Flexibacter polymorphus, a New Marine Species

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SUMMARY

A filamentous, gliding microbe isolated from a marine source is described as a new species of *Flexibacter* (Cytophagales: Flexibacteraceae). The filaments are typically multicellular. At the end of each cell there is normally a refractile granule of lipid material (not elemental sulphur, as would be the case in a *Beggiatoa* species). Under certain culture conditions, unicellular elements, helically coiled filaments or, occasionally, branched filaments may be produced. The filaments, which are not photosynthetic, are peach-coloured when grown in light: the pigment is a carotenoid spectroscopically resembling saproxanthin. The guanine + cytosine proportional content in the DNA is 29%.

The organism grows in media prepared with sea water or an equivalent saline solution. It can utilize glucose as sole carbon source; glutamate or Tryptone can serve as a suitable source of both C and N. Cobalamin is the only organic micro-nutrient essential for growth. The generation (doubling) time is 6 h at 22 °C.

INTRODUCTION

In an attempt to obtain a pure culture of a sulphur bacterium, *Beggiatoa*, from a marine source by using the method of Cataldi (1940), two different filamentous gliding organisms were isolated. Both were evidently flexibacteria. One was clearly *Saprospira grandis*, a common marine form (Lewin, 1962). The other, which forms the subject of this paper, is probably best assigned to a new species of the genus *Flexibacter*. For reasons discussed below I propose to call it *F. polymorphus*. At first it was provisionally identified as a strain of *Beggiatoa leptomitiformis*, the thinnest of the described species of *Beggiatoa* (Pringsheim, 1964), but so far all attempts to induce it to form sulphur granules – either intracellular or extracellular – have failed. It produces intracellular granules. A lipid material has been extracted and analysed by Professor R. B. Johns; his results will be presented in a separate paper. The present contribution comprises a description of the new species, some morphological and physiological features, and a tabular comparison between *F. polymorphus* and *S. grandis*.

METHODS

A piece of a colony of a littoral ascidian, *Didemnum* sp., collected near La Paz, Baja California, Mexico, in March 1971 and left decaying for some weeks in a jar of sea water in the laboratory, was observed to bear a tufted, veil-like covering of white filaments, about 1.2 µm wide. The cells contained highly refractile granules. In view of the black, anaerobic appearance of the mud below the rotting animal, and the smell of H₂S, it seemed reasonable to conclude that the granules were of sulphur and that the predominant microbe was a *Beggiatoa*.

A portion of the white weft was set on the surface of agar in a plate prepared with sea water and yeast extract (10 mg/l) and kept at room temperature. Many filaments glided and grew out and, after 24 h, portions of the marginal fringe were excised and streaked on fresh surfaces of the same agar medium, as recommended by Cataldi (1940). An unequivocal *Beggiatoa* was not isolated but, in addition to colonies of ordinary eubacteria, numerous flexibacterial colonies were obtained in this way. Some colonies were more restricted and of an orange tinge, others were more or less diaphanous and apparently colourless. The former type proved on sub-culture to be *Saprospira grandis* Gross (cf. Lewin, 1962, 1972). Colonies of the latter type were restreaked, and a single pure clone was selected for our studies.

For investigations of its physiology and nutrition, the methods employed for this organism were essentially the same as those described for studies of *S. thermalis* (Lewin, 1965) and *S. grandis* (Lewin, 1972). For comparative growth studies, cultures were generally grown in 5 ml liquid medium in test tubes (diam 18 mm), incubated with gentle shaking at 22 °C and constantly illuminated by fluorescent lamps (though light is not needed for growth). The yield was assayed periodically by determinations of turbidity in a Gilford 300-N micro-sample spectrophotometer. The absorption curve for the extracted pigments was determined with a Cary Recording Spectrophotometer (model 14). Tests for the ability of this organism to digest or assimilate cellulose, agar, gelatin or alginate, and for catalase activity or production of H₂S, were carried out according to methods described by Lewin & Lounsbery (1969). Stocks were maintained in a liquid medium with the following composition: Tryptone (Difco), 1.0 g; Casamino acids (Difco), 1.0 g; monosodium glutamate, 5.0 g; sodium glycerophosphate, 0.1 g; cobalamin, 1.0 µg; trace element mixture (see below); filtered sea water, 1 l. They were kept at room temperatures (20 to 25 °C) and transferred twice weekly so that a source of actively growing filaments was always available for use as inoculum. In semisolid media, prepared with 0.3 % agar, lysis was retarded; some cultures kept in such media at room temperatures retained viable elements for as long as one month.

Filaments were examined and photographed under phase-contrast microscopy at a magnification of 400 ×.

RESULTS

Physiology and nutrition

This organism grew in supplemented media based on natural sea water or on a synthetic sea water (NaCl, 20.0 g/l; MgSO₄·7H₂O, 5 g/l; KCl, 1 g/l; CaCl₂·2H₂O, 1 g/l), supplemented with Fe and other trace elements: final concentrations of B, Fe and Mn, each (as element) 0.5 mg/l; and of Co, Cu, Mo and Zn, each (as element) 0.01 mg/l. Tris buffer (1 g/l) was tolerated, though it was slightly inhibitory to growth. Growth occurred in media with salinities ranging from 20 to 75‰. The pH optimum was around 7.5 and growth tolerance ranged from 7.0 to 8.5. In media of lower pH the filaments tended to lyse sooner than in media at higher pH. At 22 °C the cells grew with a generation time, *T*, of about 6 h. Growth was more rapid at 32 °C (*T* = 2 h); but in warmer conditions lysis occurred earlier. Light was not required for growth and did not appreciably affect the growth rate, but illuminated cultures developed a pinkish-orange colour (see below) which was not apparent in the original material.

Supplied with glucose as sole source of carbon, cultures apparently utilized neither nitrate (NaNO₃) nor an ammonium salt (NH₄Cl) as a source of nitrogen for growth, though both were tolerated up to 10.0 g/l. Either glutamate or asparagine, in concentrations up to 2.5 g/l, constituted a satisfactory source of assimilable nitrogen.

With monosodium glutamate (0.5 g/l) as sole source of nitrogen, an increased growth yield after 4 days was obtained in cultures supplemented with glucose (0.1 to 2.5 g/l), galactose (0.1 to 1.0 g/l) or sucrose (0.5 to 10.0 g/l). Sodium lactate (5.0 g/l) augmented the yield to a lesser, though still appreciable, extent; the effects of glycerol or acetate, at all concentrations tested, were negligible.

All of our attempts to grow this organism in the absence of organic nutrients failed. It had an obligate requirement for cobalamin (optimum about 0.3 µg/l). Natural sea water, or artificial sea water supplemented with cobalamin, supported growth only if phosphate, trace elements and an organic substrate were added. Tryptone or glutamate alone was satisfactory as sole source of carbon and nitrogen. At glutamate concentrations between 0.1 and 0.6 g/l, growth yield after 3 days was proportional to substrate, though maximum yields occurred only with initial concentrations of about 5.0 g/l. Further increases of growth yield were obtained by the addition of Tryptone (optimum 2.5 g/l), Casamino acids (Difco) (up to 1 g/l) or yeast extract (up to 1 g/l). Growth was unaffected by a hydrolysate of yeast nucleic acid (cf. *Saprospira grandis*; Lewin, 1972). The addition of vitamins other than cobalamin, alone or in combination, had slight but inconsistent effects, and no other growth factor appeared to be essential.

In view of the morphological similarities between this organism and *Beggiatoa leptomitiformis* (presumably a 'sulphur bacterium'), we tried to find some special features of sulphur metabolism in this organism. Sterile-filtered $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ inhibited above 0.1 g/l, whereas thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) was tolerated up to 2.0 g/l. Neither was required for growth, and neither promoted growth or the accumulation of recognizable sulphur granules at any of the concentrations tested.

All attempts to demonstrate chemo-autotrophic growth in media containing limited amounts of organic substrates were unsuccessful. A combination of bicarbonate and thiosulphate (NaHCO_3 , 0.1 g/l; $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 1.0 g/l), although not inhibitory, permitted no growth – even in atmospheres supplemented with H_2S , CO_2 or both, and in media containing an excess CaCO_3 to keep the pH above 7.0 – unless glucose (0.2 to 2.0 g/l) and glutamate (1.0 g/l) were also added.

The addition of cysteine hydrochloride (0.2 g/l) or thiosulphate (2.0 g/l) delayed by about 2 days the onset of lysis in standing cultures. Na_2S had no such effect at any of the non-inhibitory concentrations tested.

Filaments grown under these various conditions were examined microscopically, but no distinct granules of elemental sulphur were noted. No elemental sulphur could be detected chemically in extracts of any of the several samples examined (Professor R. B. Johns, personal communications).

Morphology and motility

Form. The filaments were normally flexuous, unbranched, cylindrical, uniformly 1.2 µm wide, up to several hundred microns long, with rounded ends, generally not otherwise differentiated (Fig. 1*a, b*). Cross walls and constrictions, separating cellular elements about 3.5 µm long, were sometimes visible by phase-contrast microscopy (×400) (Fig. 2*c-f, i*). The cells retained a cylindrical form in media of lowered salinity, down to one-half sea water; but when grown in media with augmented salinity, approaching twice that of sea water, cultures also contained spindle-shaped filaments and cells inflated to 5 µm at the wider parts and tapering to 0.5 µm at the ends (Figs. 1*d, 2h, 3*). In media containing bicarbonate (NaHCO_3 , 1.0 g/l), clumps of long filaments as found in ordinary cultures were accompanied by some relatively short, highly active cellular elements, 10 to 40 µm long and

Fig. 1. Phase-contrast photomicrographs of *Flexibacter polymorphus*. (a), (b) Normal filaments in exponentially growing culture. (c) Helical filament. (d) Suspension with swollen filaments and cells undergoing lysis. (e) Walls and debris from lysed cells. Bar markers = 50 μ m.

Fig. 2. Phase-contrast photomicrographs of *Flexibacter polymorphus*. (a), (b) Normal filaments, showing granules. (c)–(f) Short filaments with barrel-shaped cells. (g) Short filament, showing progressive lysis from one end. (h) Inflated filament. (i) Short filament. (j) Helical filament. Bar marker = 50 μm .

Fig. 3. Phase-contrast photomicrographs of *Flexibacter polymorphus*; filaments showing inflated sections. Bar marker = 50 μ m.

only 0.6 μ m wide, like those of a *Cytophaga* sp. (This unexpected observation indicated a possibility of contamination, but suspensions restreaked on nutrient agar gave rise to colonies of only the usual filamentous type.) Media containing Casamino acids produced silky suspensions of filaments, whereas NaHCO_3 caused the filaments to clump; these effects were probably merely the result of different pH values in the media.

When grown at pH 7.0, many of the filaments were loosely but regularly helical (Figs. 1c, 2j). The coiling was sinistral, which conformed with the left-handed rotation of the normal gliding filaments (see below). Helical filaments were not found in subcultures grown in media at higher pH values. Another unusual and presumably abnormal feature of this organism was its tendency to produce branches, occasionally more than one on a single filament (Fig. 4a-h, j). Filaments tended to branch terminally at an angle of 120° , but only one filament in several hundred exhibited this feature. The formation of branches did not

Fig. 4. Phase-contrast photomicrographs of *Flexibacter polymorphus*. (a)–(h), (j) Filaments showing branching. (i) Filament bearing protoplast (?). Bar marker = 50 μ m.

Fig. 5. Absorption curve of carotenoid pigments extracted from *Flexibacter polymorphus*, in *n*-hexane.

seem to be correlated with any special constituents of the medium or environmental conditions during growth. Under adverse conditions the filaments tend to herniate and produce sphaeroplasts (Fig. 4*i*).

Granules. When grown at a pH above 8.0 in almost all media tested, the filaments generally contained refractile granules, 0.4 to 0.8 μm in diameter, one at each end of each cell, adjacent to the transverse walls (Fig. 2*a, b, g*). However, in young (1 day) cultures grown at lower pH (7.0 to 7.5), or in glutamate media containing Tryptone (2.5 g/l), asparagine (1 g/l) or cysteine (0.25 g/l), most cells contained no such bodies, or only one or two smaller ones. In older cultures, especially those grown in media of higher pH (8.5), the granules appeared larger, almost as wide as the filaments, and more highly refractile. According to Professor R. B. Johns (personal communication) they were composed largely of hydrocarbons or lipids. No evidence has been obtained in our strain for the presence of poly- β -hydroxybutyric acid, which was reported to occur in the form of small granules associated with those of sulphur in cultured *Beggiatoa* (Pringsheim & Wiessner, 1963; Kowalik & Pringsheim, 1966).

Colour. Although the original weft of filaments appeared white, the cultures of both species of gliding organisms isolated from it normally developed an orange colour when

grown in the light. The brightest colouration in *F. polymorphus* was observed in filament masses grown in media containing sodium glutamate (10 g/l). The pigment was readily soluble in acetone. The absorption spectrum of a solution in *n*-hexane, with a maximum at 470 nm, a secondary peak at 501 nm and a shoulder at 442 nm (Fig. 5), is very close to that of saproxanthin, a carotenoid originally isolated from *Saprospira grandis* and chemically characterized and described by Aasen & Jensen (1966).

Motility. The filaments of this organism, like those of all other flexibacteria and blue-green algae, lacked flagella. They glided on solid substrata, presumably with the aid of mucilaginous strands, which have been revealed in this strain by scanning electron microscopy (H. F. Ridgway, personal communication) and in other species by electron microscopy of negatively-stained preparations (e.g. Simon & White, 1971). The speed of gliding was usually in the range 1 to 5 $\mu\text{m/s}$ when observed by phase-contrast microscopy at room temperatures. (Actual temperatures of filaments in the illuminated part of the microscopic field were not determined.) Speeds as high as 8 $\mu\text{m/s}$ were noted in some media containing, for example, methionine (2 g/l), a substance which otherwise seemed not to affect growth. The filaments might progress in one direction for half a minute or more, or they might reverse every few seconds. As they glided they rotated; this was most readily observed in those rare filaments which bore an irregularity such as a subterminal lump or lateral 'bud'. The rate and direction of rotation were also indicated by the spiralling movements of small foreign particles borne on the superficial layer of slime. The pitch of rotation was deduced from the observation that a filament rotated once as it progressed 10 μm , e.g. a filament advancing at 5 $\mu\text{m/s}$ rotated around its own axis once in 2 s. The rotational sense was sinistral; as mentioned above, this is consonant with the left-handed coiling of some of the filaments in cultures grown in low pH media. Unless its direction of gliding was reversed, a filament tended to bend when it encountered an obstacle, forming a loop which enlarged until the obstacle could be by-passed. The swaying of filaments extending free in a liquid medium was probably attributable to rotation of embedded sections of these trichomes.

Lysis. Cultures of this organism underwent spontaneous lysis more readily than those of most flexibacteria (cf. *Saprospira grandis* or other species studied by Lewin & Lounsbury, 1969). For instance, in a 5-ml test-tube culture in a medium containing Tryptone (1 g/l) incubated at 22 °C, a weft of filaments formed in one or two days; this tended to aggregate into orange-coloured clumps on the third day, and a day or two later it dispersed to give a colourless, slightly opalescent suspension, which on microscopic examination contained no refractile filaments (Figs. 1e, 2g). In such a suspension cell lysis was complete, and no viable elements could be recovered from it.

I could readily observe the progress of lysis in a single filament by phase-contrast microscopy. Usually in sequence, starting at one end, one cell after another lost its refractility, and as it did so the walls split, permitting the individual cells to separate and drift apart. Their cytoplasm seemed to dissolve within a few seconds, leaving only the terminal granules mentioned above. (No resting endospores were formed by this or any other flexibacterium.)

Lysates contained cell walls and fragments, rhabidosomes (cf. those of *Saprospira grandis*; Lewin, 1963), and other characteristic sub-microscopic elements investigated by Ridgway & Lewin (1973).

Description of new taxon Flexibacter polymorphus

Generic characters are as given by Lewin (1969), except that cross-walls are just visible by phase-contrast microscopy. Cellulose, starch, agar, algin and gelatin are not digested. H₂S is not evolved. Catalase-negative.

Table 1. *Flexibacter polymorphus* and *Saprospira grandis*; some comparative features

	<i>F. polymorphus</i>	<i>S. grandis</i>
Morphology		
Normal filament width (μm)	1.2	0.8
Cytophaga-type cells observed	+	—
Filaments normally helical	—	+
Cross-walls visible by phase-contrast	+	—
Granules normally present	+	—
Motility		
Maximum gliding speed observed ($\mu\text{m/s}$)	8	2
Sense of rotation	Sinistral	Dextral
Lysis		
Cultures (5 ml) viable for (days)	3	10
Rhaphidosomes in lysate	+	+
Pigmentation		
In light	+	+
In darkness	—	+
Physiology		
Salinity range (‰)	20–75	20–75
pH range	7.0–8.5	6.2–8.3
pH optimum	7.5	7.5
Diphenylamine tolerance (mg/l)	2	20
DNA		
Guanine + cytosine (%)	29	48

In young, actively growing cultures, filaments are typically 1.2 μm wide and up to several hundred microns in length, with rounded ends. Cross-walls are 3.5 μm apart. Cells in late-growing and stationary phases of culture have generally one refractile granule at each end, more easily seen after lysis of cell contents. Rare filaments (one in several hundred) may bear a lateral branch, generally short (2 to 20 μm), inclined at about 120° from the main filament axis. In media of low pH (about 7.0), many filaments are slightly helical; the direction of coiling is sinistral. Gliding is relatively rapid, 2 to 8 $\mu\text{m/s}$ at room temperatures; filaments rotate sinistrally.

Ranges for growth: salinity, 20 to 75‰; pH 7.0 to 8.5; temperature, to 32 °C.

Proportional content of guanine + cytosine (GC %) in DNA: 29 % (density-gradient determination by Dr M. Mandel, personal communication).

Habitat: marine. Type strain isolated from a mass of *Beggiatoa* filaments on a decaying ascidian collected by R.A.L. at La Paz, Baja California, Mexico, in March 1971.

Differs from *Flexibacter litoralis*, another marine species, chiefly in pigment type (*F. litoralis* is pink, this species is peach coloured) and in various morphological features mentioned above.

A viable culture of this strain, designated as the type for the species, has been deposited with the American Type Culture Collection, ATCC27820.

DISCUSSION

Since our strain of *Flexibacter polymorphus* was found associated with *Saprospira grandis*, with which it has many features in common, their similarities and differences have been summarized (Table 1). The considerable difference in their GC % in the DNA would suffice to establish their specific distinction, though we should recall that *Saprospira grandis* and

S. tovisformis, which differ in relatively minor features, have GC % values as different as 48 % and 38 % respectively (Lewin & Mandel, 1970).

The problem of the relationship of such apochlorotic gliding microbes to *Beggiatoa* and its allies, among the so-called sulphur bacteria, has been reviewed by Soriano & Lewin (1965) and by Pringsheim (1963), and will not be further discussed here.

Since the only feature formally distinguishing *Beggiatoa* from members of the Flexibacteraceae (e.g. *Flexibacter* and *Microscilla*) is its possession of sulphur granules, it is important to determine whether the intracellular granules of this micro-organism contain elemental sulphur. Evidence from recent analytical work by Professor R. B. Johns (personal communication) indicates that this is probably not so. Fractions extracted with organic solvents consist mainly of hydrocarbons and lipids.

It remains to be established whether sulphur could be deposited, instead of or in addition to lipids, in these granules, under different cultural conditions. So far this has not been demonstrated.

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