Hyphomicrobium neptunium sp. n.

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A new species of *Hyphomicrobium* is described. The organism was isolated from a sample of stored seawater originating from the harbor of Barcelona, Spain. The life cycles and various morphological types are illustrated by photomicrographs. Growth takes place readily on simple peptone media with the addition of a small amount of calcium or magnesium salt, or seawater. The general physiological and cultural characteristics are recorded.

INTRODUCTION

The genus *Hyphomicrobium* with the type species *H. vulgare* was proposed by Stutzer and Hartleb in 1898. The original description was sufficiently definite to identify the genus. The authors failed to demonstrate the flagellation of the motile phase of the life cycle but otherwise gave an adequate description of the life cycle and of the cultural and general physiological characteristics of the organism. With more adequate techniques, Kingma-Boltjes (1936) studied an organism which he regarded as *H. vulgare*, and published a review of the literature to 1936. The description in Bergey's Manual, 7th Ed. (1957) is largely taken from the publication by Kingma-Boltjes. A more recent study of the organism was published by Mevius (1953). From the various publications it appears that the organism is ubiquitous in soil and in water. Isolation in pure culture has been difficult and growth was somewhat slow on all of the media used. The organism will grow on simple peptone media and also on mineral salts media with ammonium sulphate and sodium acetate as sources of nitrogen and energy.

From the combined morphological, cultural and physiological descriptions of *H. vulgare* by the various authors I have concluded that the seawater isolate is sufficiently different from *H. vulgare* Stutzer and Hartleb to justify the new species, *Hyphomicrobium neptunium*.

MATERIALS AND METHODS

A seawater sample dated Barcelona, Spain, March 1962 was received in July 1963 by air express from the Office of Naval Research, Washington, D.C. The sample was packed in dry ice and frozen when received. It was placed in a refrigerator (6–8C) and plated in November 1963. According to Robert F. Acker of the Office of Naval Research the sample had been continuously under refrigeration up to the time of shipment, a period of about 15 months. It was frozen for about 2 days while in transit and then refrigerated for another 3 months before plating.

The agar medium for isolation and general culture work consisted of peptone (Casitone, Difco) 0.2%, yeast extract 0.1%, tris buffer (tris(hydroxymethyl) amino methane) 0.05%, agar 1.5%, artificial seawater half strength, pH 7.5. The liquid culture medium had the same composition without the agar. Carbohydrate metabolism studies were made with the MOF medium of Leifson (1963) and the gelatin and nitrate studies with a single medium as described by Leifson et al. (1964). To the liquid culture medium was added a strip of filter paper to determine cellulose decomposition. To the same base medium was added 0.1% L-lysine hydrochloride for the lysine decarboxylase test and the final pH compared with that of a control without added lysine. To the seawater agar was added 0.1% soluble starch for the starch hydrolysis test with iodine, and similarly 0.1% phenylalanine for the phenylalanine deaminase test with 10% ferric chloride as indicator.

RESULTS

On an agar plate streaked with 0.1 ml of the water sample a colorless, medium sized (about 2 mm) colony had appeared after incubation at 37 C for 3 days. The colony was transferred to broth; this culture showed some motile organisms and, on flagellar staining, two kinds of bacteria. The culture was consequently replated and two distinctly different colony types were found. These were replated and a pure culture of *Hyphomicrobium* was obtained along with a culture of a nonflagellated rod, which was discarded.

Morphological characteristics. The distinctive feature of the organism is its characteristic life cycle. If we start with a round to oval, nonflagellate structure about 1 μ in diameter, which may be called the mother cell, we may observe the following events: A stalk, usually one but at times several, grows out; the end of the stalk, which may be very short or several microns long, swells and forms into an oval body somewhat smaller than the parent; on this daughter cell develops a single flagellum, usually on the distal pole but occasionally on the side; the stalk divides, usually close to the daughter cell, and the daughter

cell swims away; after some time the daughter cell loses its flagellum, rounds up somewhat and the cycle is repeated. This is what I have, for want of a better term, labelled the daughter cycle. This cycle is illustrated by photomicrographs 1*a–h*. The mother cell with the remaining part of the stalk continues to develop; the end of the stalk swells; a new daughter cell is formed and the mother cycle goes to completion. This cycle is illustrated by Figs. 1*A–D*. Each completed daughter cycle produces one additional mother cell and each com-

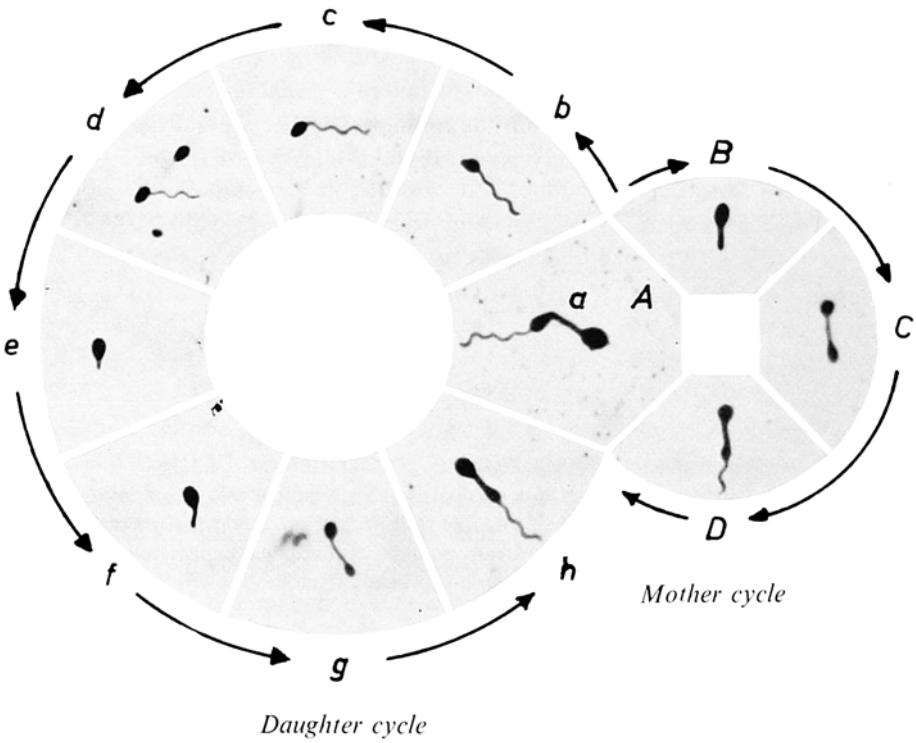

Fig. 1. *Hyphomicrobium neptunium*. 2000 ×.

Figs. 1*a-h* show several stages in the *daughter* life cycle. Normally phase 1*d* is aflagellate as illustrated by the upper of the two organisms. From a study of many preparations I have formed the impression that the flagellum takes a lateral position just prior to its disappearance and the beginning of stalk formation. However, the situation is rather uncertain. Fig. 1*a*-1*A* shows the usual type of separation of daughter cell from mother cell. Fig. 1*b* shows the usual short residual stalk of the daughter cell.

Figs. 1*A-D* show several stages in the *mother cycle*. The relative time required for completion of the respective cycles has not been determined nor has it been determined if there is any definite time relationship between the cycles.

pleted mother cycle produces one additional daughter cell. With the completion of both cycles the population has doubled. Should the daughter cycle stop, the mother cycle would produce an accumulation of daughter cells. Should the mother cycle stop, the daughter cycle would produce an accumulation of mother cells.

The mother cell usually develops only one stalk, but, more frequently from solid media than from liquid media, two or more stalks may develop as illustrated in Figs. 2*b*, 2*c* and 2*d*. From young cultures in the seawater nutrient broth the stalks have averaged from 1 to 2μ between mother and daughter cells. Occasionally the stalk is practically absent as illustrated in Fig. 2*e*. An unusually long stalk is illustrated in Fig. 2*f*. In a few instances a daughter cell may develop a stalk before it has separated from the mother cell. The second daughter cell which develops on this stalk may be flagellate as shown in Fig. 2*g*.

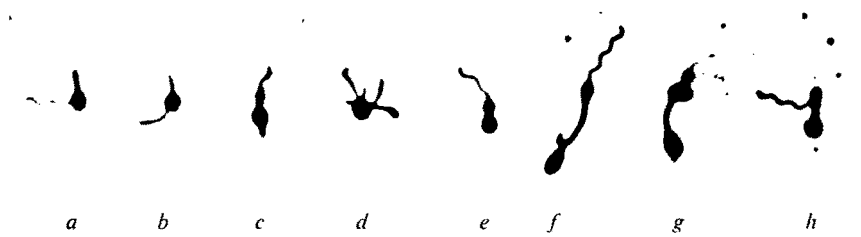

Fig. 2. *Hyphomicrobium neptunium*. 2000 ×.
a, Either a daughter cell with a long residual stalk or a mother cell with a residual flagellum. *b*, *c* and *d*, Examples of multiple stalk formation. *e* and *f*, Examples of very short and very long stalks. *g*, A rare example of multiple daughter cells. *h*. A daughter cell with a lateral flagellum on a short stalk from mother cell.

The flagellated daughter cell normally has a single polar flagellum and shows very lively motility. The flagella are not altered by formalin and have an average wavelength of 1.3μ. I have seen only one instance of 2 flagella at the same pole and never any cells with a flagellum at each pole. There is no evidence of binary fission of the soma. Where the daughter cell is formed so close to the mother cell that the two are touching, as in Figs. 2*c* and 2*e*, this should not be regarded as budding. A slender stalk is formed first and, if it is not over one micron in length, the terminal swelling (daughter cell) may extend to the mother cell and give the appearance of a bud.

Fig. 2*a* may be paradoxical and may represent either a daughter cell with a long residual stalk and a lateral flagellum, or a situation in which the flagellum of the daughter cell did not degenerate prior to progression into the stalked phase. Fig. 2*h* is included to show that occasionally the flagellum of the daughter cell may be, or appear to be, located laterally.

Physiological characteristics. Colonies on agar are colorless, raised, semi-translucent and rarely exceed 1 to 1.5 mm in diameter. When old they may take on a somewhat brownish color. On agar slants the confluent growth is colorless, smooth and quite opaque. In broth the growth is uniform without pellicle formation except in old cultures. Oxygen relations, aerobic; temperature relations, mesophilic with an upper limit at about 40 C, optimum between 30 and 37 C, and good growth at 20 C. The optimum pH for growth is about 8.0 with a growth range from pH 6.0 to pH 9.5. The normal range for satisfactory or good growth is pH 7 to pH 9. The organism thus definitely prefers a somewhat alkaline medium, which apparently is also true for *H. vulgare.* The organism is not osmophilic or halophilic like most marine bacteria in that the osmotic pressure or salt concentration has to be raised above that required by the ordinary non-marine bacteria. Growth does not take place in liquid media composed of peptone (Casitone) 0.2% and yeast extract 0.1%, but does take place on agar media of the same basic composition. Good growth takes place when the above liquid medium is supplemented with 0.01% to 0.3% magnesium chloride or calcium chloride. The agar itself apparently satisfies the requirement for magnesium or calcium. A definite requirement is thus shown for magnesium or calcium and this requirement is satisfied by very low concentrations of these salts. When to a good growth medium composed of Casitone 0.2%, yeast extract 0.1%, magnesium chloride 0.1%, at pH 8, is added 0.2% sodium citrate, growth is completely inhibited, confirming the requirement for the magnesium ion. Mineral salts media with ammonium sulphate and sodium acetate, which have been reported as supporting good growth of *H. vulgare*, have not supported macroscopic growth of *H. neptunium.*

With none of the carbohydrates tested, including glucose, sucrose, lactose, xylose, maltose and mannitol, was detectable acid produced in the MOF medium. Gelatin was not liquefied, starch and cellulose were not hydrolysed. Cultures in the lysine medium showed a reaction more acid than the control cultures. This may indicate, perhaps, a deamination of the lysine. Nitrate was reduced to nitrite. The test for catalase was positive and that for phenylalanine deaminase was weakly positive. Vitamin-free casamino acids (Difco) supplemented with magnesium chloride or seawater supported good growth. The organism does not seem to require any preformed vitamins.

Habitat. Isolated from seawater. May be a marine type but could exist wherever the environment is somewhat alkaline, and where magnesium or calcium ions and also some organic matter such as amino acids are present.

Type strain. A culture of laboratory strain 14–15 has been deposited in the American Type Culture Collection, Washington, D.C.

DISCUSSION

It is interesting to note that earlier studies with *H. vulgare* have reported the close association of this organism with nitrifying bacteria. The culture of *H. neptunium* was obtained first as a mixed culture with a capsulated non-flagellate rod. Mixed cultures from single colonies are not common but do occur. No evidence of attachment of *H. vulgare* to the nitrifying bacteria has

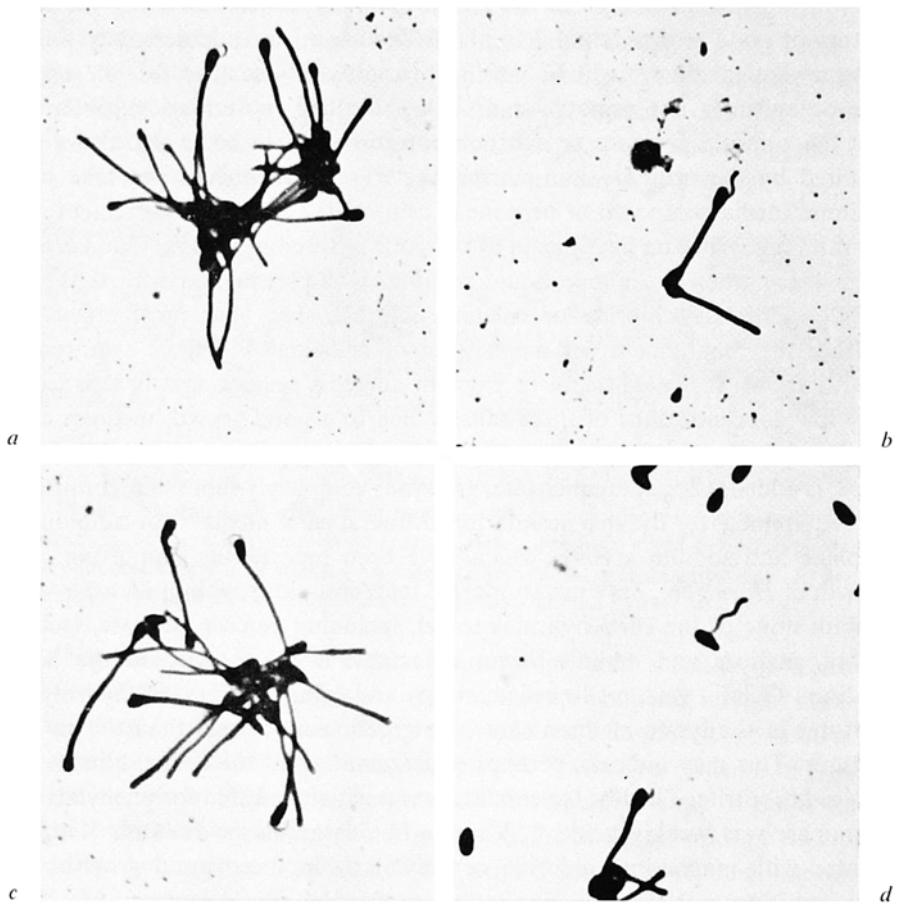

Fig. 3. *Hyphomicrobium vulgare*. 2000 ×. Note the tendency to form clusters. *a*, One daughter cell with a short (juvenile) laterally located flagellum. *b*, A daughter cell with a more mature flagellum. *c*, Two daughter cells with juvenile flagella and one daughter cell with a mature flagellum of typical curvature. The latter daughter cell appears ready to break away from the stalk and I have the impression that the daughter cell becomes detached when the flagellum is mature. *d*, The detached and typically flagellated daughter cell.

been reported in the literature and *H. neptunium* has shown no evidence of attachment to other bacteria.

Morphologically *H. vulgare* and *H. neptunium* are sufficiently similar to be placed in the same genus. From descriptions in the literature and from personal observations on one culture of *H. vulgare* received from Kingma-Boltjes, the two are sufficiently different to justify separate species. *H. vulgare* grows very slowly in the various media used, produces fairly long filaments between mother and daughter cells and frequently forms clusters in the shape of rosettes. *H. neptunium* grows rapidly in simple peptone media, filaments between mother and daughter cells are short, and aggregates have been rare in the media used. The flagellum of the attached daughter cell of *H. vulgare* generally has a lateral location while that of *H. neptunium* is generally located on the pole distal to the stalk. The detached daughter cells show the same difference in flagellar location. The length of the stalk and, perhaps, other morphological features of *H. vulgare* may vary with the cultural conditions but this has not as yet been studied.

To illustrate the morphological differences between the two species, photomicrographs of *H. vulgare*, Fig. 3*a–d*, are presented. These photomicrographs were taken from slides prepared from one week-old liquid cultures incubated at 20 C. As stated previously, the organism was received from Kingma-Boltjes and cultured in media of his formulation: tap water, $NaNO_3$ 0.1%, Na_2HPO_4 0.1%, NaCl 0.05%, $MgSO_4$ 0.05%, $FeSO_4$ 0.001%, KCl 0.001%, Na-acetate 0.1%.

The specific growth requirement of *H. neptunium* for magnesium or calcium is somewhat unusual if it is regarded as a bacterium. This requirement is not apparent when agar media are used because of the magnesium and/or calcium present in the agar. *H. neptunium* will grow on agar media composed of Casitone 0.2% and yeast extract 0.1%, but not in broth of the same basic composition. Most bacteria which can grow on such a simple agar medium can also grow in broth of the same basic composition, and show no specific requirement for magnesium or calcium beyond what may be present in the peptone or yeast extract. Unlike typical marine bacteria, *H. neptunium* is not halophilic.

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