

A NEW STRAIN OF *PARATETRAMITUS JUGOSUS* FROM LAGUNA FIGUEROA, BAJA CALIFORNIA, MEXICO

LAURIE K. READ¹, LYNN MARGULIS¹, JOHN STOLZ¹, ROBERT OBAR²,
AND THOMAS K. SAWYER³

¹Department of Biology and ²Department of Chemistry, Boston University, Boston, MA 02215, and

³National Marine Fisheries Service, U. S. Department of Commerce, Oxford, MD 21654

ABSTRACT

A euryhalic, moderately temperature tolerant, fast growing strain of the amoebomastigote¹ *Paratetramitus jugosus* was isolated from the North Pond flat laminated microbial mat at Laguna Figueroa, Baja California del Norte, Mexico. The morphology was studied with phase contrast, differential interference contrast, scanning, and transmission electron microscopy. On the basis of its life cycle characteristics, growth rate, salt and heat tolerance, fluorescence excitation and emission spectra, and isozymes, the organism was determined to be a new strain, *P. jugosus baja californiensis*. This new strain, unlike the type specimen (ATCC 30703), grows vigorously on half strength sea water and slowly at 0.51 *M* (nearly 3 per cent) sodium chloride. It tolerates the hypersaline conditions of the evaporite flat that prevail when the North Pond mats are dominated by *Microcoleus* and other bacteria, growing well during periods of influx of fresh water. Its cysts survive complete dryness of the sediment for at least three years.

The microbial mats in which this *Paratetramitus jugosus* has been found are thought to have Archean analogues over 3 billion years old. The discovery of resistant abundant small eukaryotes within a setting dominated by bacteria may be important for the interpretation of the Proterozoic microbial fossil record.

INTRODUCTION

We report here the isolation and identification of an extremely fast growing, hardy, desiccation resistant new strain of the amoebomastigote (=amoeboflagellate)¹ *Paratetramitus jugosus* from the North Pond of Laguna Figueroa, Baja California del Norte, Mexico (Fig. 1).

The living microbial mats of Laguna Figueroa, Baja California, have been compared with the 3400 million year old carbon-rich Fig Tree cherts of the Swaziland System of rocks from South Africa (Margulis, *et al.*, 1980). These laminated rocks, as well as others also deposited over 3 billion years ago from western Australia (Lowe, 1980), show a textural and paleoecological resemblance to the flat laminated bacterial mats of Baja California. Fossils of bacteria have been found in some of the most ancient cherts (Knoll and Barghoorn, 1977; Awramik *et al.*, 1983) and there is a continuous record of such microfossils from over three billion years ago to the present. However, the time of appearance of the first eukaryotic microorganisms

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¹ The terms "flagella" and "flagellate" are ambiguous since they refer to both flagellin-containing bacterial structures and tubulin (9 + 2) eukaryotic structures, and the organisms which bear them, respectively (Margulis, 1980). In this paper we restrict "flagella" and "flagellate" to the bacteria we describe and use "undulipodia" for the eukaryotic structures and "mastigote" for the organism that bears them. We replace "amoeboflagellate" with "amoebomastigote."

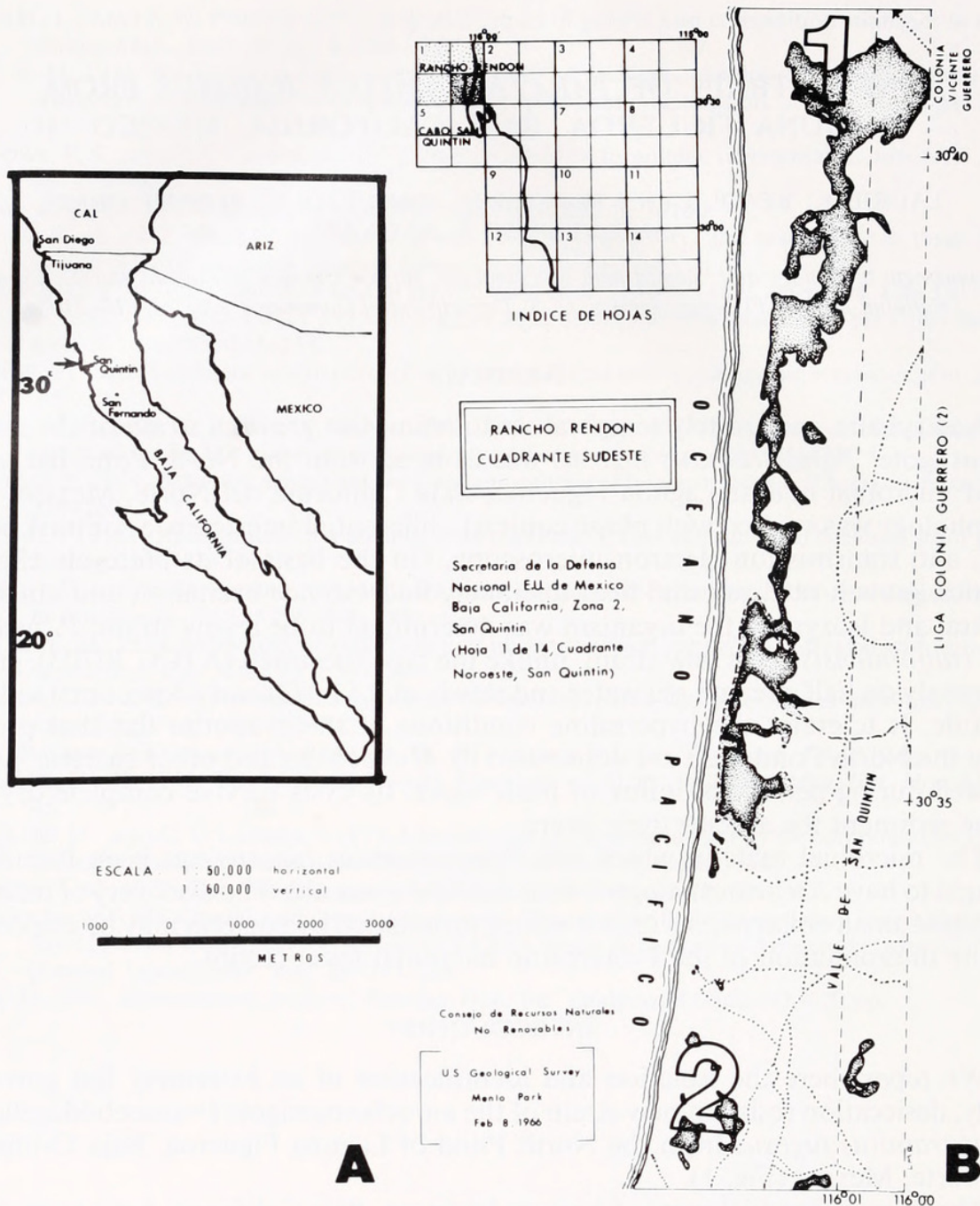


FIGURE 1. Map of Baja California del Norte. A. Location of Laguna Figueroa and field sites. B. The organism has been isolated from North Pond (site 1) and South Salinas (site 2).

in the fossil record is not known with certainty (Francis *et al*, 1978). The first protists are thought to have appeared before 1400 million years ago (Knoll, 1982). We initiated this study on the protistan composition of the bacterial mats to identify the major eukaryotes in ecosystems overwhelmingly dominated by bacteria in the hope of providing a better interpretation of the fossil record of laminated mats and microorganisms preserved in cherts.

During the spring of 1979 an unusually severe flood occurred at Laguna Figueroa, submerging the mats under one meter of fresh water until late August. The flood water, which contained terrigenous sediment from the neighboring alluvial plains, subsided by the late summer of 1979 but the rains of winter 1979-80 were even more severe. From December 1979 until late summer 1981 the mats were

continuously flooded with fresh water. Never during the entire summer of 1980 did the *Microcoleus* community emerge and grow. These episodes drastically altered the composition of the mats from their relatively stable former state (described by Horodyski *et al.*, 1975, 1977). When the fresh water finally subsided, the productive cyanobacterial community was replaced by a different community of heterotrophs and purple photosynthetic sulfur bacteria, mainly by thiocapsoids (Margulis *et al.*, 1983; Stolz, 1983a). From both submerged samples of the *Microcoleus* mat and from reemerged samples, *Paratetramitus jugosus* amoebae were recovered in impressive numbers from every mat sample transferred onto permissive plates.

Several features of this amoebomastigote including its morphology, fluorescence, as well as salt, heat, and desiccation tolerance are described here. The Baja California isolate is compared with the original American Type Culture Collection strain (ATCC 30703).

MATERIALS AND METHODS

Growth and isolation

The two kinds of media used in this study (modified K and manganese acetate, Table I) were taken to the field study site at North Pond, Laguna Figueroa, Baja

TABLE I

<i>Media</i>	
<i>Modified K medium</i>	
MnSO ₄ · 4H ₂ O	0.1 g
Bacto-Peptide	1.0 g
Yeast extract	0.25 g
Agar	7.5 g
500 ml ASW (autoclaved together)	
<i>Manganese acetate medium</i>	
Mn(C ₂ H ₃ O ₂) ₂ · 4H ₂ O	0.002 per cent (w/v) in ASW
(unless other concentration specified, <i>i.e.</i> , from 2×10^{-5} to 10^{-3})	
<i>Artificial sea water (ASW)</i>	
CaCl ₂ · 2H ₂ O	1.45 g
MgSO ₄ · 7H ₂ O	12.35 g
KCl	0.75 g
NaCl	17.55 g
Tris buffer (1.0 M, pH 7.5)	50 ml
Distilled water	950 ml
<i>Tris buffer</i>	
HCl (conc)	33.3 ml
Trizma Base	60.55 g
bring to 500 ml with distilled water	
<i>Sawyer medium</i>	
Malt extract	0.1 g
Yeast extract	0.1 g
Difco agar	10 g
Distilled water	1000 ml

California (Margulis *et al.*, 1980). About 1 mm² samples of the flat laminated microbial mat (Margulis *et al.*, 1980; Stolz, 1983a) were placed directly on sterile plates.

After 48 hours plates were then covered with about 1 ml of sterile distilled water to resuspend the organisms and initiate a new growth cycle. After vigorous growth occurred, cysts and amoebae were repeatedly subcultured on fresh medium by streaking with a sterile platinum loop. In the final step of the isolation the organisms were inoculated onto plates of modified K or manganese acetate medium with 2.4 per cent sodium chloride and checked for uniformity of cyst morphology. They were then transferred from this medium to different conditions for study.

The organisms were routinely grown on modified K medium or manganese acetate (McAc) medium (both of which contain half-concentrated sea water), or on nonnutrient fresh-water agar with or without *Klebsiella* as food (Sawyer medium, Table I). Growing cultures were kept at room temperature or in an incubator at 30°C. The major food source for *P. jugosus* was a gram positive, flagellated, facultatively aerobic rod which grew readily on modified K and MnAc media, and was called the B bacillus.

Mastigotes were obtained by adding distilled water to agar plates of young cultures 24–48 h old. Samples taken on the following day revealed that approximately one third of the organisms had transformed into mastigotes, generally with more than 2 undulipodia each.

American Type Culture Collection (ATCC) *Paratetramitus jugosus* strain no. 30703 was obtained in axenic medium no. 1034 (ATCC catalog, 1982). MnAc plates containing 0.1 M NaCl were inoculated with the food inoculum dominated by the B bacillus and the ATCC *P. jugosus*. The ATCC *P. jugosus* grew better on this medium with the food bacillus than on medium no. 711, the one routinely used (ATCC catalog, 1982).

Storage of live material

The isolate was most easily preserved by storage of desiccated agar plates at 4°C. Over the past 3 years cultures have been resuscitated within two or three days by replating on modified K or MnAc medium. Healthy cultures have also been reestablished from desiccated field samples or desiccated plates. A portion of the dry sample was placed on fresh medium and flooded with about 1 ml of sterile distilled water for at least 10 minutes.

P. jugosus also survived freezing. About 2 ml of sterile distilled water was placed on each of several plates, 48–72 h old, containing healthy cultures of rounded forms and cysts. The organisms were pipetted into centrifuge tubes, spun in a desk top centrifuge at medium or high speed for about 10 min, and then resuspended in Page's salt solution (Table I, ATCC catalogue, 1982, p. 633). The cyst concentration was from 10⁶ to 10⁷ organisms per ml as determined by a counting chamber. The amoeba suspension was then diluted by half with Tris buffer (Table I) to which 15 per cent dimethylsulfoxide (DMSO) had been added to yield a final DMSO concentration of 7.5 per cent (v/v). The DMSO-buffer-*P. jugosus* suspension was divided into 1 ml plastic capped vials and frozen at –70°C. For resuscitation of the culture contents of the vials were poured onto plates containing fresh medium.

Light microscopy

Living amoebae and cysts were observed using wet mounts with bright field Nomarski, phase contrast and fluorescence optics (Nikon Optiphot and Fluorophot). Agar coated slides were prepared to observe growing cultures. Alcohol-cleaned slides

were dipped into hot 1.5 per cent agar, and the undersides were wiped clean with sterile cheesecloth. The cooled slides were inoculated down the center by streaking the slide with a sterile platinum needle. Sterile cover slips, held up with bits of plasticene, were placed on the inoculated preparations. The slides were incubated in sterile Coplin jars or petri plates to which a few drops of sterile distilled water were added from time to time. Growing amoebae and food bacteria could be maintained for at least a week under these conditions with very little contamination. Measurements of live amoebae and cysts were made with a calibrated ocular micrometer. Fifty amoebae and fifty rounded forms including mature cysts were measured.

Nuclear division patterns were studied after staining with Kernechtrot (Darbyshire *et al.*, 1976). Prior to staining, blocks of agar containing amoebae and cysts were transferred, upside down, into distilled water on microscope slides. These were allowed to sit for about 45 min, in which time the amoebae swam into the water and away from the agar. The agar was removed and the amoebae were fixed for 15 s in Nissenbaum's fixative (Nissenbaum, 1953) and treated with saturated iodine alcohol. The fixed amoebae were then stained for 8 min in Kernechtrot (0.1 g in a 5 per cent aqueous solution of $\text{Al}_2(\text{SO}_4)_3$). The preparations were dehydrated in ethanol (70, 95, and 100 per cent) and xylene. for Protargol staining, the methods of Zagon (1969) were used with modifications as suggested by Eugene Small, University of Maryland (pers. comm.).

Electron microscopy

For transmission electron microscopy, amoebae and cysts were fixed, embedded and observed according to the methods described in Margulis *et al.*, 1983.

For scanning electron microscopy organisms were suspended in distilled water to produce mastigotes. The distilled water from suspensions harvested from several petri plates was collected and the organisms were washed 12 times in 0.5 artificial sea water (ASW, Table I) using a desk top centrifuge. The resuspended organisms were fixed for 5 min in Parducz's fixative (6 parts 2 per cent osmium tetroxide in 0.5 ASW to 1 part saturated HgCl_2 in distilled water) and washed 10 times in distilled water. Amoebae were affixed to broken pieces of coverslip with 1 per cent polylysine in distilled water. They were then dehydrated in a series of alcohols, dried in a critical point dryer (Denton DC31), evaporated with a vacuum evaporator (Denton DV502) and observed using SEM (AMR 1000) at 10 Kv at the University of Massachusetts at Boston.

Salt tolerance

Growth of *P. jugosus* as a function of salt concentration was measured between 0.0 and 0.60 M NaCl. An inoculation of 0.1 ml of the suspended culture in 5 ml of distilled water was plated on each test plate. From the 3rd until the 26th day plates were scored every 2 to 3 days for appearance of cysts relative to their food bacteria. Using a dissecting microscope, outlines of areas covered by bacteria only were compared to outlines of areas of bacterial colonies riddled with cysts. The outlines were pencil-copied onto filter paper, cut out and weighed. The weight of each cyst-covered outline was divided by the weight of the bacteria-covered outline to yield relative amounts of amoeba growth. Since *P. jugosus* growth is limited to the very surface of the plate and the results were consistent from experiment to experiment, we felt this procedure was adequate to estimate the relative growth as a function of salt and temperature. Growth was defined as continued production

of amoebae and cysts after three transfers 9 days apart. These experiments were repeated three times for the new Baja California isolate, and twice for the ATCC strain.

Heat tolerance

Growth of the amoebae as a function of temperature was measured by incubating plates made with MnAc media at temperatures from 4°C to 48°C. Ability to survive high temperatures was tested by suspending samples of *P. jugosus* and their food bacteria in distilled water and exposing them to elevated temperatures in water baths for 10 min. The samples were then poured onto plates containing MnAc media and incubated at 30°C to check for growth, which was defined as in the salt experiments.

Fluorescence

Chlorophyll fluorescence was used routinely to aid in the identification of cyanobacteria in mixed cultures on media designed to enrich for photosynthetic microbes. On such plates the yellow-green fluorescence associated with the cysts of *P. jugosus* was observed. The Nikon Fluorophot microscopic observations were documented with a 35 mm mounted camera back and supplemented by measurements of the excitation and emission peaks using a Perkin-Elmer MPF-44A Fluorescence Spectrophotometer. Approximately 0.2 ml of concentrated mature cysts from plates about two weeks old was spread on alcohol cleaned microscope slides which were secured in the spectrophotometer either with tape or with a model no. 063-0502 solid sample holder attachment.

Enzyme analysis

Starch gel electrophoretic techniques for enzyme patterns were conducted under a contract with the American Type Culture collection, Rockville, MD (Nerad and Daggett, 1979). Both strains were tested for three isoenzyme systems: propionyl esterase, leucine aminopeptidase and acid phosphatase (Daggett and Nerad, 1983).

RESULTS

Field studies and isolation

Recognition of cysts. Many of the mat samples plated in the field in 1979 and 1980 showed sporadic clumps of cysts. Some field sample plates were overrun by cysts and others apparently lacked them entirely. Unidentified cysts appeared in low numbers on media designed to enrich for manganese oxidizing bacteria in the summer of 1980. Samples of mixed bacteria and cysts were prepared for transmission electron microscopy. A separate ultrastructural study of mat organisms, coccoid chlorophytes grown on photosynthetic medium containing no carbon source (ASN III, Rippka *et al.*, 1979), also revealed cysts. These were very similar to those previously seen in the bacterial cultures (Fig. 2). Cysts on the photosynthetic medium were well fixed and more abundant than those on heterotrophic media (Margulis *et al.*, 1983). Easily overlooked by light microscopy, the cysts could be differentiated from the coccoid algae by their fluorescence spectra. Transfer of cysts onto fresh low nutrient heterotrophic media (Table I) resulted in a higher yield of clearly distinguishable cysts. Characterization of the cyst ultrastructure led to the recognition of the same cysts *in situ* from 1977 laminated mat dominated by the cyanobacterium *Microcoleus chthonoplastes* (Stolz, 1983a, b).

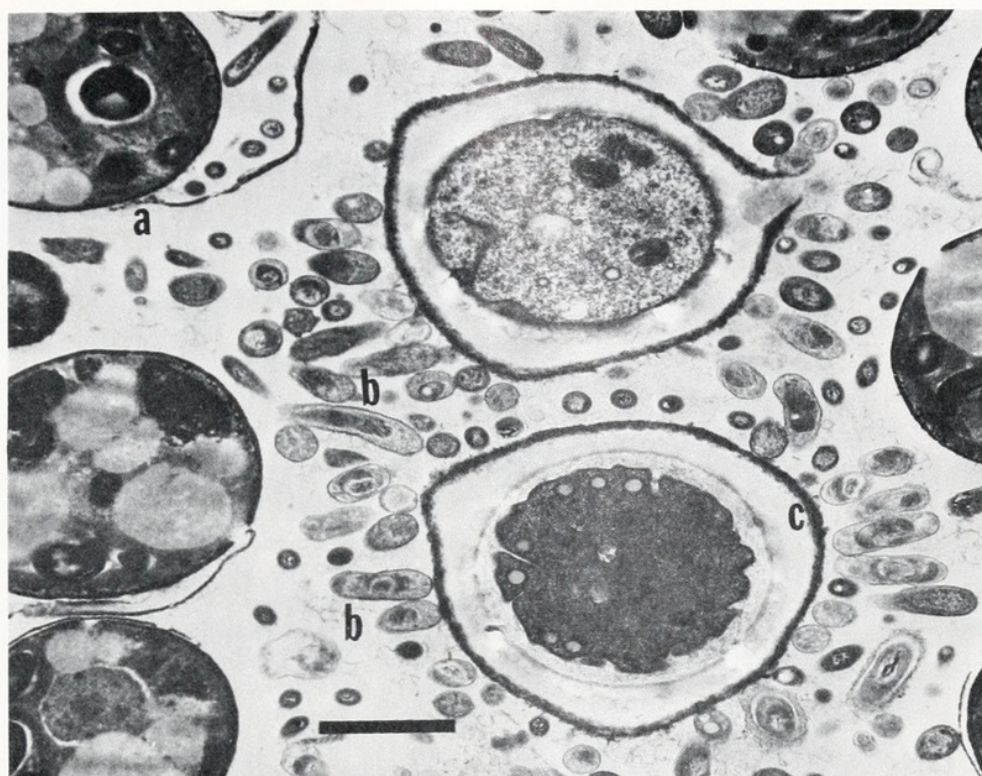
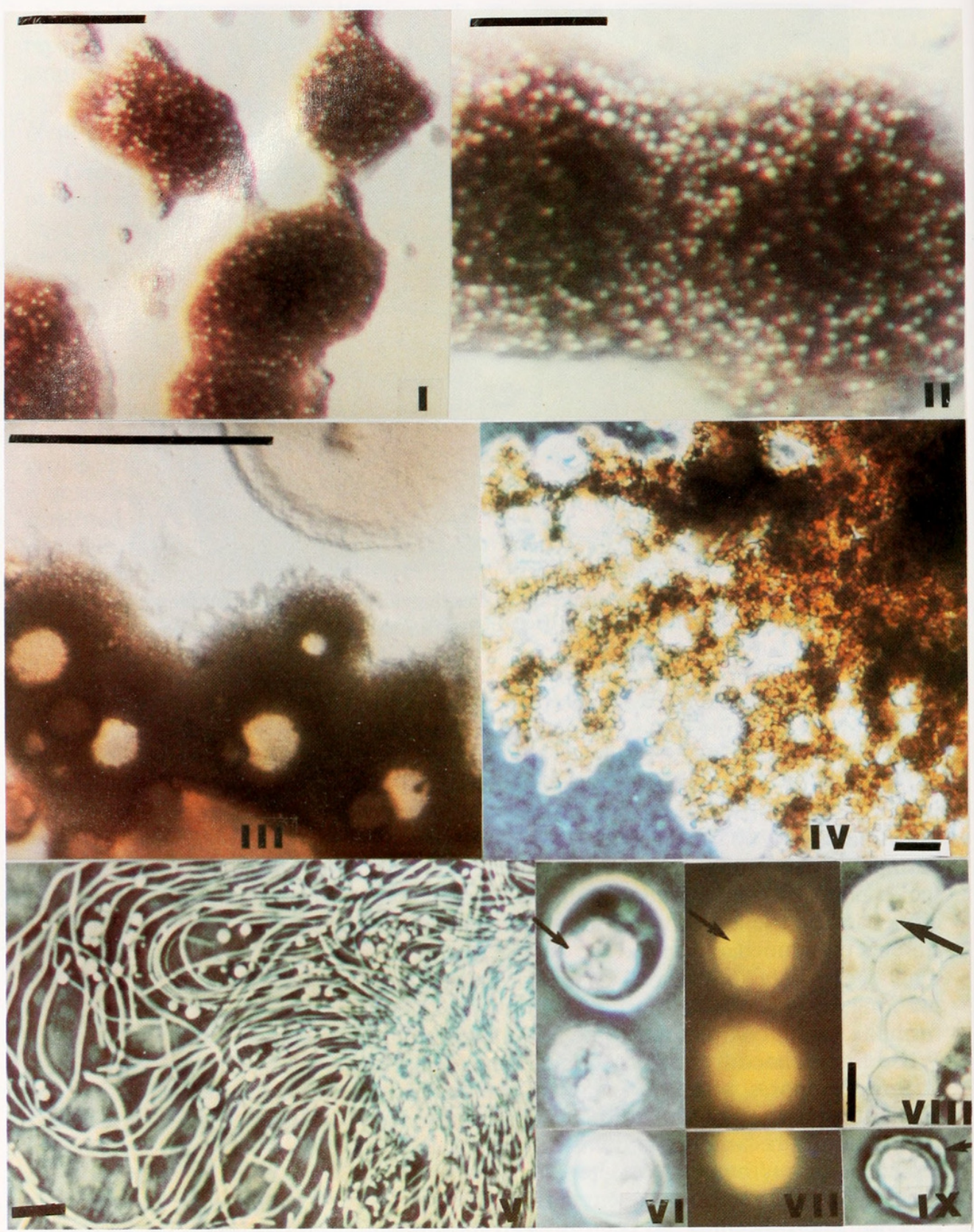


FIGURE 2. TEM of cysts in an algal culture, showing different stages of cyst development. Note pore in younger, lighter cyst. a = alga, b = bacterium, c = cyst. Bar = 2 μ m

The amoebomastigote was subsequently recognized easily within 48 h on several types of mixed culture plates: manganese acetate, K medium, or various photosynthetic media either fresh from the field or in transferred or stored samples. The appearance of white spots in dark colonies of manganese oxidizing bacilli (color plate I, II, III) were taken as a presumptive test for the presence of cysts. With higher magnification (200 $\times$ or greater) the numerous amoeba cysts were seen among clumps of manganese-coated spores (color plate IV) and distended food bacteria (color plate V). The abundance of bacilli decreased as they were fed upon by the amoebae and the area covered by white spots (which are the cysts) increased over time as the amoebae digested the bacteria.

The food bacillus. The B bacillus was easily recognized: it measured about 4 μ m long and 1 μ m wide (Fig. 3A). It produced subterminal spores (Fig. 3B, C) and formed smooth colonies that were beige and became brown centered in a few days. When first isolated from the Laguna Figueroa mats in 1980 it oxidized manganese, coating its spores within 4–7 days of incubation (Margulis *et al.*, 1983). During subculture this ability to oxidize manganese was lost. In the presence of *P. jugosus* these colonies became spotted with white cysts, then riddled with cysts, and finally replaced entirely by cysts (color plate I, II, III, IV). However at least three other types of bacteria were also present in this “B + cyst” inoculum in far smaller numbers (Fig. 4). The B bacillus has been isolated in pure culture on at least two occasions by taking advantage of the spores’ resistance to temperatures up to 85°C for at least ten minutes. This treatment killed the amoebae and cysts and all but one or occasionally two of the bacterial types in the inoculum. The B bacillus colonies were then easily picked and transferred to sterile plates and maintained indefinitely. When inocula of *P. jugosus* were introduced into a pure culture of B bacilli, however, they brought with them several other types of bacteria, presumably by adherence to their



COLOR PLATE

- I. Dark bacterial colonies riddled with light cysts after 2 days growth. Bar = 600 μ m.
- II. Dark bacterial colonies riddled with light cysts after 4–5 days growth. Bar = 1.2 μ m.
- III. Colonies of manganese oxidizing (dark) and other heterotrophic bacteria taken directly from the field. The “plaques” or cleared areas represent the growth of *P. jugosus* within colonies of manganese oxidizing bacteria. At the lower left an entire dark colony has been converted to cysts. Bar = 100 μ m.
- IV. At higher power cysts can be seen among the manganese coated bacterial spores. Bar = 10 μ m.

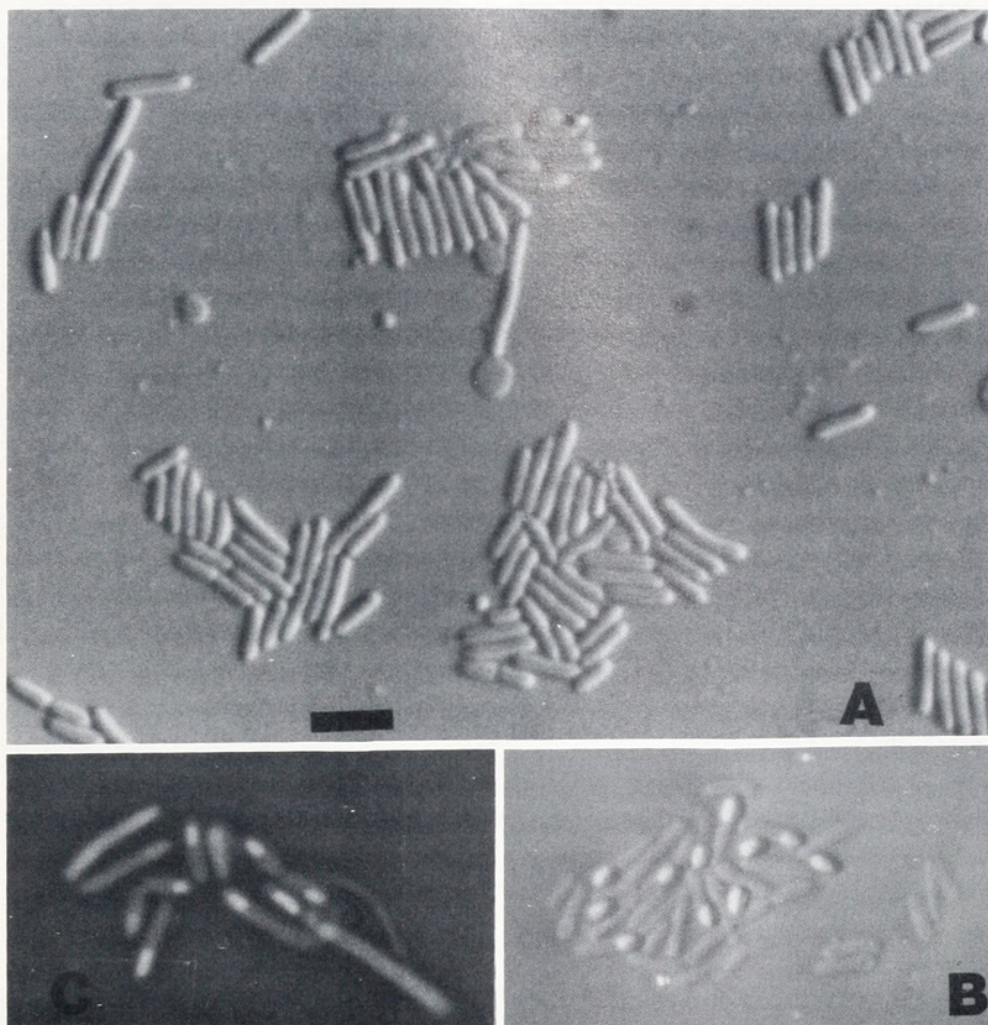


FIGURE 3. B bacillus colonies with cysts. A. Agar slide preparation of a young culture prior to spore formation (less than 1 day old) Nomarski differential interference microscopy. B. Subterminal spores phase contrast microscopy C. Same as 3B. but Nomarski optics. Bar = 5 μm .

cysts. For this reason the *P. jugosus* cultures contained several bacterial types but in fewer numbers than the B bacillus.

Morphology

Amoebae. The amoeboid form was monopodial when moving forward (Fig. 5A–C). When stationary the amoebae often exhibited bulging forms typical of vahlkampfiids (Fig. 6A–C). Monopodial forms range in length from 12–24 μm , averaging 17.2 μm . This fell in the lower part of the size range reported by Darbyshire (*et al.*, 1976) for other strains of *P. jugosus*. The average length:breadth ratio was 3.2:1. Occasional binucleate amoebae were seen, but fewer than the 7 per cent reported

V. Spaghetti-like masses of B bacillus in young culture infected with ectoplasmic forms, phase contrast Bar = 20 μm .

VI. Phase contrast white light micrograph of fluorescent cysts. Bar = 5 μm .

VII. Matching fluorescence micrograph. Note dense granules which may correspond to autolysosomal or even chromatin bodies (arrows) (see figure 8D). Bar = 5 μm .

VIII. Mature cysts, note the binucleate cyst (arrow), phase contrast. Bar = 5 μm .

IX. Mature cyst with pore (arrow). Bar = 5 μm .

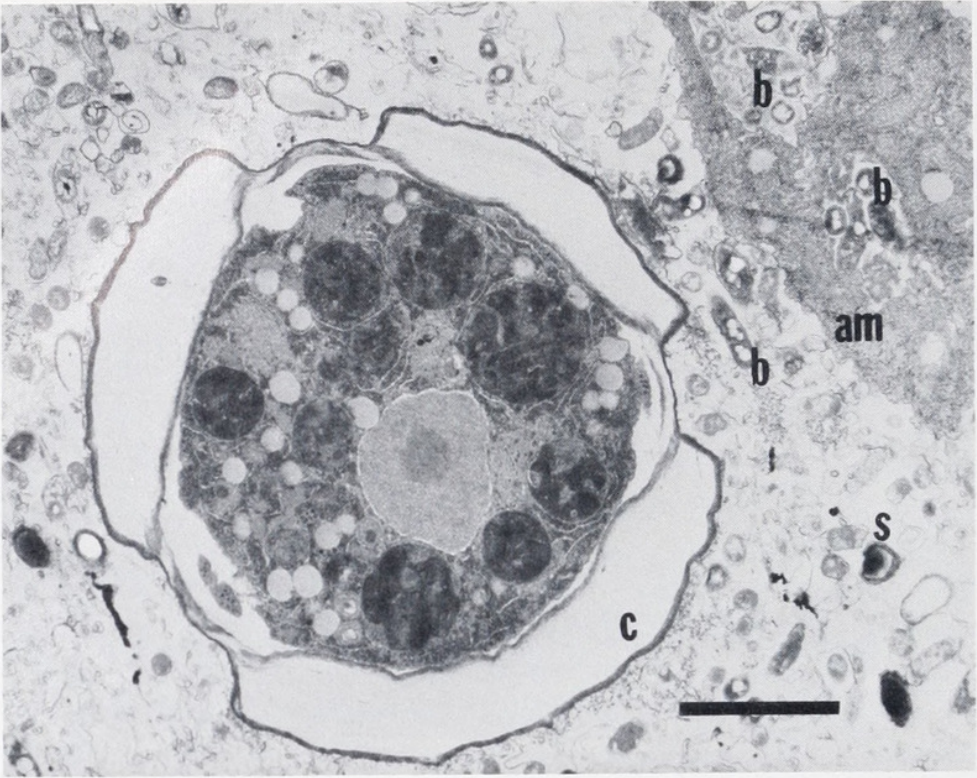


FIGURE 4. TEM of a cyst in a mixed bacterial culture. Bar = 2 μ m. Amoeba at upper right. am = amoeba, b = bacteria, c = cyst, s = spore.

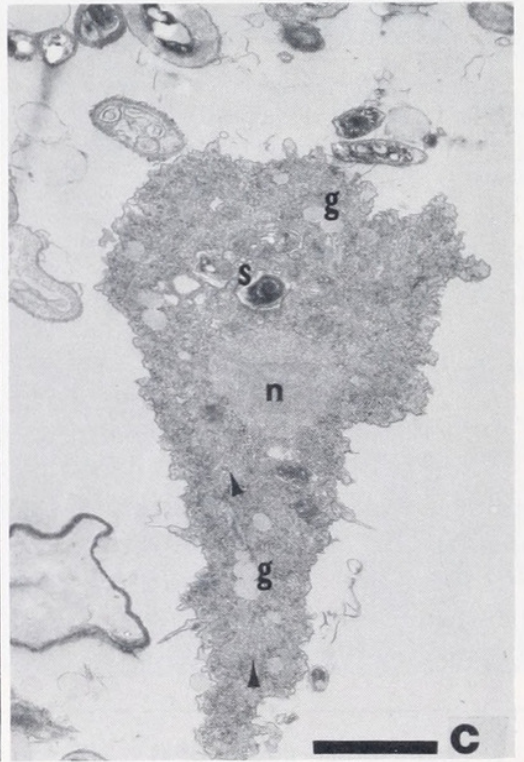
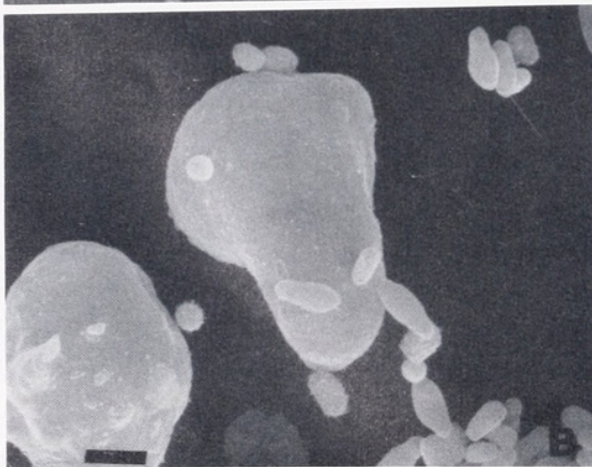
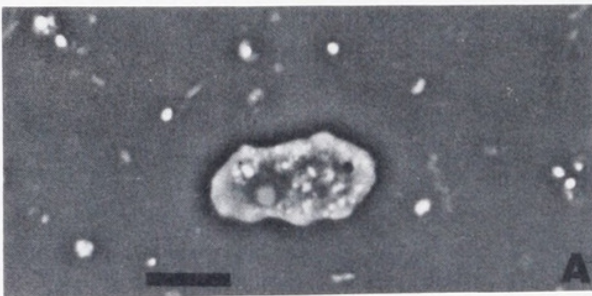


FIGURE 5. Monopodial amoebae. A. Phase contrast Bar = 5 μ m. B. SEM Bar = 2 μ m. C. TEM Note mitochondria with tubular cristae and granules. arrows = mitochondria, g = granules, n = nucleus, s = spore. Bar = 2 μ m.

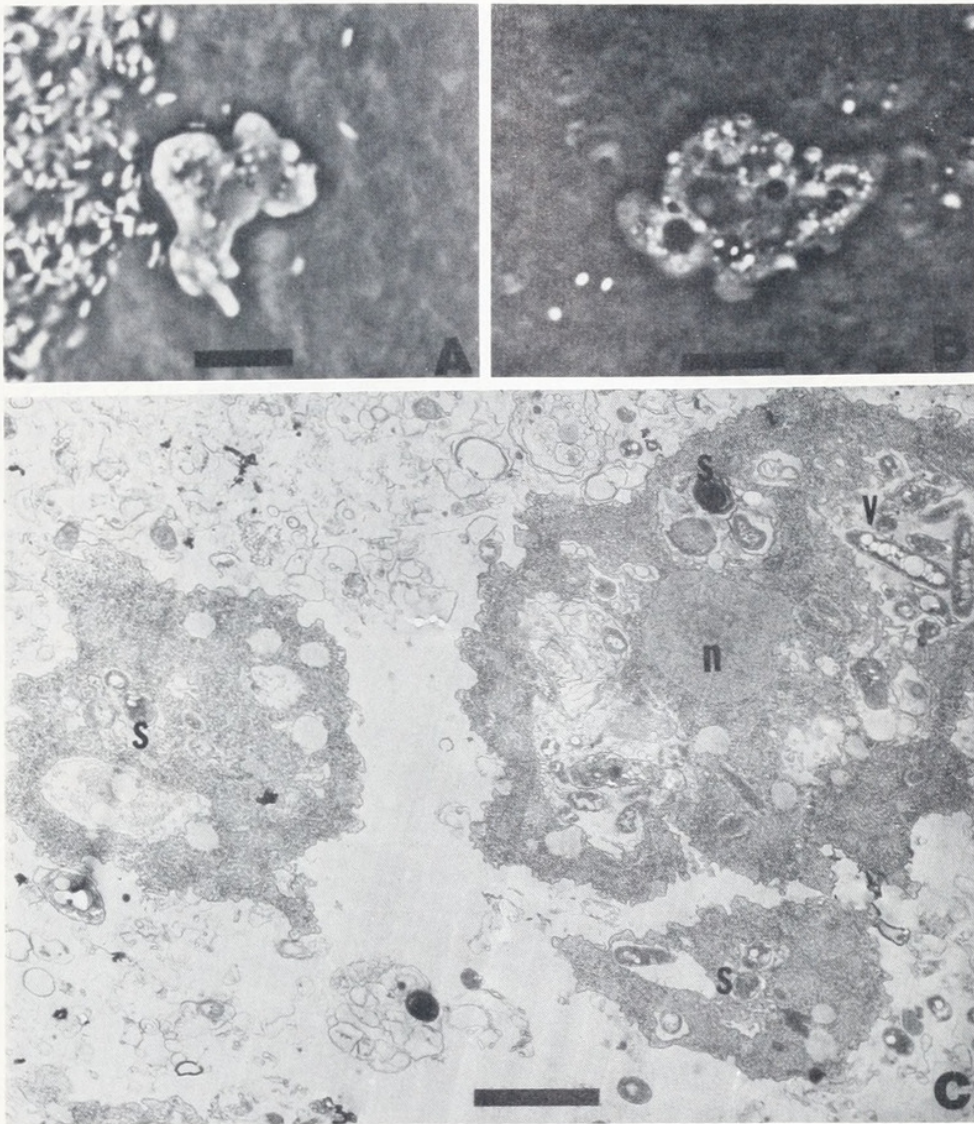


FIGURE 6. Irregular vahlkampfiids. A. Feeding, phase contrast, Bar = 5 μ m. B. Contractile vacuole (black) and nucleus phase contrast, Bar = 5 μ m. C. TEM with bacteria in food vacuoles. n = nucleus, s = spore, v = food vacuole, Bar = 2 μ m.

by Page (1967, 1976) for some strains. The cytoplasm contained many granules and conspicuous vacuoles which contained bacteria, interpreted to be food vacuoles.

Ectoplasmic and small rounded forms. In actively growing cultures rounded forms with thin or indistinct walls and often with an outer clear ectoplasmic layer were by far the most obvious forms on the plates (Fig. 7A–H). These forms ranged from 3–15 μ m and often had large vacuoles containing bacteria, bacterial spores and cytoplasmic granules. Except for occasional bulging the rounded forms were stationary. In those with a distinct ectoplasm, the inner granular cytoplasm was observed in various positions of protrusion beyond the ectoplasmic layer (Fig. 7E). We surmise that the bulging cytoplasm protrudes through an organized opening. Up to three such pore-like openings per rounded form could be distinguished by phase microscopy (color plate IX) and in electron micrographs (Fig. 4).

Small rounded forms from less than 3 to about 5 μ m in diameter were extremely conspicuous in young healthy cultures and more mature ones which had been flooded with distilled water to stimulate more growth. The small forms even outnumbered the larger cyst-sized forms, especially in low salt medium. We compared

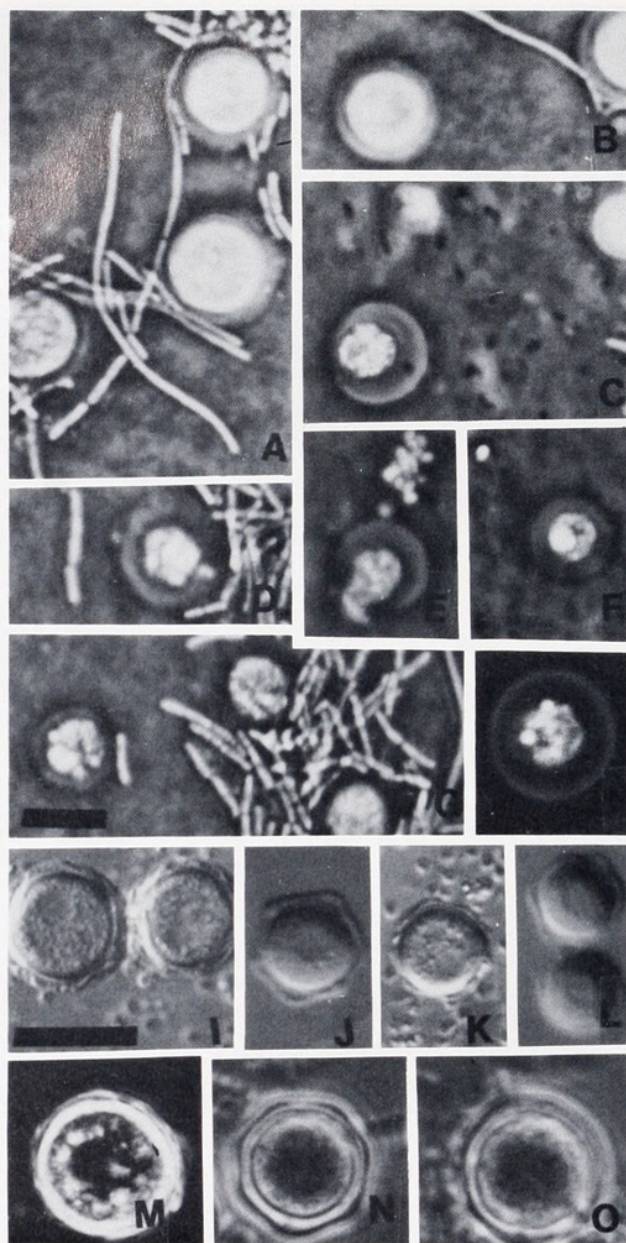


FIGURE 7. A-H. Ectoplasmic forms and affected bacteria, all of these are common in active cultures, Bar = 5 μ m. I-O. Mature cysts. I-L. Nomarski optics. M-O. Phase contrast. Bar = 5 μ m.

the ATCC *P. jugosus* in axenic liquid medium immediately after it was received. That culture also was filled with small bodies and rounded forms. We suggest that these forms are stages in the life cycle of *P. jugosus* and certainly not contaminants, possibly precysts or encysting amoebomastigotes.

Mature cysts. The mature cysts which appeared on the second or third day generally had a distinct smooth round endocyst and an irregular ectocyst (Fig. 7 I-O). At some points the ectocyst contacted the endocyst. Cysts averaged 8 μ m in diameter and ranged from 5.5–10 μ m. The majority of the cysts were uninucleate but binucleate cysts were seen occasionally (color plate VIII). Mature cysts tended to become smaller as they aged and desiccated further. They also became more and more fluorescent as they desiccated. Fluorescent materials seen as granules in moistened cysts may be transferred to the walls as maturation proceeds. Bodies traditionally referred to as "autolysosomal bodies" in electron micrographs of rounded forms are assumed to be related to breakdown of cell material and to rapid wall

formation (Page, 1981). However, these bodies, which apparently contained ribosomes, and material that resembled chromatin may be related to the rapid proliferation of *P. jugosus*. These bodies were very conspicuous in rewet cysts and growing cultures, like the nucleus they stained green with acridine orange. It is our judgment, whatever their nature and development, that these intracellular bodies seen in the electron micrographs (Fig. 8A–C) correspond to the bright bodies seen with phase contrast microscopy (Fig. 8D) and are the source of fluorescence observed on the light micrographic level (color plate VI, VII).

Mastigotes. Mastigotes were never observed on routine culture plates. We were not aware of the ability to form a mastigote stage until it was brought to our attention by F. C. Page of the Culture Centre of Algae and Protozoa, Cambridge, England. Page, on the basis of the morphology of live cultures sent to him, kindly identified the organism as *P. jugosus*. When suspended in distilled water overnight about one third of the organisms transformed into mastigotes overnight. The mastigote stage persisted for 1–2 days. Mastigotes were spherical, or more frequently, elongated in shape; they had 2, sometimes more, forward directed undulipodia (Fig. 9). They tend to be smaller than the amoebae. Bacterial spores could be seen in food vacuoles through the transparent mastigotes. Whether or not the mastigote form actively feeds is unknown; undigested bacteria and spores may have been residues from feeding immediately prior to transformation.

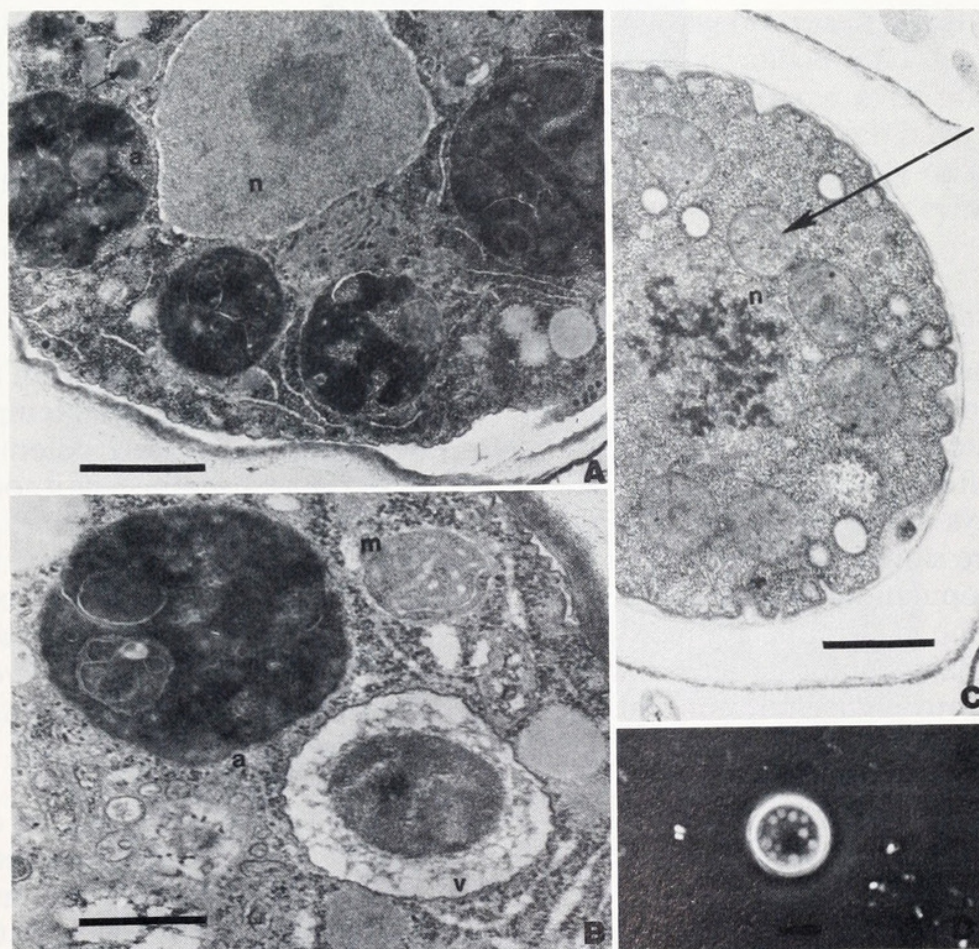


FIGURE 8. Intracellular inclusions in cysts. A. TEM of "autolysosomal bodies" (a) and mitochondria (m). Note the unidentified crystals inside the mitochondria (arrow). Bar = 1 μ m. B. Cysts contain food vacuoles (v) and "autolysosomal" bodies with ribosomes. Bar = 0.5 μ m. C. Cyst with bodies that may contain chromatin (arrow), n = nucleus. Bar = 1 μ m. D. Phase contrast light micrograph. Bar = 1 μ m.

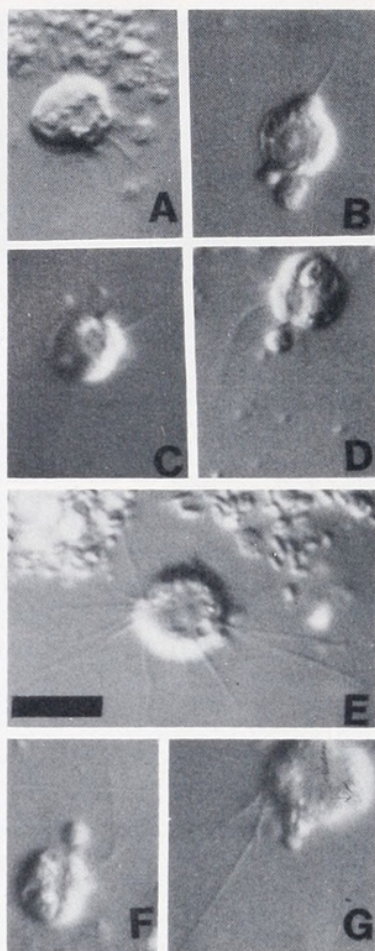


FIGURE 9. Mastigotes. A-G The number of undulipodia per cell vary from 1 to as many as 11. Bar = 5 μ m.

Growth and reproduction

The new strain of *P. jugosus* grew extremely rapidly. New isolates from the field entirely covered the B bacillus colonies with cysts within 3 days at room temperature (color plate I-IV). About sixty cysts per colony developed from plated colonies using a loop. After about a year in culture the growth rate slowed somewhat: it took from 4 to 5 days to entirely replace the food colonies with cysts. The ATCC *P. jugosus* grew more slowly, not forming visible cysts at all until after the 12th day. It never formed populations as dense as the Baja California isolate on any media tested (for example the maximum number of cysts per colony was about 30 in the same test that the Baja California strain developed about 60 cysts per colony). Even after transfer from higher salt concentration (0.1 M NaCl) the ATCC *P. jugosus* grew very slowly and to low cell densities on our routine MnAc media (Fig. 16).

We interpret the rounded forms to be active feeding stages. In young cultures virtually devoid of monopodial amoebae the effects of *P. jugosus* on bacteria were easily seen. Motility was lost and the bacteria became severely clumped and elongated. Apparently *P. jugosus* arrested bacterial cell division, for when infected with *P. jugosus* the bacilli would grow to up to 10 times their normal length and in some cases spaghetti-like masses of unhealthy-appearing bacteria were seen (color plate V, Fig. 10A, compare with Fig. 3). Thread-like material in which bacteria were embedded could be seen in scanning electron micrographs (Fig. 10B). The material,

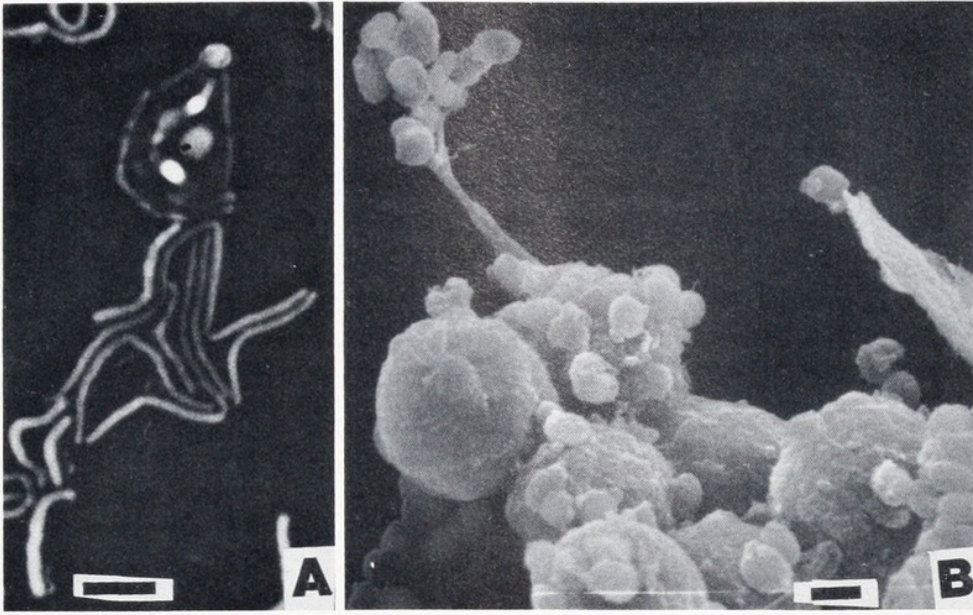


FIGURE 10. B bacillus infected with *P. jugosus* A. Swollen, elongated bacteria with ectoplasmic forms, phase contrast. Bar = 20 μm . B. Ectoplasmic forms, bacteria and apparent exudate SEM. Bar = 2 μm .

consistently seen as cotton-like fluff in all active amoebae cultures and absent in pure cultures of the B bacillus, may be part of the feeding process. Typical engulfing by pseudopods was rare as it was for at least one other vahlkampfid described by Page (1967). It is likely that *P. jugosus* can digest bacillus spores, even manganese-coated ones. As *P. jugosus* grow on older colonies of bacteria which have all transformed to manganese-encrusted spores, cyst-ridden plaque-like holes on plates replace the bacteria. Electron micrographs show bacterial spores in the cytoplasm of the amoebae (Fig. 11).

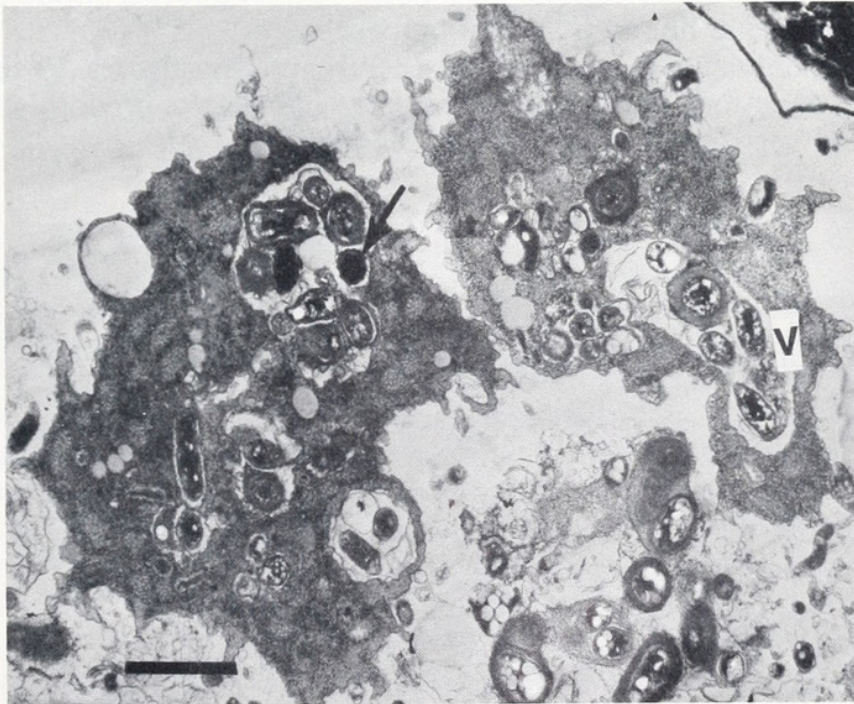


FIGURE 11. TEM of two vahlkampfid amoebae with spores (arrow) and bacteria in food vacuoles (V). Bar = 2 μm .

When divisions were seen they were promitotic, characterized by the persistent nuclear envelope as seen in two other vahlkampfiids (Fig. 12). However, in over two years of continuous and frequent observation including close monitoring of agar slide cultures, divisions were rarely observed. Samples taken from 1–2 day old cultures at frequent intervals (1 to 2 hours) failed to reveal any divisions after examination with oil immersion microscopy. Indeed, there were few amoebae. Similarly, amoebae stained with Kernechtrot or Protargol (Fig. 13) showed only a few figures that could be interpreted as in division. However, these same active cultures were replete with great numbers of small round bodies. These bodies contained yellow-green fluorescent granules (such fluorescence is absent in pure cultures of food bacillus). We suggest that these bodies may be involved in reproduction, thus explaining the scarcity of vegetative amoebae and of their mitotic figures. The frequency of amoebae was highest on moist plates. Generally fewer than 10 percent of the forms in growing young cultures were amoebae and sometimes none at all were seen. Protargol staining confirmed this observation. Small bodies appeared entirely purple whereas only the nuclei of vegetative amoebae retained the stain. The large round bodies contained purple nuclei and cytoplasmic bodies which also stained (Fig. 13, A, B). As the cysts desiccated and matured, the entire round bodies, large and small, stained heavily (Fig. 13 C–I). Both wall material and chromatin stain heavily. On many occasions small round bodies associated with cysts were observed (Fig. 14). These tiny rounded amoebae-like forms were often clumped in groups of seven or eight. Their abundance and association with cysts and large amoebae suggest they may be the product of a rapid series of standard mitoses or multiple fission. Some of the released bodies were fecal pellets that were seen in the amoebae (Fig. 15A) and in the medium (Fig. 15B). Fecal bodies, which are striped and contain partially digested bacteria (Fig. 15C), could be distinguished from the small amoeboid-like bodies. Nothing short of a sequential, carefully timed ultrastructural study of development will solve the question. However, the astonishingly fast reproductive rate, paucity of dividing amoebae, and the omnipresence of spherical bodies which appear to contain chromatin suggest another mode of division in addition to promitotic binary fission of amoebae.

Apparently, only amoebae transform directly into mastigotes. Plates containing abundant amoebae formed mastigotes whereas old plates, predominantly mature cysts, produced very few mastigotes when flooded.

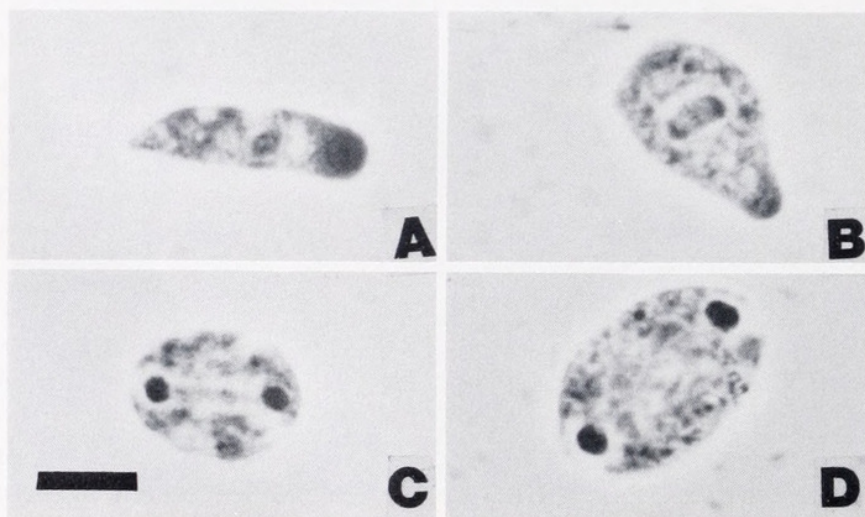


FIGURE 12. Mitosis (A–D Bar = 5 μ m): A. Interphase, B. Metaphase, C. Anaphase, D. Telophase.

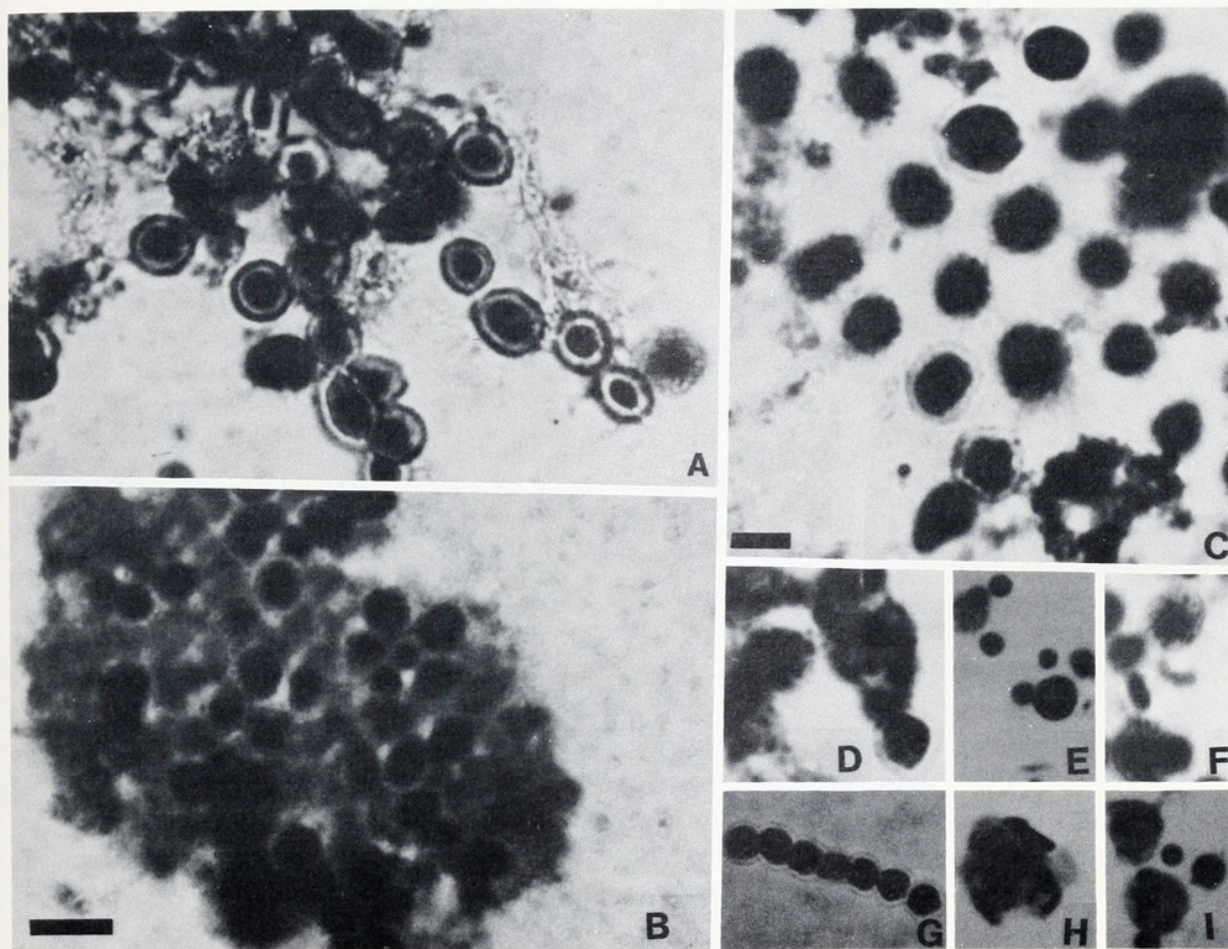


FIGURE 13. Protargol stained preparations. Bar = 5 μ m. A. 12 day old cultures. B. 65 day old cultures. C-I. Ectoplasmic, small rounded, granulated forms all of which are typical of young cultures. Bar = 5 μ m.

When old plates with mature cysts were moistened and carefully observed, amoebae could be seen to emerge from the encysted form. On one occasion a single cyst was seen to convert to the monopodial amoeba form in about 10 minutes.

Salt

Although the growth of this *P. jugosus* isolate was most rapid in media made with distilled water, it also grew well in half strength sea water medium (1.7 per cent NaCl). Furthermore growth occurred in NaCl concentrations up to 0.50 *M* (2.92 per cent). Figure 16A shows typical data from one of three experiments in which growth was shown to be an inverse function of NaCl concentration. When the inoculum size was large, growth was more vigorous even on 0.4 *M* (2.34 per cent) NaCl, and the cysts covered the food bacterial colonies within 7 days. There was even some continued growth (through three transfers) at 0.55 *M* (transferred from 0.05 *M*). The ATCC *P. jugosus* strain also grew optimally in media without NaCl, but did not grow in concentrations of salt above 0.3 *M* (Fig. 16B).

Cyst morphology changed as a function of salt, as is common in encysting amoebae (Fig. 17). Presumably due to shrinkage of the cell, the space between the endo- and the ectocyst widened at higher concentrations of salt and the cysts became more refractile (Fig. 17C, F).

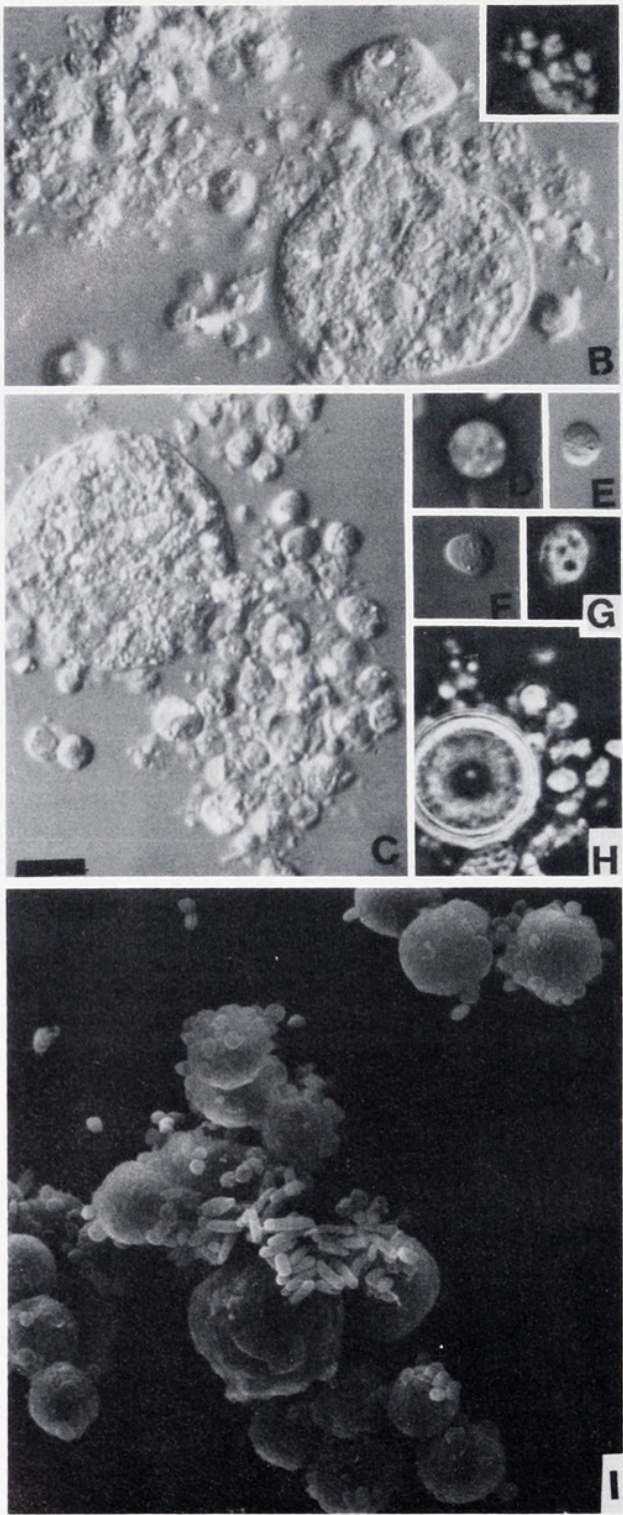


FIGURE 14. Small bodies released? (A., D., E., F., G., Phase contrast; B., C., Nomarski optics. Bar = 5 μ m). A. Clump of newly released amoebae? B. and C. enormous cyst from which (A., D. E., F., G., H.) have young amoebae been released? I. SEM spherical small amoebae? Bar = 2 μ m.

The small, round wall-less forms so conspicuous in actively growing cultures were less frequent at higher salt concentrations. The correlation of these bodies with media that support the most rapid growth rate reinforces the hypothesis that these bodies are directly involved in reproduction.

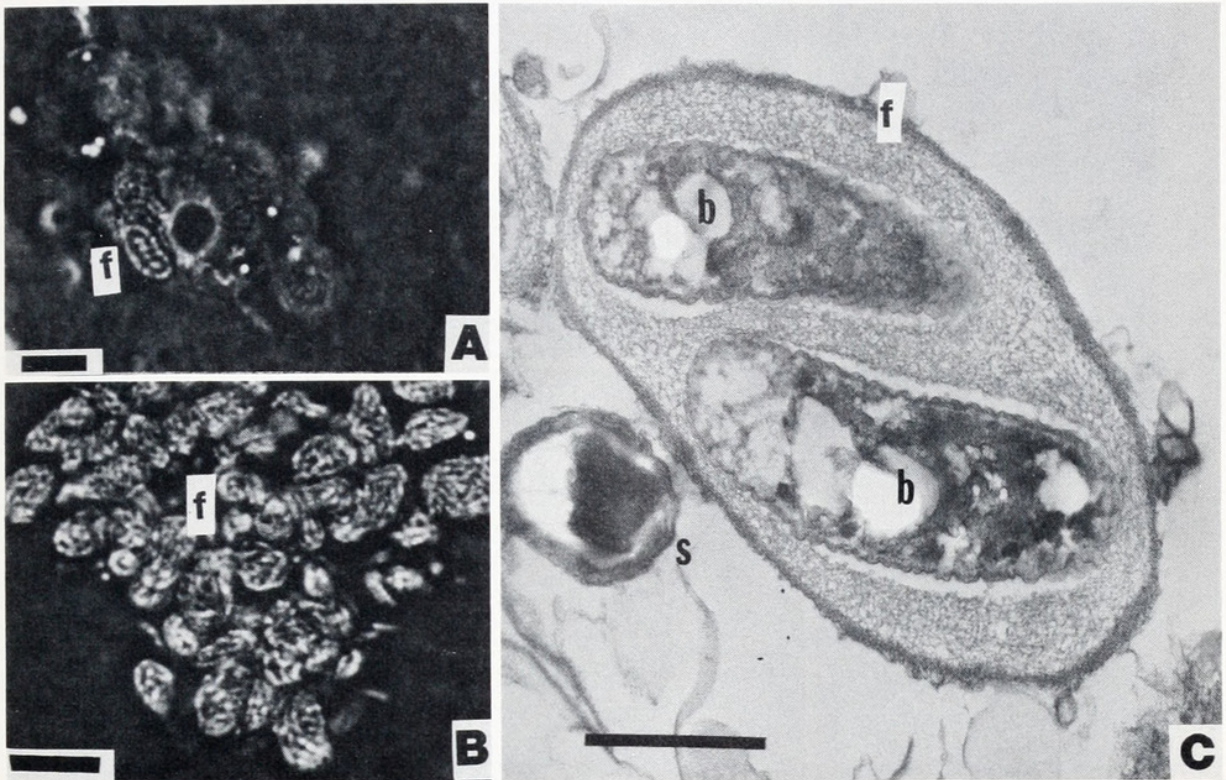


FIGURE 15. Fecal pellets. A. Amoeba containing fecal pellet (Bar = 1 μm). B. Clumps of fecal pellets (f) (Bar = 1 μm). C. TEM thin section of fecal pellet with disintegrating bacteria (b). s = spore. Bar = 0.5 μm .

Temperature

The Baja California strain of *P. jugosus* grew well between 20 and 36°C. It did not grow at 37°C. It survived temperatures up to 56°C for 10 minutes, but did not survive heat treatment for 10 minutes at 59°C. The ATCC *P. jugosus* also survived temperatures up to 56°C for ten minutes. However, it did not grow when incubated at 36°C, but grew well at 30°C. No temperatures were tested between these two points.

Fluorescence

Yellow-green fluorescence emission from vahlkampfiids is unreported. Yet in our studies of Baja California microbial communities we have seen this phenomenon consistently not only in small amoebic cysts but in larger unidentified acanthamoebids. From the fluorescence data in Table II it can be seen that the two strains of *P. jugosus* differ from one another in their emissive properties. Little is known about the chemical basis or possible significance of this fluorescence, but the possibility of its use as a tool in diagnostics is obvious.

Fluorescence in these amoebomastigotes was strongly correlated with life cycle stage. Amoebae do not fluoresce, yet the small rounded bodies had faint fluorescence. The larger round bodies usually contained 2–6 strongly fluorescing bodies that measured from 1–3 μm (Color plate VI, VII). Mature cyst walls fluoresced most strongly; the cysts themselves fluoresced more and more strongly as they desiccated. Although Page (1967) did not report fluorescence data he did describe cytoplasmic granules in *Vahlkampfia ornata*. Dense spherules (1.5 to 2 μm in diameter) were

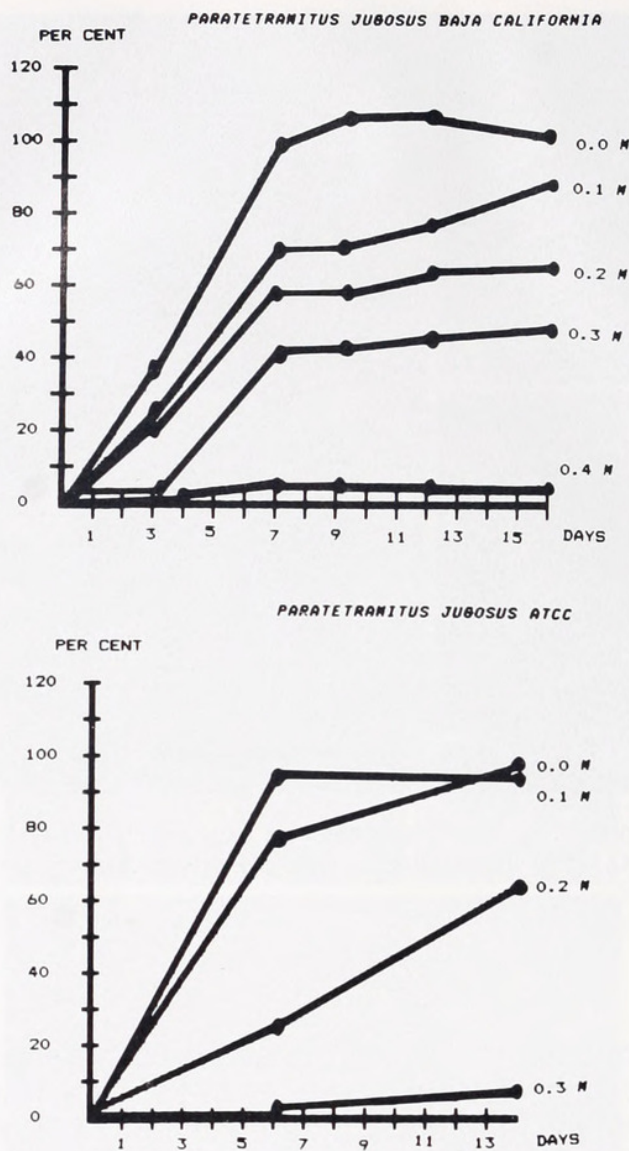


FIGURE 16. Growth of *P. jugosus* as a function of salt. A. The Baja California strain: area of colony covered by cysts in media made up from 0.0 to 0.4 M (2.34 per cent) sodium chloride. B. The ATCC strain: area of colony covered by cysts in media made up from 0.0 to 0.3 M sodium chloride.

present in immature *V. ornata* cysts whereas mature cysts showed only fine granulation. There is most likely a relationship between the fluorescent material, the granules, and the conspicuous autolysosomal bodies seen in electron micrographs (Fig. 8).

Isoenzymes

The electrophoretic mobility pattern for three different enzymes of *P. jugosus* from Baja California was compared with that from the *P. jugosus* from the ATCC. The patterns for propionyl esterase and leucine aminopeptidase were nearly identical. However, there were conspicuous differences between the two stains with respect to their alkaline phosphatase (Fig. 18).

DISCUSSION

Paratetramitus jugosus was proposed by Darbyshire (*et al.*, 1976) as a new genus and species of amoebae isolated from a stream near Moscow, Idaho. Before the

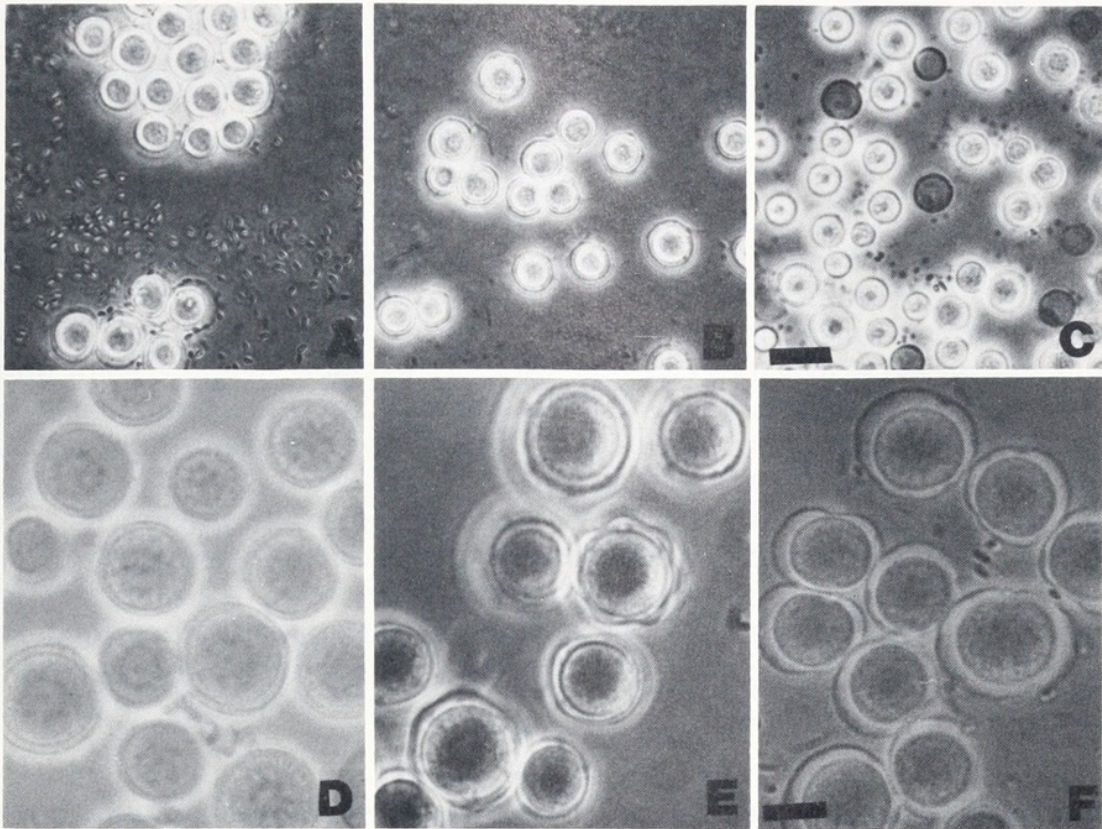


FIGURE 17. Cyst morphology as a function of salt concentration. A. and D. 0.5 per cent. B. and E. 1.2 per cent. C. and F. 2.4 per cent. A., B., and C. Bar = 10 μ m. D., E., and F. Bar = 5 μ m.

mastigote stage had been seen, *P. jugosus* had been introduced by Page (1967) into the literature as *Vahlkampfia jugosus*. These amoebae have a closed nuclear division pattern (promitosis), eruptive monopodial pseudopods, and temporary amoebomastigote stages. They belong to the family Vahlkampfiidae (Page, 1976).

Different geographical strains have been isolated from Scottish soil samples, fresh water lakes and streams in England and in the United States (Darbyshire *et al.*, 1976), and from a Czech swimming pool (Cerva, 1971). All of the strains originally identified as *Vahlkampfia jugosus* also transformed into mastigotes and thus were reclassified by Page (1976) as *Paratetramitus jugosus*.

We believe that the differences in growth rate, salt and heat tolerance, isoenzyme pattern, fluorescence emission maxima, and extraordinary desiccation resistance constitute enough difference to recognize this protist as a new strain, *Paratetramitus jugosus baja californiensis*.

On the coastal evaporite flat of Laguna Figueroa the protist survives but does not grow during normal periods of cyanobacterial organic mat deposition and ex-

TABLE II

Fluorescence of mature cysts

Strain	Maxima	
	Excitation (nm)	Emission (nm)
<i>Paratetramitus jugosus</i> BC	488	611
<i>Paratetramitus jugosus</i> ATCC	488	592

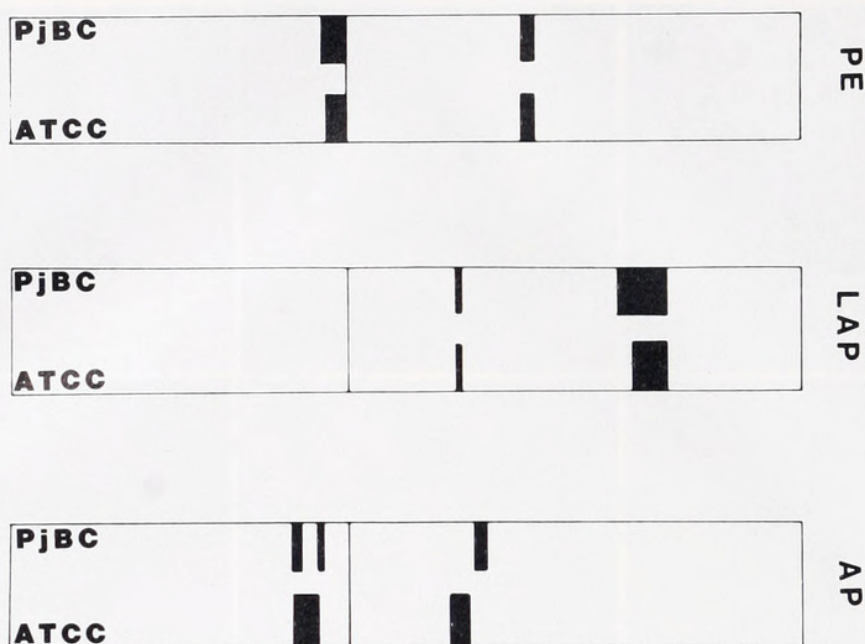


FIGURE 18. Isoenzymes: PE = propionyl esterase, LAP = leucine aminopeptidase, AP = acid phosphatase. Starch gel electrophoresis, movement right to left.

tremely high evaporation rates. Once a year during winter-spring rains, however, conditions become ideal for the rapid growth of *P. jugosus*; blooms become obvious. The unusual weather conditions of 1979–1980 conspired to retain such superb growing conditions for *P. jugosus* that even after severe desiccation three years later it was the dominant organism enriched from mat material in the laboratory.

During the winter and spring of 1982 there was extremely little or no rainfall at the field site. As the flood water evaporated, the mat condition became more saline, recolonization by halophilic bacteria began, and the growth of *P. jugosus* diminished, decreasing the frequency with which the protist was isolated in the summer of 1982. We conclude that this amoebomastigote is highly adapted to the transient appearance of fresh water.

As described elsewhere in detail (Margulis *et al.*, 1983; Stolz, 1983a, b), during the summer of 1982 the laminated microbial mat which developed from before 1965 thru 1979, although covered by several centimeters of terrigenous sediment due to the flood, was found buried from 10–15 cm below the surface of newly forming mat. Between the older laminated mat and the new growth we observed a smooth organic-rich mud smelling of sulfide which contained remains of cyanobacterial sheaths, and heterotrophic bacteria of many kinds. It also contained the abundant *P. jugosus*. If silicified, this smooth black mud layer, as it lithified to chert, would likely preserve entrapped microbes and their remains. Because of their high population densities, euryhalinity and environment of deposition, hardness, and resistance, *P. jugosus* is likely to have a high preservation potential. Indeed it is possible that it has already been reported in the microbial fossil record as “acritarchs” or other problematica (Knoll, 1982; Vidal and Knoll, 1983).

Only further study can solve the mystery of the mode of reproduction in young, relatively dry cultures on agar plates that contain many spheres and nearly no amoebae. Multiple fissioning of some kind that produces small spheres may occur. In studies of similar small amoeboid forms from oysters, Hogue (1914) diagrammed amoebal multiple fission uncannily similar to what we have seen. Even though it is likely that Hogue’s studies were plagued by mixed cultures of protists we think

her suggestion of multiple fission deserves reconsideration. The ubiquitous small spheres may be the active feeding and multiplying forms of *P. jugosus*. Either very rapid mitosis or multiple fission to produce small spherical forms occurs.

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