterized primarily by multiplication through a process of bud formation at the end of a filament (hypha), rather than by fission. Although their morphological similarities suggest a close relationship, they appear to be physiologically dissimilar. *H. vulgare* is reported to be an aerobic organotroph, whereas *Rh. vannielii* is described as an anaerobic photoorganotroph. A comparative study of the morphology and physiology of each of these organisms has therefore been undertaken in order to establish any possible interrelationships.

The present investigation describes methods for the enrichment and isolation of different hyphomicrobia from soil and water habitats. Some morphological observations on newly isolated strains are compared with those of known cultures of *H. vulgare*.

A. Materials and Methods

Sources of Hyphomicrobium: The inoculum for enrichment experiments with air as the gas phase was a freshly mixed soil sample (upper 3 cm; sand, peat, and lawn soil; pH 6.6). In other enrichment experiments, with CO in the gas phase, the soil sample contained lawn soil and sandy loam (pH 6.8). Hyphomicrobia were also readily enriched from other types of soils (compost, rotten manure, flower pot soil and the shore of a pond 10 cm above the water table).

Hyphomicrobium spp. were found in water from taps in the laboratory, in a water bath in a photographic dark room, on water surface films of a Warburg apparatus, and in constantly illuminated water baths used for the culture of purple bacteria; hyphomicrobia could be isolated from the air of a variety of laboratories, and were found in samples of sea water taken from the shore at Woods Hole, Massachusetts, U.S.A.

A culture of *H. vulgare*, "*NQ*," was obtained from Dr. J. R. QUAYLE Sheffield, England; it was originally isolated by MEVIUS jr. (1953) from Elbe River water and designated as strain "*B*". Another culture of the "*B*" strain of MEVIUS jr. („*MEV*") was obtained from Dr. R. NÄVEKE Braunschweig, Germany.

Mineral media. Salts were dissolved in 1000 ml of dist. water; the pH was adjusted with NaOH or HCl. Sterilization: 25 min/121 °C.

"340": $Na_2HPO_4 \cdot 7H_2O$ 0.2 g; KNO_3 0.4 g; $MgSO_4 \cdot 7H_2O$ 4.8 mg; $MnCl_2 \cdot 4H_2O$ 0.1 mg; $FeCl_3 \cdot 6H_2O$ 0.2 mg; pH 6.4 or 7.2.

"337": KH_2PO_4 1.36 g; $Na_2HPO_4 \cdot 7H_2O$ 2.13 g; $(NH_4)_2SO_4$ 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; $CaCl_2 \cdot 2H_2O$ 10 mg; $FeSO_4 \cdot 7H_2O$ 5 mg; $MnSO_4 \cdot 4H_2O$ 2.5 mg; $Na_2MoO_4 \cdot 2H_2O$ 2.5 mg; pH 7.0.

"349": KH_2PO_4 0.68 g; $Na_2HPO_4 \cdot 7H_2O$ 1.42 g; KNO_3 1.01 g; $MgSO_4 \cdot 7H_2O$ 0.123 g; $CaSO_4 \cdot 2H_2O$ 0.017 g (Merck No. 2161; dissolved separately); $FeSO_4 \cdot 7H_2O$ 0.014 g; pH 7.2; after NÄVEKE (1963).

Organic media. For the isolation of hyphomicrobia from water bath samples and sea water we used medium 337 supplemented with 1.8% Difco purified Noble agar and 0.675% methylamine hydrochloride (sterilized separately).

The isolation from enrichment cultures and further purification was usually done in disposable petri dishes with medium 337 or 340 containing agar and methylamine. Bacto nutrient agar or—broth, with 2 g/l NaCl and 10 g/l glucose added, pH 6.4 or 7.5, sterilized 20 min/121 °C, were used in checks for contamination. Stock cultures were kept on methylamine agar slants in screw cap tubes.

All chemicals were reagent grade; cotton was purchased from Fisher Sci. Comp. (No. 7-895). Gases: CO came from Matheson Co., Inc.; He, O_2 and CO_2 were from Ohio Chem. & Surg. Equipment Co. and were of highest purity.

Enrichment cultures. 250 or 300 ml flasks, loosely plugged with cotton, containing 50—100 ml of the sterilized mineral medium, were inoculated with 0.5 g of soil or 0.5—1.0 ml of liquid samples. For enrichment under a defined atmosphere flasks were kept in desiccators sealed with Dow Corning "Silicone lubricant."

Microscopy. All cultures were examined directly under phase contrast. Stained preparations were made by air drying samples of the pellicle or medium, staining with carbol fuchsin for 3 min (Ziehl's method, Manual of Microbial Methods 1957), rinsing with distilled water, and dried by blowing air over them. Phase contrast slides for observations of living cells were prepared in the following way (HIRSCH 1957): microscope slides were cut into 3 equal parts and the center portion replaced by attaching to the sides a 50×22 mm Corning cover slip with water glass. After drying at 30° C the slides were used in the normal fashion.

Micrographs were taken on Kodak Plus X Pan or Schleussner KB 14 or 17 film with a Zeiss photomicroscope employing bright field for stained preparations or phase contrast for living cells.

B. Results

I. Enrichment experiments

Hyphomicrobium was previously isolated from enrichment cultures for *Nitrobacter* spp. (RULLMANN 1897; STUTZER and HARTLEB 1897, 1898; KINGMA BOLTJES 1936) or from enrichments for *Nitrosomonas* spp. (KINGMA BOLTJES 1936; MEVIUS jr. 1953). Judging from the variety of nitrogen sources available in these enrichments (NO_2^-, NO_3^- and NH_4^+), we concluded that the N-source probably did not contribute greatly to the enrichment of *Hyphomicrobium* spp. The type of carbon source available

during the time of enrichment might, however, have influenced the appearance of hyphomicrobia. Although the enrichment media employed in these studies lacked added C-sources, CO_2 and other gaseous components of the laboratory air could have been utilized. Formate or methanol was added to enrichment media employed by KINGMA BOLTJES (1936), and ZAVARZIN (1960).

Table 1. *Initial conditions employed for enrichments of Hyphomicrobium spp.* Mineral salts medium 340 (pH 7.2) in 125 ml Erlenmeyer flasks, kept in the dark at 25—28°C, see methods

Atmosphere	added nitrogen source (1.0 g/l)	temperature (°C)	inoculum (appr. 0.5 g)	optimal growth of hyphomicrobia (weeks)	remarks
air	$NaNO_3$	25	manure soil	4	$CaCO_3$ added; purification under an atmosphere of CO/O_2
air	$NaNO_3$	25	mud from 10 cm above the water table of a pond	5	
air	$Ca(NO_3)_2$	30	surface soil of a flower pot, top soil of grass lawn	6	
methanol	$NaNO_3$	30	compost soil	7	
acetic acid	$(NH_4)_2HPO_4$ + $NaNO_3$	28	manure soil	4	purification under an atmosphere of CO/O_2

Initial attempts were made to enrich hyphomicrobia under conditions shown in Table 1. Stalked, budding bacteria were seen in all of the pellicles which developed on the surface of the medium. Optimally, *Hyphomicrobium* multiplied after 4—7 weeks, and pure cultures were readily obtained from these enrichments.

The influence of temperature, pH of the medium, and light on the development of hypomicrobia in enrichment cultures was also followed (Table 2). After 10 days incubation at 20° or 37°C a thin colorless pellicle, which subsequently thickened, was formed on the surface of the medium in most samples. Microscopic examination revealed the presence of *Hyphomicrobium* and other microorganisms. After 24 days slides were prepared from all flasks and the occurrence of hyphomicrobia estimated (Table 2; and see Fig. 1). The greatest number was found in flasks incubated at 37°C. Hyphomicrobia also developed at 20°C, but under these conditions they were more numerous in the dark than in the light. Only

one flask incubated at 5°C contained *Hyphomicrobium* in the pellicle. Thus, the temperature, and to a certain extent, light influenced the appearance of *Hyphomicrobium* in the enrichments, while the pH, in the range tested, had little or no effect.

Table 2. *Growth of Hyphomicrobium spp. after 24 days in enrichment cultures under various conditions*

50 ml of medium 340 in 125 ml Erlenmeyer flasks, covered with glass beakers. Inoculum: a mixed soil sample of pH 6.6

	pH 6.4		pH 7.2	
	Illumination: dark	light	Illumination: dark	light
Temperature: 37°C	++++	+++++	+++++	+++++
20°C	++++	+++	++++	++
5°C	(−)	(−)	(−)	++

Growth estimates: No hyphomicrobia: (−); less than 1% of the total microbial population of the pellicle: ++; 5—10%: +++; 10—40%: ++++; 40—80%: +++++

Since it is known that hyphomicrobia grow in media without added C- or N-sources (STUTZER and HARTLEB 1898; KINGMA BOLTJES 1936), it was thought that the enrichment might be suitably influenced by the presence of a specific atmosphere. The introduction of a knallgas/CO_2 mixture did not seem to stimulate the hyphomicrobia; but it was found that under CO/O_2/CO_2/helium (75:10:0.5:14.5 or 30:15:0.5:54.5) stalked bacteria of the *Hyphomicrobium* type developed regularly at 30°C. Thin pellicles on the surface of the medium thickened rapidly and became dry, folded and white. There was no pellicle formation in flasks kept in desiccators with air as the gas phase.

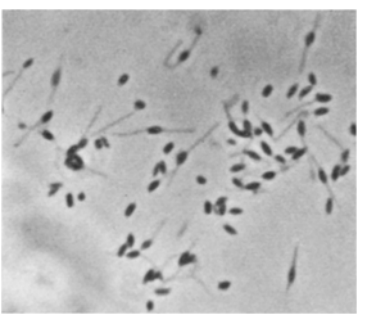

Fig. 1. *Hyphomicrobium* spp. from an enrichment culture. Grown for 16 weeks on mineral medium, pH 6.4 at 37° C

After 9 weeks the desiccators with the CO/O_2/CO_2 atmosphere were opened and an underpressure was observed. Slide preparations showed that most of the organisms in the pellicles were bacteria other than hyphomicrobia. Several types of stalked or budding bacteria were present, including *Caulobacter* spp., though they were far less numerous than in the case of the enrichments under air.

Subculture on methylamine agar yielded typical colonies of hyphomicrobia. It thus appears that the presence of CO did not markedly inhibit the growth of *Hyphomicrobium* spp. in enrichments; it remains to be determined if CO can be used as a source of carbon and energy.

Attempts were made to enrich *Hyphomicrobium* from sea water samples in order to obtain halophilic forms. Surface water from "Woy Dock", Woods Hole/Mass. or from a salt water inlet at the harbor of Woods Hole was either cultured directly on methylamine agar, or inoculated into the mineral medium 337 containing methylamine and incubated at 20° and 30° C, with and without illumination. After 5 days of incubation, bacteria other than hyphomicrobia predominated; *Hyphomicrobium* spp. as well as other budding bacteria developed only after continued incubation. These are being isolated and purified at the present time. Hyphomicrobia also developed on mineral medium 337 lacking an added carbon source.

II. Isolation from the enrichment cultures

It proved relatively simple to enrich for *Hyphomicrobium* with mineral salts media lacking added organic C- and N-sources, thus taking advantage of its ability to grow from impurities in the atmosphere. The subsequent isolation procedures were satisfactorily carried out with mineral agar 337 containing methylamine or methanol as the added C-source.

The pleomorphic appearance of *Hyphomicrobium* spp. made it difficult to estimate the purity of cultures. Cells of different age, with and without hyphae, and swarmers, were regularly seen in pure cultures. Observations of the microscopic appearance of the cultures was our initial check for purity. These were then subcultured in nutrient broth and agar at pH 6.4 and 7.5 (with and without the addition of glucose and NaCl), and on mineral salts media without an added C-source, at various temperatures. The microscopic appearance was then observed. In the case of the *H. vulgare* strains (*NQ* and *MEV*) turbidity of nutrient broth was indicative of contamination, since pure cultures only formed pellicles. The other hyphomicrobia produced slight turbidity and a sediment, but pellicles were not formed.

III. Morphology of H. vulgare (NQ and MEV) and of newly isolated Hyphomicrobium spp.

1. General considerations

The degree of morphological variation in a given culture of our budding, stalked bacteria was initially somewhat disturbing. This was due in part to the morphological changes which occurred during growth (Fig. 7).

The recognition of *Hyphomicrobium* in enrichment cultures and observations on its morphology were also difficult, because it was similar in appearance to filamentous stages of saprophytic *Mycoplasma* strains, L-forms of soil bacteria, plasma threads of *Labyrinthula*, reticulopodia of foraminifera, and to *Caulobacter* spp., which also developed (Figs. 2–4).

The only observed process of multiplication in hyphomicrobia is budding (KINGMA BOLTJES 1936; ZAVARZIN 1960). Rarely did cells appear to divide by transverse fission (Figs. 5, 6 and 28); in these instances careful observation indicated the presence of a short hypha, or in many cases, one cell was considerably smaller than the other, an indication that it had arisen by budding.

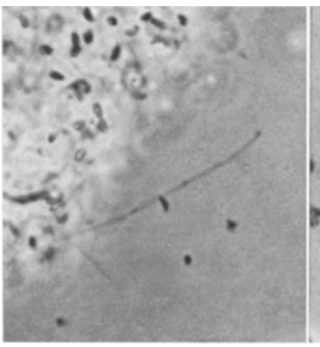

Fig. 2

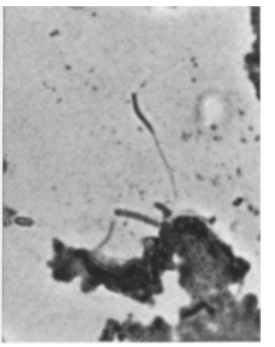

Fig. 3

Fig. 2. An organism resembling *Hyphomicrobium*. The plasma thread undulated and changed in length; small granules inside of it travelled along the "hypha". Enrichment culture, grown $4^1/_2$ months under light at 37° C. Mineral medium 340, pH 7.2

Fig. 3. A stalked bacterium of the *Caulobacter* type. From a pellicle on tap water in a gas burette, grown for 3 weeks at 20° C

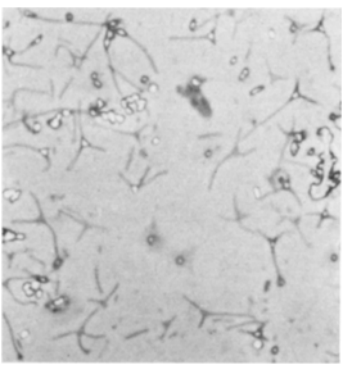

Fig. 4. Microorganisms resembling *Hyphomicrobium* and thought to be saprophytic *Mycoplasma* cells. Enrichment culture kept for 4 months at 20° C and in the dark; mineral medium pH 6.4; air as the atmosphere. Stained preparation

Multiplication does not appear to result in the formation of two morphologically equivalent cells; the mother cell is quite different from the bud or swarmer. Since, as will be shown later, morphogenesis was affected by the medium, the ratio, of mother cells to swarmers, and hence daughter cells, greatly affected the microscopic appearance of the cultures.

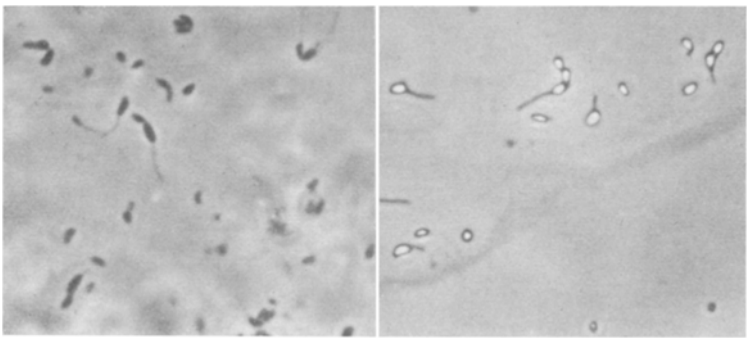

Fig. 5 Fig. 6

Fig. 5. *H. vulgare NQ*, grown for $2^1/_2$ months with acetamide as the carbon source. Note that some rods appear to be dividing by fission

Fig. 6. *H. vulgare NQ*, grown on methylamine agar 337. Colonies pressed on slide and stained. Note chain formation

2. The morphology of H. vulgare (NQ and MEV)

a) General observations. Since cross walls were not observed between rod and filament, the term "cell" is used to refer to the entire organism, i.e. the rod together with any hyphae or developing buds.

The rod is normally bean-shaped; its width is rather constant (0.5—0.75 μ), but the length (0.6—5.0 μ) varies with age and conditions of growth (Figs. 7—15). Giant forms (0.75—2.0 × 2.0—5.0 μ), are occasionally found among the normal cells. The rod usually contains one and occasionally 2—3 highly refractile granules, which consist of poly-β-hydroxybutyrate ("PHB"; Figs. 12—14; HIRSCH and CONTI 1964) and are situated at one end of the rod in the region of the hypha. Such inclusions were rarely observed in swarmer cells. Slightly elongated cells containing two PHB granules were seen occasionally in older cultures. They closely resembled the organism described by HENRICI and JOHNSON (1935) as *Blastocaulis* (Fig. 21).

The hyphae have a rather constant diameter of 0.2—0.3 μ, but are of varying length (Figs. 8—15). Primary side branches are formed at right angles to the main hypha, although other angles were also formed. Swarmers developed from the hyphal ends. Some added carbon sources initiate the formation of a short true mycelium with both primary and secondary side branches (Fig. 12).

Under certain cultural conditions the formation of dense and inflated portions of the hypha, which appeared to be intercalary buds, were observed. In contrast to *Rh. vannielii*, no preformed points of breakage (sites of cross walls) were found.

The bud of *H. vulgare* is bean- or slightly pear-shaped; it is generally smaller and more slender than the mother cell, and is often turned at a

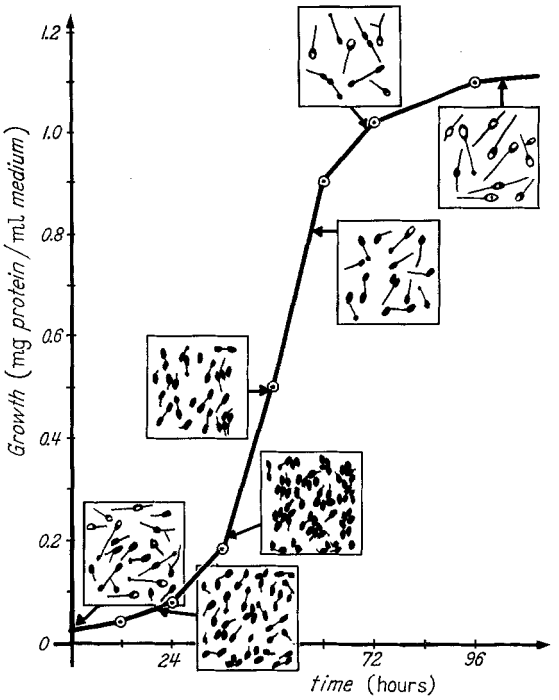

Fig. 7. Morphological changes during the growth cycle of *H. vulgare NQ*, samples from a stirred, aerated culture with methylamine as the C- and N-source, and $(NH_4)_2SO_4$ as an additional N-source. Schematical drawing after stained preparations

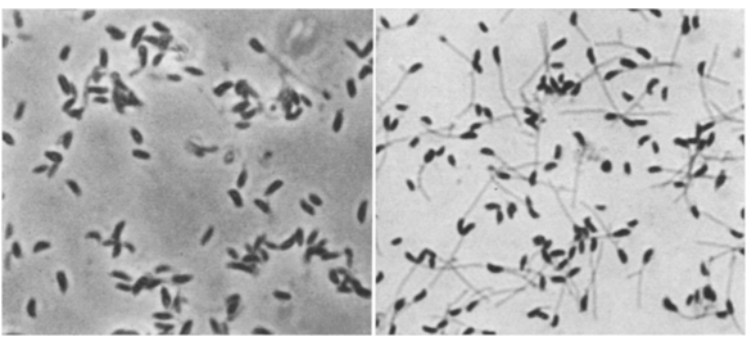

Fig. 8 Fig. 9

Fig. 8. *H. vulgare NQ*, grown for 4 weeks on methylamine mineral medium 337

Fig. 9. *H. vulgare NQ*, grown for 9 weeks on mineral medium 337 under $CO/O_2/CO_2$. Stained

90° angle to it. A motile bud is referred to as a "swarmer." The single flagellum (generally subpolar) is easily lost (UHLKEN 1959; ZAVARZIN 1960). The bud can either tear free as a swarmer or remain connected with the mother cell; in some cases chain or cross formation can be observed

(Figs. 6 and 25). The mechanism of attachment of swarmers to surfaces (glass, other cells) is not fully understood, however, we were able to observe an inhibitory influence of light upon this attachment (HIRSCH and CONTI 1964).

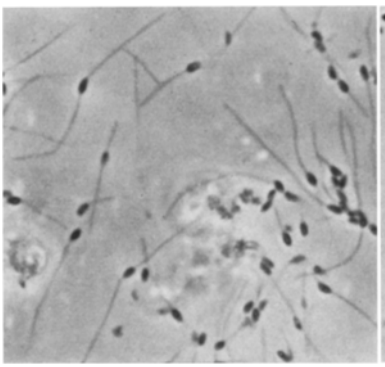

Fig. 10

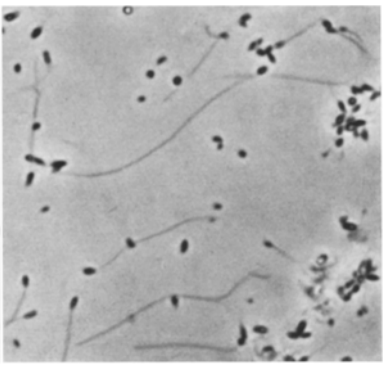

Fig. 11

Fig. 10. *H. vulgare MEV*, grown for 9 weeks on mineral medium with bicarbonate as the added carbon source

Fig. 11. *H. vulgare NQ*, grown for 7 weeks on formamide

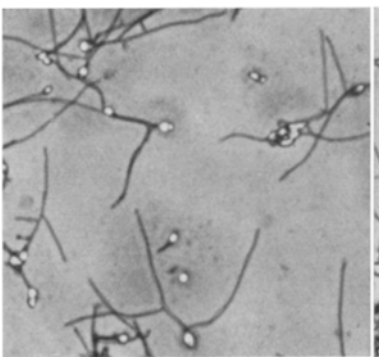

Fig. 12

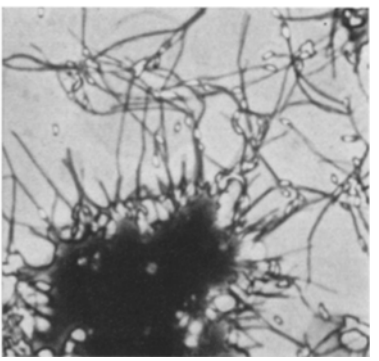

Fig. 13

Fig. 12. *H. vulgare NQ*, grown for 8 weeks on acetate mineral medium. Stained with carbol fuchsin. The unstained, highly refractile granules in the rods are PHB

Fig. 13. *H. vulgare NQ*, grown for 8 weeks on urea mineral medium. Stained

b) Factors influencing the morphology of H. vulgare NQ. It has been established that the shape and size of the rod is relatively constant, but the length and degree of branching of the hypha is dependent on external factors (RULLMANN 1897; STUTZER and HARTLEB 1898; KINGMA BOLTJES 1936; ZAVARZIN 1960). The effects of a variety of factors on the appearance of the hyphae were therefore investigated.

The influence of the age on hyphal appearance in a culture grown with vigorous stirring and aeration was followed. Under these conditions

H. vulgare NQ grew as homogeneous turbidity or as small flakes. The change in appearance of the cells during the various phases of growth is shown in Fig. 7.

Preliminary experiments indicated that *H. vulgare NQ* is osmotolerant, and salt concentration (up to 9 g/l) had little or no effect on its morphology. Similarly, the temperature (+ 5, 20, 30 or 37° C), light intensity, and the partial pressure of oxygen during growth did not significantly influence its morphology.

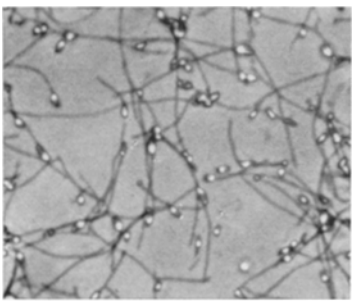

Fig. 14. *H. vulgare NQ*, grown for 8 weeks on hydantoic acid. Stained

The added carbon source, however, had a marked effect on the appearance of the cells. Cultures were grown statically, with NH_4^+ as the N-source, and with different C-sources. The observations, summarized in Table 3 and illustrated in Figs. 8—14, generally indicate that rapid growth due to the presence of an appropriate C-source stimulates the formation of buds and swarmer cells, and the hyphae, if present, are short. Poor growth results in a striking decrease in the number of daughter cells, whereas elongation of the hypha is marked.

Other experiments with the N-source varied, and methanol as the added C-source, gave similar results (Table 4). Cells grown on methanol/NH_4^+

Table 3. *Morphological effects of the carbon source on cells of H. vulgare NQ, grown for 2—3 months on a mineral medium (337) with NH_4^+ as the N-source*[1]

Carbon source	average size in microns		observed branching	swarmer formation	growth
	rods	hyphae			
Methylamine-HCl[1]	0.7×1.5	0.2 × 1.5	seldom	many	excellent
Methanol[3]	0.7×1.5	0.2 × 2.0	rarely	many	good
Sodium formate[1]	0.6×1.0	0.15×15	rarely	very few	fair
Carbon monoxide[4]	0.8×1.5	0.2 × 8.0	often	few	poor
Sodium bicarbonate[1]	0.7×1.2	0.2 ×20	often	very few	fair
Sodium acetate[2]	0.7×1.0	0.15×10	occasionally	few	fair
Sodium glycerate, or sodium malate[2]	0.7×1.5	0.2 ×10	seldom	few	good
Sodium lactate[2]	0.8×2.0	0.2 × 5.0	often	many	good
None[1]	0.5×1.0	0.15×15	seldom	very few	fair

[1] Concentrations: C-source/20 mM, N-source 10 mM.

[2] C-source 0.2% (w/vol); $(NH_4)_2SO_4$ as the N-source (conc. 4 mM); mineral salts medium 337.

[3] Concentration 0.5% (vol/vol).

[4] Mineral salts medium 337; gas phase 30—75% CO, 15—10% O_2, 0.5% CO_2, rest helium.

Table 4. *Morphological effects of the nitrogen source (conc. 0.1 M) on cells of H. vulgare grown for 2 months on a mineral salts medium 337 with methanol (conc. 0.5 vol-%) as the carbon source; methanol also present in the atmosphere*

Added nitrogen source	average size in microns		branching of hyphae	swarmer formation	growth	remarks
	rods	hyphae				
$(NH_4)_2HPO_4$ (sec. ammonium phosphate)	1.2×2.0	0.3 × 2.0	occasionally	none	fair	—
$NaNO_2$ (sodium nitrite) or $NH_2OH \cdot HCl$ (hydroxylamine hydrochloride)	0.8×1.8	0.2 × 2.0	none	none	poor	rods pale, convoluted and granulated
$NaNO_3$ (sodium nitrate)	0.7×1.5	0.2 × 2.0	rarely	many	good	—
$CH_3NH_2 \cdot HCl$ (methylamine hydrochloride)	0.7×1.5	0.2 × 1.5	rarely	many	excellent	—
1. $HCONH_2$ (formamide), or	0.7×1.5	0.2 × 3.0	rarely	many	excellent	—
2. CH_3CONH_2 (acetamide)	0.6×1.2	0.15×35.0	rarely	very few	excellent	—
NH_2CONH_2 (urea)	0.7×2.0	0.2 × 4.0	often	many	excellent	—
$NH_2CONH \cdot CH_2COOH$[1] (hydantoic acid)	0.7×1.5	0.2 × 6.0	rarely	many	good	—
$HOOC \cdot CHNH_2 CH_2COOH$[1] · (aspartic acid)	0.7×1.5	0.2 × 6.0	often	few	good	—
None	0.5×1.0	0.15×12.0	rarely	very few	fair	—

[1] Concentration 0.2% (w/vol).

were used as an inoculum. The appearance of hyphomicrobia grown on formamide (Fig. 11) or acetamide was most interesting. Although growth was quite good, a significant number of cells had very long hyphae. Only a few swarmers were observed, and the majority of hyphomicrobia had very short hyphae. It thus appears that growth of the hypha of most of the cells from the inoculum was stimulated by the formamide or acetamide. It may well be that the majority of the cells with short or no hyphae are progeny of the swarmers initially formed, or present, in the inoculum.

3. Morphology of newly isolated strains of hyphomicrobia

The 11 new strains are morphologically distinguishable from *H. vulgare NQ* or *MEV*. The rods are significantly larger (Figs. 15 and 16) and distinctly pear-shaped (Figs. 16, 20 and 26). The hyphae tend to appear wrinkled, and intrahyphal granulation is frequent (Figs. 20 and 23).

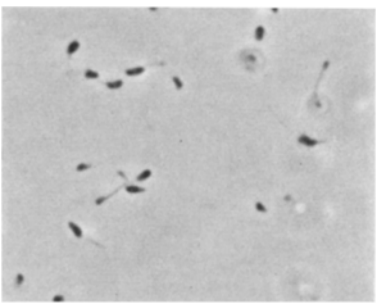

Fig. 15. *H. vulgare NQ*, grown for 4 weeks on methylamine agar. Cells suspended in tap water. Compare size with *Hyphomicrobium* spec. *H* in Fig. 16

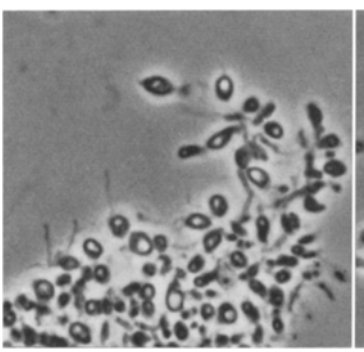

Fig. 16

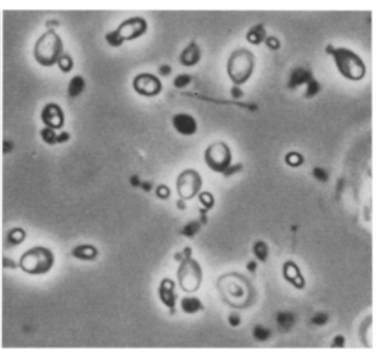

Fig. 17

Fig. 16. *Hyphomicrobium* spec. strain *H*, grown for 4 weeks on methylamine agar, suspended in water. Compare size with *H. vulgare* in Fig. 15. Note PHB granules

Fig. 17. *Hyphomicrobium* spec. strain *D*, grown for 8 weeks on methanol with $(NH_4)_2HPO_4$ as the N-source. Note bud formation and large PHB granules

The morphological changes induced in strains *NQ* and *MEV* by the nutritional conditions were also exhibited by all of the new strains. The morphological effects of varying the nitrogen source were much more pronounced than in the case of strains *NQ* or *MEV*. When NH_4^+ was added as the N-source, the rods of strains *D* (*524*) and *H* (*526*) were considerably enlarged (2.5—4.0 × 3.0—6.0 μ; Fig. 17). Formation of intracellular inclusions (PHB and metachromatic granules) was also enhanced. If present, hyphae were very short (1—3 μ) and rarely branched. Consequently, most cells closely resembled in appearance budding cells of the *Saccharomycetes*.

The new strains can be initially grouped as follows on the basis of morphology and enrichment procedure:

Group 1. Strains isolated from soil, under daylight, with mineral medium (pH 6.4) at a temperature of 20° C: *B (522)*, *E (525)*, *H (526)*, *K (529)*, *L (530)*. Rods generally are large [1.2 (0.9—5.0) × 0.7 (0.6—3.0) μ), pear-shaped, and during initial stages of

Fig. 18

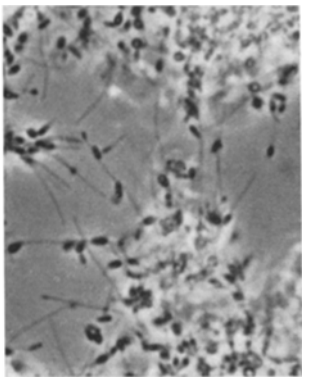

Fig. 19

Fig. 18. *Hyphomicrobium* spec. strain *H*, grown for 8 weeks on methanol, with formamide as the N- and additional C-source

Fig. 19. *Hyphomicrobium* spec. strain *H*, grown for 8 weeks on methanol, without any added N-source

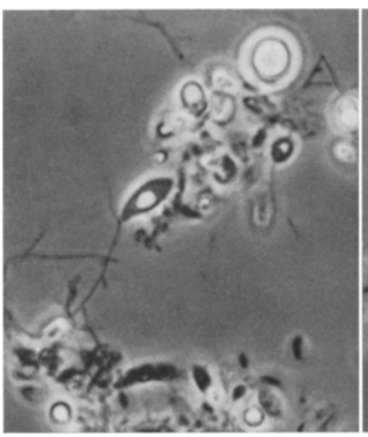

Fig. 20

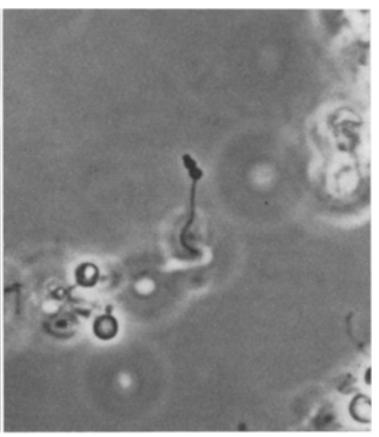

Fig. 21

Fig. 20. *Hyphomicrobium* spec. strain *H*, grown for 8 weeks on methanol, with methylamine as the N- and additional C-source. Note giant cells with and without PHB granules

Fig. 21. *Hyphomicrobium* spec. strain *H*, grown for 8 weeks on methanol, with urea as the N- and additional C-source. Note *„Blastocaulis"* form of the budding cell

formation quite slender. As growth proceeds, the cells shorten and PHB accumulates (Figs. 22 and 23). Some rods have a protrusion distal to the hypha. Occasionally large rods (4.0—5.0 × 1.5—2.0 μ; Fig. 20), which sometimes appeared transparent and contained optically dense granules near or at the center, were observed. In rare

cases, budding of the mother rod in older cultures gave rise to "*Blastocaulis*-like cells" (Figs. 21 and 28). This was more frequent in cultures with $(NH_4)_2HPO_4$ as the added N-source. Cells from the inoculum did not change their shape as growth proceeded.

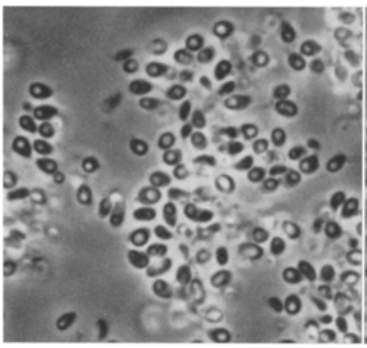

Fig. 22

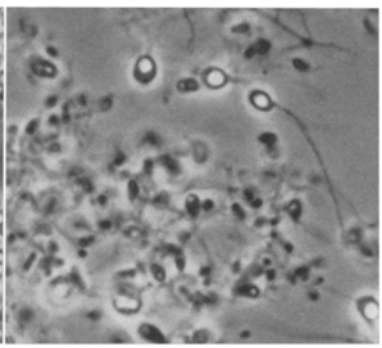

Fig. 23

Fig. 22. *Hyphomicrobium* spec. strain *E*, grown for 5 weeks on methylamine agar 337. Cells suspended in water. Note the short pear-shaped cells

Fig. 23. *Hyphomicrobium* spec. Strain *B*, grown for 6 weeks on methylamine agar. Cells suspended in water. Note the granulated hyphae and pear-shaped cells with PHB granules

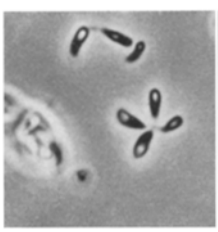

Fig. 24

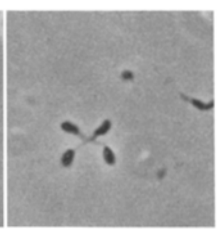

Fig. 25

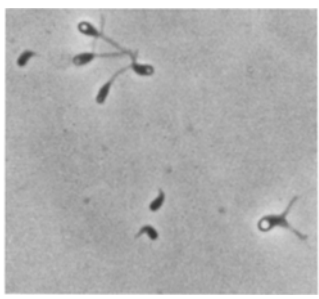

Fig. 26

Fig. 24. *Hyphomicrobium* spec. strain *C*, grown for 10 weeks on methylamine. Cross formation, compare with Fig. 25

Fig. 25. *H. vulgare NQ*, grown for 4 weeks on methylamine. Cross formation; compare with Fig. 24

Fig. 26. *Hyphomicrobium* spec. strain *D*, grown for 5 weeks on methylamine. Swarmer cells

Hyphae are well developed and often extensively branched, forming a true mycelium, with the side branches usually attached perpendicularly (Figs. 18 and 27). The hyphae are curved and granulated, much more so than those of *H. vulgare NQ*. Intercalary buds were regularly formed, and in some cases chains of as many as 6 rods were observed. Swarmers are elongated and cone-shaped; the thin end closest to the hypha is often bent (Fig. 26). The swarmers are usually motile, in some cases even when hyphae were present.

Group 2. Two strains [*O* (*545*) and *Q* (*547*)], isolated from soil under a $CO/O_2/CO_2$ atmosphere morphologically resembled strains of groups 1 and 3.

Group 3. Strains isolated from soil in the dark with mineral medium of pH 7.2 at 20° C: *C* (*523*), *D* (*524*) and *G* (*527*). Morphology similar to organisms of group 1 (Figs. 26—28), but the rods generally are longer [1.9 (1.5—3.5) × 0.9 (0.7—2.5 μ)]. Frequently cells grow perpendicularly to one another (Figs. 24 and 25).

Group 4. Strain *P (546)* isolated from soil under a $CO/O_2/CO_2$ atmosphere, like strains of group 3, resembles *H. vulgare NQ* in shape, but the rod is larger (1.5—2.5 × 0.7—1.3 μ). Cells in cultures of this strain store only small amounts of PHB.

A detailed description of cultural and biochemical characteristics of the new strains, together with a discussion of their taxonomic position is in preparation.

C. Discussion

Enrichment and isolation. The ubiquitous presence of budding, stalked bacteria should make the enrichment and isolation a simple matter. However, *Hyphomicrobium* is usually initially overgrown by faster developing organisms unless the conditions are selective. Enrichment in dilute media without added C-sources other than the inoculum overcame this difficulty. *H. vulgare* was also isolated in this manner by STUTZER and HARTLEB. Enrichment and selection was further enhanced by incorporating methylamine into the medium employed for subsequent subcultures. Primary enrichment with methylamine favors rapid growth of organisms of the *Pseudomonas* type which readily utilize C_1-compounds. The influence of light and CO on the growth of *Hyphomicrobium* in the enrichments will have to be examined further. Although the hyphomicrobia do not seem to use CO as a carbon source, they survived in an atmosphere containing 75% CO and were able to form swarmers. The inhibitory effect of light on the attachment of hyphomicrobia to surfaces is also of interest.

Budding bacteria, similar in appearance to *Hyphomicrobium* have been found in enrichments from sea water. GUILLARD and WATSON (1962) reported the isolation of hyphomicrobia from water samples derived from the Sargasso sea. These organisms were detected in cultures of diatoms after treatment with antibiotics. Budding bacteria were also present in our enrichments from sea water and characteristically hypha when present, were very short. In many respects they resembled *Pasteuria* spp. as described by HENRICI and JOHNSON (1935).

Morphology. Unfortunately, the original cultures of *H. vulgare* from STUTZER and HARTLEB are no longer available. Another isolate, strain *B* from MEVIUS jr. (1953), which is considered to be *H. vulgare* (BERGEY's Manual 1957), was compared with our new strains. Differences were found in size, shape, and granulation in the cells, as well as in the mode of growth on liquid media. Further detailed studies of the cultural and biochemical properties may enable one to determine the taxonomic position and status of the new strains.

There has always been the question as to what is the "cell" of *Hyphomicrobium.* The argument that it is essentially mycelial in character, and that the "rod" is a type of spore (STUTZER and HARTLEB 1897, 1898) is contradicted by the fact that viable mycelia without rods were never

found, but rods can exist and multiply without mycelia or hyphae. Therefore, the rod can be considered as the original "cell," and the hypha simply as a proliferation or outgrowth from the cell, analogous to the rhizoid of *Rhizopus niger* or the runners of strawberries. Slow growth (due to unfavourable growth conditions) increases the length of the hypha, whereas the formation of swarmers is inhibited. Suitable growth conditions result in the opposite; formation of swarmer cells is rapid, and

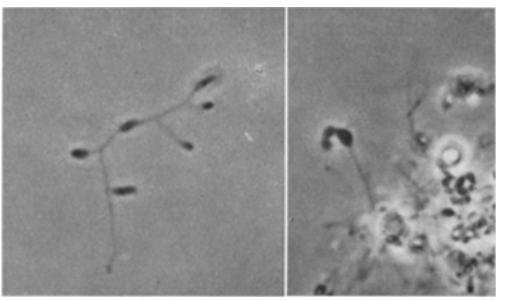

Fig. 27 Fig. 28

Fig. 27. *Hyphomicrobium* spec. strain *D*, grown for 11 weeks on methanol, with ammonium sulfate as the N-source. Small mycelium

Fig. 28. *Hyphomicrobium* spec. strain *D*, grown for 8 weeks on methanol, without any added N-source. Note direct budding from the rod

hyphae, if present, are quite short. When long hyphae are present, swarmer cells are often formed on short side branches or in an intercalary position.

Our observations on the morphology of *H. vulgare* (*NQ* and *MEV*) generally confirm those of STUTZER and HARTLEB (1898), KINGMA BOLTJES (1936), MEVIUS jr. (1953) and ZAVARZIN (1960). The most obvious differences between the new strains and those of *NQ* and *MEV* are their growth as turbidity without pellicle formation and their larger size. In all strains the rods tend to adhere to one another, probably by means of a mucoid-like substance.

There is a marked morphological similarity between *Hyphomicrobium* and *Caulobacter* species. The stalk (hypha) is in both cases a living part of the cell and not a secretion. They both form uniflagellated swarmer cells, and exhibit a variety of different morphological forms which is rare among true bacteria. These organisms also undergo a process of multiplication resulting in the formation of two unequal cells; one retains the stalk, the other is usually flagellated. In *Hyphomicrobium* we refer to the parent cell as the "mother cell," because repeated swarmer formation can occur without morphological effects. The swarmer, usually flagellated, is considered to be a "daughter cell." STOVE and STANIER (1962) refer to analo-

gous types of cells in *Caulobacter* as "sister cells," primarily because of their formation through a process of transverse fission.

A few morphological differences between hyphomicrobia and *Caulobacter* spp. should be emphasized. *Hyphomicrobium* aggregation is accomplished by adhesion of the rods, with the hyphae radiating outwards. *Caulobacter* forms "rosettes" by attachment of the hyphal ends by means of a "holdfast material." In *Caulobacter* the outgrowth of a stalk is obligatory for the formation of a swarmer cell, whereas multiplication in *Hyphomicrobium* does not depend on the presence of a stalk (hypha). Of course, *Caulobacter* spp. divides by transverse fission only, whereas *Hyphomicrobium* divides by means of a budding process.

The morphological resemblance of *H. vulgare NQ* and *MEV* to *Rh. vannielii* has been previously mentioned. The new strains exhibit even greater similarity to *Rh. vannielii*. Their large size, and the tendency of their hyphae to branch and form chains of rods (Fig. 27) illustrate this point. The effects of nutritional conditions on the morphology of *Rh. vannielii* are under investigation.

The occurrence, nature and distribution, as well as taxonomy of budding and stalked bacteria should be re-evaluated in view of the wide variety of morphological forms encountered in this and other studies (see ZAVARZIN 1961).

Summary

A variety of procedures for the enrichment and subsequent isolation of stalked, budding bacteria of the genus *Hyphomicrobium* is described. Although ubiquitous, *Hyphomicrobium* was easily overgrown in enrichments by other microorganisms. This difficulty was partially overcome by 1. enrichment in dilute mineral salts media lacking added carbon sources, and 2. isolation from these enrichments on mineral media with added methylamine. The effect on the enrichments of replacing air by a variety of gas mixtures was also followed.

The presence of hyphomicrobia in enrichments was not significantly influenced by the nitrogen source, light intensity, or pH of the medium, whereas temperature had some effect.

Enrichments from soil with mineral medium under air yielded 8, and enrichments under $CO/O_2/CO_2$ yielded 3 additional strains of *Hyphomicrobium* spp. which were morphologically different from *H. vulgare*.

A study of the factors effecting morphogenesis in *H. vulgare* resulted in the observation that length and branching of hyphae can be influenced by nutritional conditions. Unsuitable carbon or nitrogen sources decreased the number of swarmers formed and increased the growth (length) of the hyphae. Optimal conditions favoured rapid bud formation, and hyphae when present, were very short.

H. vulgare, grown in the presence of $(NH_4)_2HPO_4$ as the N-source, exhibited a yeast-like morphology with a significant increase in size. Hyphae, when present, were only poorly developed, and buds were formed directly from the mother cell.

H. vulgare, as well as the 11 new strains, contain two types of granules in the rods: large, refractile granules consisting of poly-β-hydroxybutyric acid, and small, dense, metachromatic granules located centrally.

The morphology of the 11 new strains is compared to that of the two known strains of *H. vulgare*.

Zusammenfassung

Eine Anzahl verschiedener Anreicherungs- und Isolierungsmethoden für gestielte, knospende Bakterien der Gattung *Hyphomicrobium* wird beschrieben. Obgleich weit verbreitet, wurde *Hyphomicrobium* in den Anreicherungen doch leicht von anderen Organismen überwachsen. Diese Schwierigkeit ließ sich teilweise dadurch umgehen, daß in verdünnten Mineralsalzmedien ohne zugegebene C-Quellen angereichert wurde, teilweise dadurch, daß die Isolierungen von diesen Anreicherungen auf Methylamin-haltigem Mineralagar vorgenommen wurden. Der Einfluß verschiedener Gasmischungen auf den Ausgang der Anreicherungen wurde fernerhin untersucht.

Das Auftreten von Hyphomikrobien in Anreicherungen ließ sich nicht wesentlich durch die Art der N-Quelle, durch die Lichtintenistät oder den pH-Wert der Nährlösung beeinflussen, während jedoch die Einhaltung einer bestimmten Temperatur von Wichtigkeit war.

Anreicherungen aus Bodenproben mit Mineralnährlösung unter Luft ergaben 8, und Anreicherungen unter $CO/O_2/CO_2$ weitere 3 neue Stämme von *Hyphomicrobium* spp., die alle von *H. vulgare* morphologisch verschieden waren.

Eine Untersuchung der die Morphogenese bei *H. vulgare* beeinflussenden Faktoren ergab, daß die Länge und Art der Verzweigung der Hyphen durch die Art des Nährbodens beeinflußt werden konnte. C- oder N-Quellen, die nur wenig genutzt werden konnten, verringerten die Schwärmerbildung und vermehrten das Längenwachstum der Hyphen. Günstige Wachstumsbedingungen förderten dagegen eine rasche Knospenbildung; die Hyphen waren dann — wenn überhaupt vorhanden — nur kurz.

Wenn *H. vulgare* mit $(NH_4)_2HPO_4$ als einziger N-Quelle gewachsen war, hatten die Zellen hefeähnliche Gestalt und waren deutlich vergrößert. Wenn dann überhaupt Hyphen gefunden wurden, waren diese nur schwach entwickelt; Knospen wurden zumeist direkt von der Mutterzelle gebildet.

Sowohl *H. vulgare*, als auch die 11 neuen Stämme, enthielten zweierlei Granula im Stäbchenteil der Zellen: große, stark lichtbrechende Granula enthielten Poly-β-hydroxybutyrat, und kleine, dichtere und metachromatische Granula, die meist im Zentrum der Stäbchen lokalisiert waren.

Die Morphologie der 11 neuen Stämme wurde mit der zweier bekannter Stämme von *H. vulgare* verglichen.

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References

BERGEY's Manual of Determinative Bacteriology, VII. Ed. Baltimore 1957.

DUCHOW, E., and H. C. DOUGLAS: *Rhodomicrobium vannielii*, a new photoheterotrophic bacterium. J. Bact. **58**, 409—416 (1949).

ENGEL, H., u. W. MEVIUS jr.: Beiträge zur Kenntnis der Hyphenstruktur von *Hyphomicrobium vulgare*. Ber. dtsch. bot. Ges. **64**, 208—212 (1951).

GUILLARD, R. R., and S. W. WATSON: A new marine bacterium. Oceanus **8**, 22—23 (1962).

HENRICI, A. T., and D. E. JOHNSON: Studies of fresh water bacteria. II. Stalked bacteria, a new order of *Schizomycetes*. J. Bact. **30**, 61—86 (1935).

HIRSCH, P.: Stoffwechselphysiologische Untersuchungen an *Nocardia petroleophila* n.sp. Diss. Hamburg, Germany, 1957; auszugsweise Arch. Mikrobiol. **29**, 368—393 (1958).

—, and S. F. CONTI: Biology of budding bacteria. II. Growth and nutrition of *Hyphomicrobium* spp. Arch. Mikrobiol. **48**, 358—367 (1964).

KINGMA BOLTJES, T. Y.: Über *Hyphomicrobium vulgare* Stutzer et Hartleb. Arch. Mikrobiol. **7**, 188—205 (1936).

Manual of Microbial Methods. Amer. Soc. Microbiol. 1957.

MEVIUS, W., jr.: Beiträge zur Kenntnis von *Hyphomicrobium vulgare* Stutzer et Hartleb. Arch. Mikrobiol. **19**, 1—29 (1953).

NÄVEKE, R.: Pers. Communication (1963).

RULLMANN, W.: Über ein Nitrosobakterium mit neuen Wuchsformen. Zbl. Bakt. **3**, 228—231 (1897).

STOVE, J. L., and R. Y. STANIER: Cellular differentiation in stalked bacteria. Nature (Lond.) **196**, 1189—1192 (1962).

STUTZER, A., u. R. HARTLEB: Bemerkungen zu der Mitteilung von Dr. W. RULLMANN: Über ein Nitrosobakterium mit neuen Wuchsformen. Zbl. Bakt. **3**, 621—622 (1897).

— — Untersuchungen über die bei der Bildung von Salpeter beobachteten Mikroorganismen. I. Abhandlungen Mitt. Landw. Inst. d. Königl. Univ. Breslau **1**, 75—100 (1898).

UHLKEN, A.: Untersuchungen über die Begeißelung von *Hyphomicrobium vulgare* Stutzer et Hartleb. Dipl.-Arbeit. Hamburg 1959.

ZAVARZIN, G. A.: The life cycle and nuclear apparatus in *Hyphomicrobium vulgare* Stutzer et Hartleb. Mikrobiologiya (Transl.) **29**, 24—27 (1960).

— Budding bacteria (A Review). Mikrobiologiya (Transl.) **30**, 774—791 (1961).

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