Reference: *Biol. Bull.* **177:** 110–129. (August, 1989)

Ecology and Life History of an Amoebomastigote, *Paratetramitus jugosus,* from a Microbial Mat: New Evidence for Multiple Fission

MICHAEL ENZIEN, HEATHER I. McKHANN[1], AND LYNN MARGULIS[2]

Boston University, Department of Biology, 2 Cummington Street, Boston, Massachusetts 02215

Abstract. Five microbial habitats (gypsum crust, gypsum photosynthetic community, *Microcoleus* mat, *Thiocapsa* scum, and black mud) were sampled for the presence of the euryhaline, rapidly growing amoebomastigote, *Paratetramitus jugosus.* Field investigations of microbial mats from Baja California Norte, Mexico, and Salina Bido near Matanzas, Cuba, reveal that *P. jugosus* is most frequently found in the *Thiocapsa* layer of microbial mats.

Various stages of the life history were studied using phase-contrast, differential-interference, and transmission electron microscopy. Mastigote stages were induced and studied by electron microscopy; mastigotes that actively feed on bacteria bear two or more undulipodia*. A three-dimensional drawing of the kinetid ("basal apparatus") based on electron micrographs is presented.

Although promitoses were occasionally observed, it is unlikely that they can account for the rapid growth of *P. jugosus* populations on culture media. Dense, refractile, spherical, and irregular-shaped bodies were seen at all times in all cultures along with small mononucleate (approximately 2–7 μm diameter) amoebae. Cytochemical studies employing two different fluorescent stains for DNA (DAPI, mithramycin) verified the presence of DNA in these small bodies. Chromatin-like material seen in electron micrographs within the cytoplasm and blebbing off nuclei were interpreted to be chromatin bodies. Our interpretation, consistent with the data but not proven, is that propagation by multiple fission of released chromatin bodies that become small amoebae may occur in *Paratetramitus jugosus.* These observations are consistent with descriptions of amoeba propagules in the early literature (Hogue, 1914).

Received 12 December 1988; accepted 31 May 1989.

[1] Current address: Department of Biology, University of California at Los Angeles, Los Angeles, CA 90024.

[2] Current address: Department of Botany, University of Massachusetts, Amherst, MA 01003.

* The term mastigote refers to an organism that bears undulipodia ("eukaryotic flagella"), *i.e.,* microtubular motility organelles with [9(2) + 2] microtubules in transverse section (Margulis, 1980; Margulis and Sagan, 1985).

Introduction

We report here field and laboratory studies of a rapidly growing, hardy, encysting, and desiccation-resistant amoebomastigote, *Paratetramitus jugosus.* Responsive to recent changes in systematics, we provide the classification of *P. jugosus* (Page, 1983) in the Protoctista (rather than animal) kingdom (Table I) (Margulis, 1988; Margulis *et al.,* 1989). Our strain *P. jugosus bajacaliforniensis* (Read *et al.,* 1983), isolated from sediments of a microbial mat in an evaporated marine salt pond, is unusually euryhaline for the genus. All other isolates were taken from freshwater and soil environments (Darbyshire *et al.,* 1976).

Approximately once a year since a two-year episode of flooding that began in 1979, *P. j. bajacaliforniensis* has been taken from both submerged and re-emerged field samples of the *Microcoleus* mats at North Pond and at the south salinas area of Laguna Figueroa, Baja California Norte, Mexico (Fig. 1). *P. jugosus* amoebae were recovered in impressive numbers from nearly every mat sample transferred onto permissive plates in the years during and just after the floods (1979–80). Even after the recession of the flood waters and the return of the region to its more typical dry conditions, we have collected fresh isolates of *Paratetramitus jugosus* at the same geographic

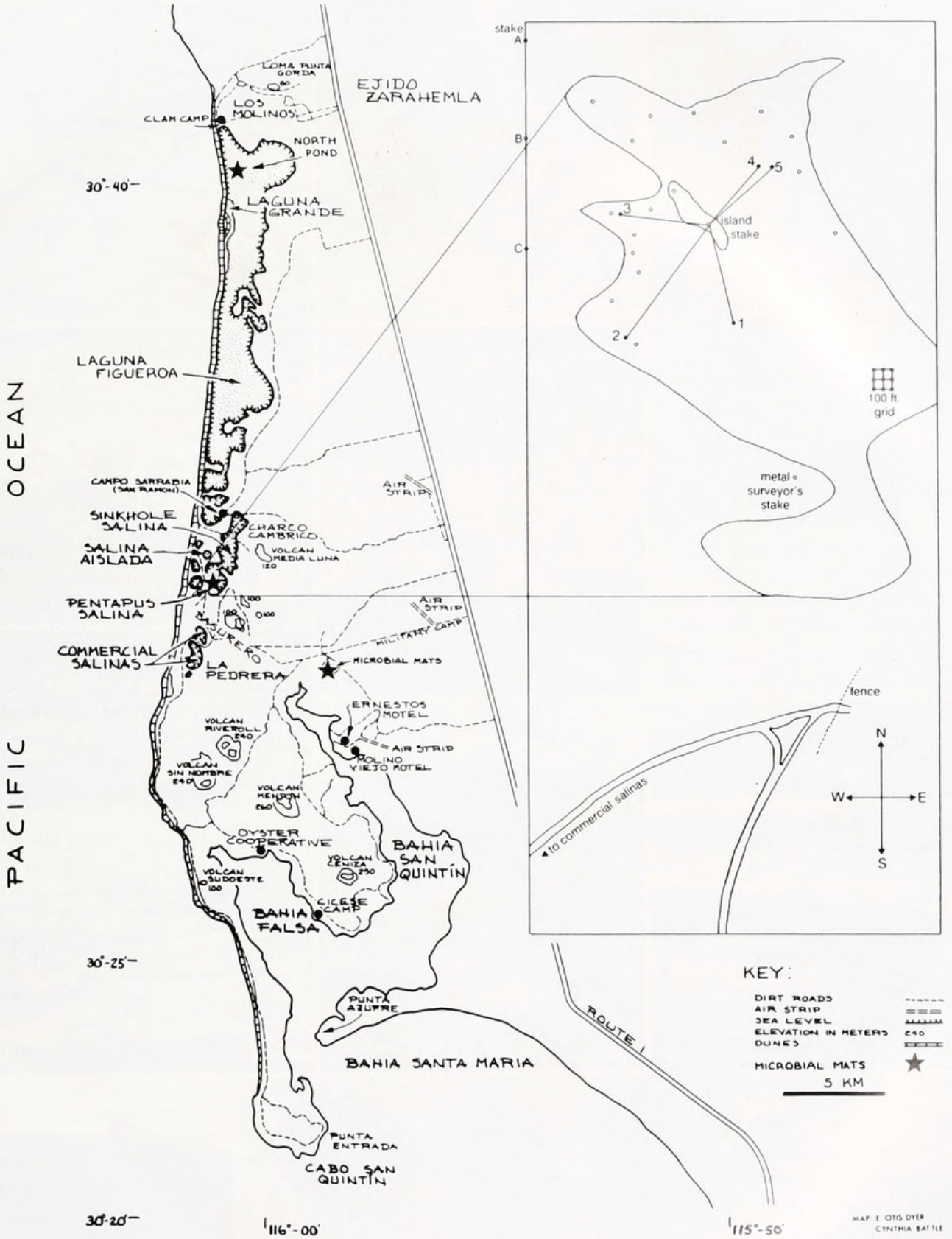

Figure 1. Map of field site in Baja California, Mexico. Samples were collected from both North Pond and Pentapus Salinas. Inset map indicates specific sampling sites at Pentapus Salinas. Sites 1–5 correspond to Figures 2A–E.

locations. A continuation of previous work (Read *et al.*, 1983), this study verifies the occurrence of *P. jugosus* in similar microbial habitats, *e.g.*, in Cuba and Mexico, and presents effective methods of ecological sampling to demonstrate its presence.

In live microscopic preparations of *P. jugosus*, every

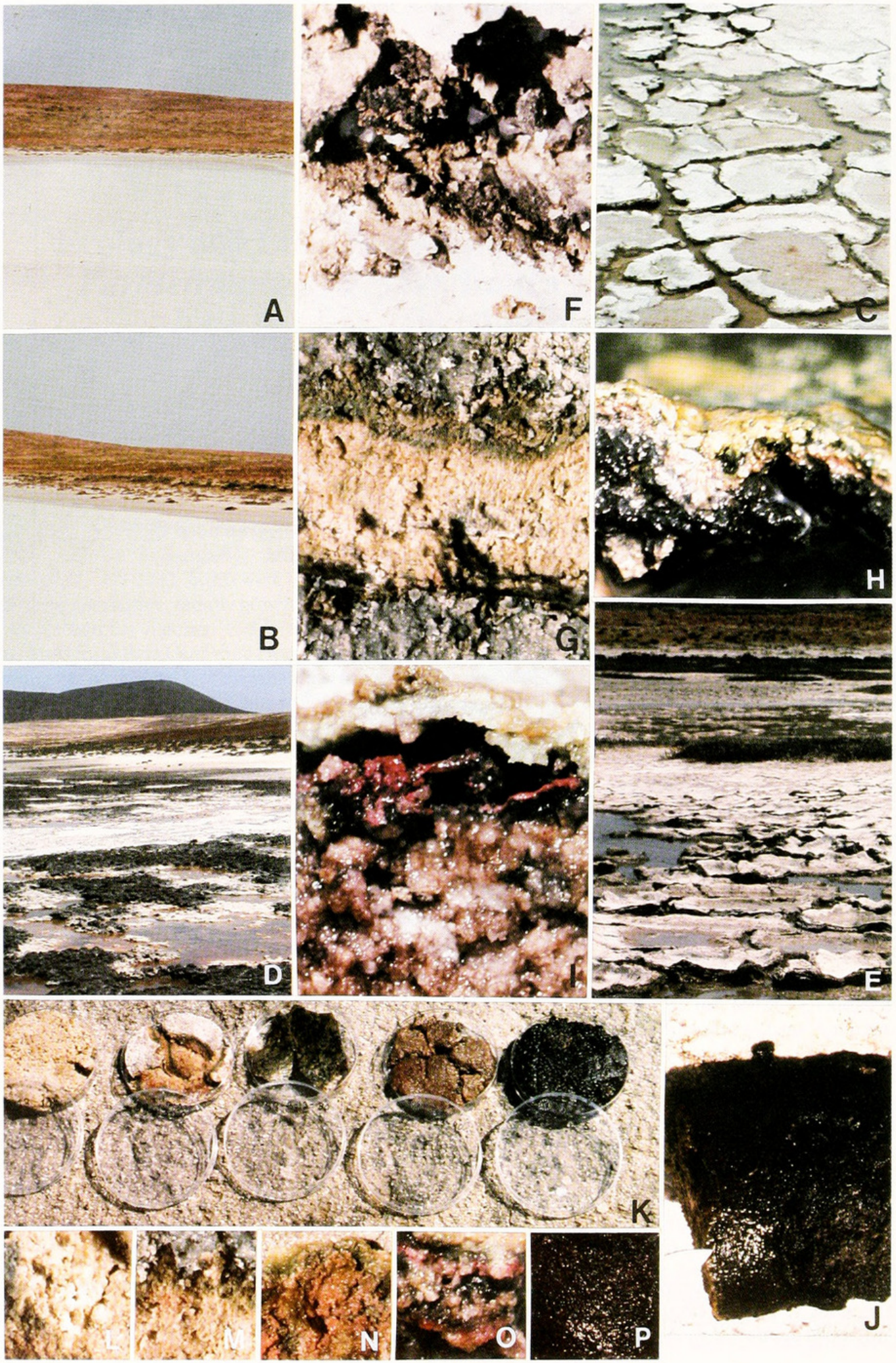
A
F
C
B
G
H
D
I
E
K
J
L
M
N
O
P

Table I

Classification of Paratetramitus jugosus

		Taxon references
KINGDOM	Protoctista	Margulis *et al.*, 1990
PHYLUM	Zoomastigina	Margulis *et al.*, 1990
CLASS	Amoebomastigota	Margulis *et al.*, 1990
ORDER	Schizopyrenida	Singh, 1952 in Page, 1983
FAMILY	Vahlkampfiidae	Jollos, 1917; Zulueta, 1917 in Page, 1983
GENUS	*Paratetramitus*	Darbyshire *et al.*, 1976
SPECIES	*jugosus*	Page, 1967
strains	*bajacaliforniensis*, Cuba	Read *et al.*, 1983 this paper

stage in the reproductive life history has been sought; yet, in spite of continued attempts, the complete process of standard vahlkampfid promitotic division has never been observed. [Promitosis is defined as karyokinesis followed by cytokinesis in which the nuclear membrane is preserved throughout; *i.e.*, closed nuclear division with an intranuclear spindle (Page, 1983; Raikov, 1982).]

The presence of small spherical and irregular shaped bodies (2–7 μm diameter) always found in our cultures, chromatin-like bodies, nuclear blebbing, and apparent cytoplasmic "budding," led us to hypothesize the existence of multiple fission in the reproduction of this small eukaryote and thus to examine the localization of DNA in actively growing and reproducing cultures. Electron microscopic studies and cytochemical observations on amoebae stained with fluorescent dyes specific for DNA (DAPI, mithramycin) coupled with DNase enzyme treatments, led us to our interpretation of the life history of this amoebomastigote.

"Chromidia," *i.e.*, chromatin bodies in large vahlkampfid amoebae parasitic on oysters, were described by Hogue (1914) from live and fixed material using nonspecific nuclear stains; apparently ours is the first cytochemical study since that time that employs DNA-specific stains of comparable structures.

Materials and Methods

Field studies

Sampling for most of the fieldwork reported here was done at Pentapus Salina (inset, Fig. 1), a small member of the south salina complex of Laguna Figueroa, Baja California Norte, Mexico. Unlike the larger salinas to the south, Pentapus is not used for commercial salt production. Although more microbial community-dominated surfaces could be distinguished than were studied, five of those easiest to recognize were chosen as sources for sampling. Each corresponds to major surface cover at the five survey sites shown on the inset map of Pentapus. Descriptively named for the appearance of the surface sediment they are (Fig. 2): (1) gypsum crust (black-and-white sediment only, no macroscopic evidence for green or red phototrophic bacterial communities); (2) gypsum photosynthetic mat (green-layered pink-underlain community of phototrophic bacteria beneath the white gypsum crust); (3) *Microcoleus* mat (cohesive microbial mat community, often polygonal at surface, dominated by the sheathed, green, filamentous cyanobacterium *Microcoleus chthonoplastes*); (4) *Thiocapsa* scum (purple-pink, dense, gelatinous community dominated by the purple phototroph *Thiocapsa sp.*); and (5) black mud (sulfurous, homogeneous black mud sample taken subaqueously from standing water in the channels or from beneath the *Microcoleus* mat). Additional samples were collected from sediment rich with sulfur-oxidizing bacteria (Fig. 3A) and from microbial mats from a mangrove area in Salina de Bido, Cuba (Fig. 3B, C).

Figure 2. Field sites in Pentapus Salina. A–E. Scenes. A. Gypsum crust, Pacific Ocean is at other side of vegetation-covered dune. B. Gypsum mat develops where enough water seeps under the dune to support it. C. *Microcoleus chthonoplastes* stratified microbial community tends to split into polygons covered with evaporite minerals on the surface in tidal channels on the seaward side of the salina. D. *Thiocapsa* scum community tends to have rumpled sediment cover and black coloration due to complex communities of coccoid cyanobacteria and other microbes in the oxygenic surface layers. E. Black muds reside in many places, but at site 5 where water is abundant, they approach the surface and can be seen without cutting into the sediment beneath the cyanobacterial community. F–J. Unaided-eye views of typical sediment profiles from sites 1–5. F. Gypsum crust which grades directly from oxidized calcium sulfate to black sulfide-rich mud tends to be dry, lacking evidence of colored phototrophic bacterial communities indicating low populations of these organisms. G. Gypsum mat in which laminae of green cyanobacterial populations are underlain by a paper-thin layer of purple phototrophs. H. Fully developed *Microcoleus* mat community (Stolz, 1983) I. *Thiocapsa* scum, which contains several types of purple phototrophs including the salmon-colored *Thiocapsa pfennigii* and the orange-colored microbe *T. roseopercicina*. J. Amorphous sulfurous gelatinous mud, which underlies most mat communities, contains many heterotrophs and evidence for degradation of the phototrophic community. K. Samples of each of the five community types are placed in sterile Petri dishes for further study. L–P. Close-up of samples used as source inocula from each of the five field sites: L. Gypsum crust, M. Gypsum mat, N. *Microcoleus* mat, O. *Thiocapsa* scum, P. Black mud. See Table II for results from each of the five sites.

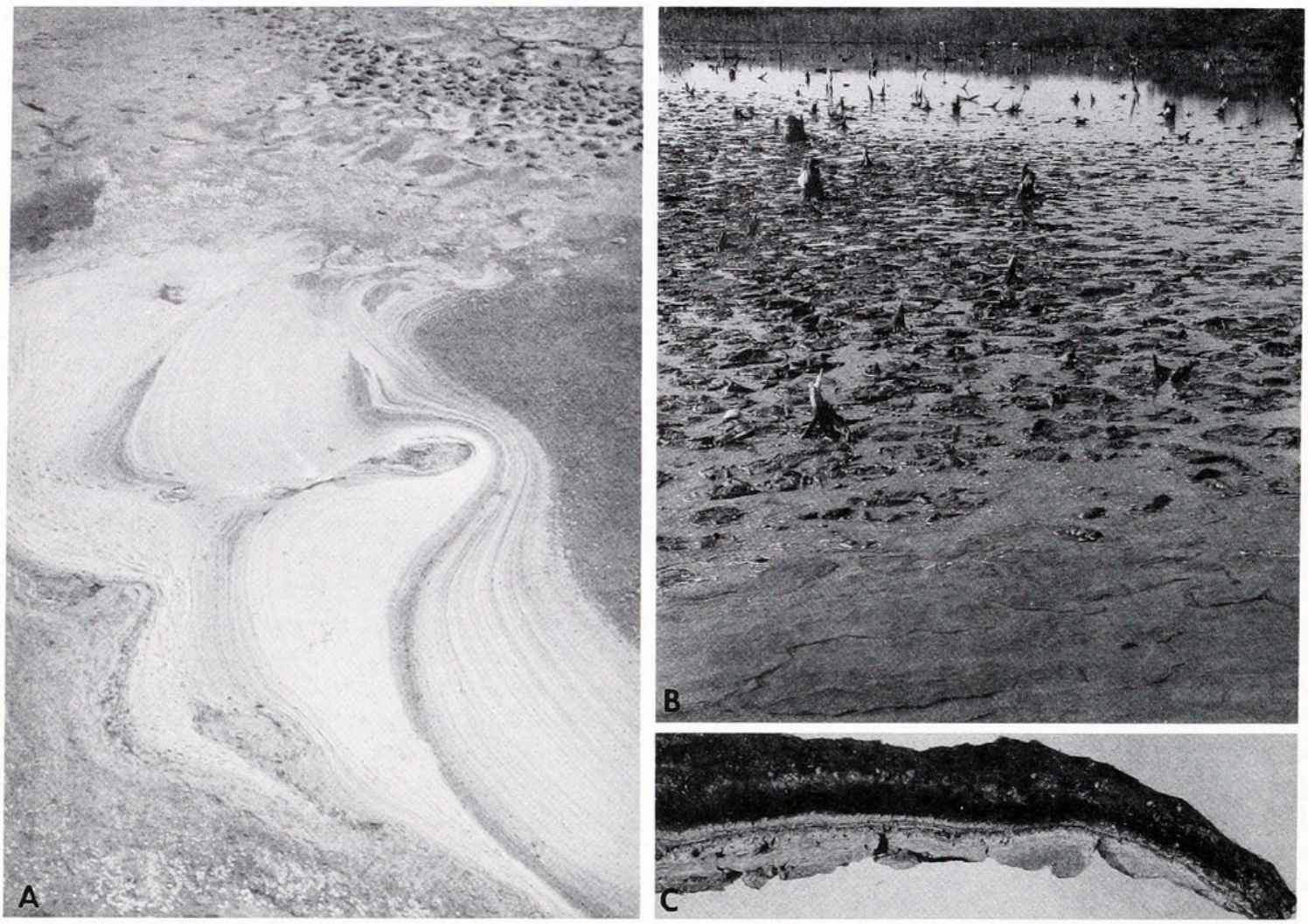

Figure 3. A. Salt pond scum community dominated by sulfide oxidizing bacteria to the west of stake marked 4 on inset of Figure 1. B. Salina Bido, Matanzas Cuba, site of collection of the Cuba strain of *Paratetramitus jugosus*. Mangrove trees form the barrier between the mats and the open ocean. C. *Microcoleus* mat from Salina Bido, hand sample.

With the help of two Earthwatch teams (1983) the Laguna Figueroa sites were extensively surveyed and marked with a permanent stake ("metal surveyor's stake," Fig. 1). Wooden stakes were placed as indicated by the open circles, including three reference stakes ("stakes A, B, C," Fig. 1). Sediment samples from the five sites were placed on petri plates to enrich for *P. jugosus* in early May; the experiments were repeated six weeks later in June 1983. Collections involving samples from the five different sediment types at Pentapus were twice made again in March and October 1988.

Agar plates were prepared with two kinds of thinly poured sterile enrichment media: "modified-K" and "manganese-acetate" (MnAc) (Margulis *et al.*, 1980; Read *et al.*, 1983). These plates were taken to the field study site where a sample about 1 mm^3 of each of the five sediment types was placed directly at the center of both kinds of sterile plates.

Immediately after samples were in place, they were covered with approximately 1 ml of sterile distilled water to suspend the organisms and initiate reproduction and perhaps excystment. When vigorous growth was evident, plates were monitored and scored for the presence of cysts and amoebae. These were subcultured as needed onto fresh medium by streaking with a sterile platinum loop.

The organisms were routinely grown at room temperature on either modified K or MnAc media, both of which contain half-concentrated seawater. The food source for *P. jugosus* is a gram-positive, flagellated, facultatively aerobic rod (designated "B bacillus") that grows readily on both media. Because it is morphologically indistinguishable, the B bacillus is likely to be a strain of the organism reported by Gong-Collins (1986).

Maintenance and storage of stock cultures is described in Read *et al.* (1983).

Light microscopy

Culture slides. Hanging-drop culture slides were made for observation of live *P. jugosus*. A very small drop of

Table II

Paratetramitus jugosus *on field plates*

Observation scored on	Day 1		Day 8		Day 10–11		Day 16–18		Day 19		Day 28		Day 30	
MEDIUM	K	MnAc	K	MnAc	K	MnAc	K	MnAc	K	MnAc	K	MnAc	K	MnAc
Sites														
1. Gypsum crust	−	−			+	+	+	+						
2. Gypsum mat	−	−			−	+	−	+						
3. *Microcoleus* mat	−	−			−, −, −	+, +, +	+, +, +	+, +, +						
4. *Thiocapsa* scum	−	−			−	+, +, −	−, +	+, +, +						
1. Gypsum crust	−	−	−	−	−, +	+, +	−, +	+, +	−	+	−	+, −	−	+
2. Gypsum mat	−	−	−	+	−, +	+, +, +	−, −	+, +	−	++	−	−	−	++
3. *Microcoleus* mat	−	−	++	+	++, −	+, +, N	++, +	+, +	++	+	−, F	+, −	++	++
4. *Thiocapsa* scum	−	−	++	+	+, −	+, +, −	++, −	+, +	++	+	++	+	+	+
5. black mud	−	−	−	−	−	−	+	−	−	−	++	++	−	−

Top: May–June 1983

Bottom: March 1988, except for day 28 which corresponds to August experiment

Key: −, no or very few cysts; +, cysts abundant; ++ at 25× magnification every field on plate has cysts; N, F nematodes, fungi obscure readings

Notes: *Acanthamoeba* sp. cysts (larger and more crenulated than those of *Paratetramitus*) also tend to appear on plates where *P. jugosus* cysts are abundant. Commas between entries indicate entirely different sets of experiments; each entry represents the value on 1 to 4 plates.

MnAc medium was placed on a glass coverslip, inoculated with amoeba cysts, and then covered with a depression slide. Petroleum jelly was used to adhere and seal the coverslip to the slide. Fresh preparations were viewed immediately with a Nikon Diaphot inverted microscope. The excysting amoebae grew on the coverslips mounted over the inverted glass depression slides. After amoebae were detected with the inverted scope, the slides were flipped upright and observed at higher magnification with the Nikon Fluophot phase-fluorescence microscope.

Amoebomastigote transformation. Monoprotist cultures were grown from fresh microbial mat material collected by the above method at Salina de Bido, Matanzas, Cuba. The cultures, started within two weeks of collection, contained various types of bacteria, including food bacteria, and were used to obtain amoebomastigote transformation. Plates were flooded with distilled water and allowed to stand for 10 min. The water was then pipetted off and placed in a sterile test tube. Several drops of this aqueous suspension were placed on a K plate, and the plate was monitored for the presence of amoebae and mastigotes by light microscopy over a 48-h period.

Nuclear fluorescent staining

Fixation. Cultures necessary for monitoring the reproductive processes of *P. jugosus* had to contain growing amoebae. Active trophic amoebae were acquired by the following technique: five glass coverslips were aseptically placed in a circle on a MnAc plate. The center of the circle made by the coverslips was then inoculated with a monoprotist culture of *P. jugosus* and flooded with sterile MnAc medium (lacking agar) up to the edge of the coverslips. One to two drops K medium (lacking agar) were added to the surface of the five coverslips. The amoebae were incubated at room temperature for one to four days, or until the growing edge of the culture could be detected on the surface of the coverslips using an inverted microscope. The coverslips were removed and immediately immersed in Columbia jars containing a modified Carnoy's fixative (70% ethanol, glacial acetic acid, in a 3:1 ratio). The coverslips containing the amoebae were then rinsed twice in 70% ethanol and stored at 4°C in 70% ethanol until they were stained.

Unlike the agar culture slides used previously (Read *et al.*, 1983) it was not necessary to coat the coverslips with agar. No step in the staining procedure was needed to insure the adherence of the amoebae to the coverslips. Apparently enough adhesive substance from the K medium and any proteinaceous substances secreted by or from lysed bacteria caused excellent adhesion of fixed amoebae. Eliminating the necessity of coating coverslips with Parlodion® or other substances decreased the amount of background debris including spurious fluorescence in stained samples.

Staining. Two fluorescent DNA stains were used in this study: 4′-6-diamidino-2-phenylindole (DAPI) and mithramycin (both purchased from Sigma Chemical Co., St. Louis, Missouri). Staining procedures were those of Coleman *et al.* (1981). Stain concentrations were 0.5 μg/ml DAPI in McIlvaine's pH 4.4, or 50 μg/ml mithramycin in McIlvaine's pH 7.0 with 10 mM $MgSO_4$. Fixed coverslips were rehydrated briefly through an ethanol series, washed twice in dH_2O, and then twice in the appropriate buffer.

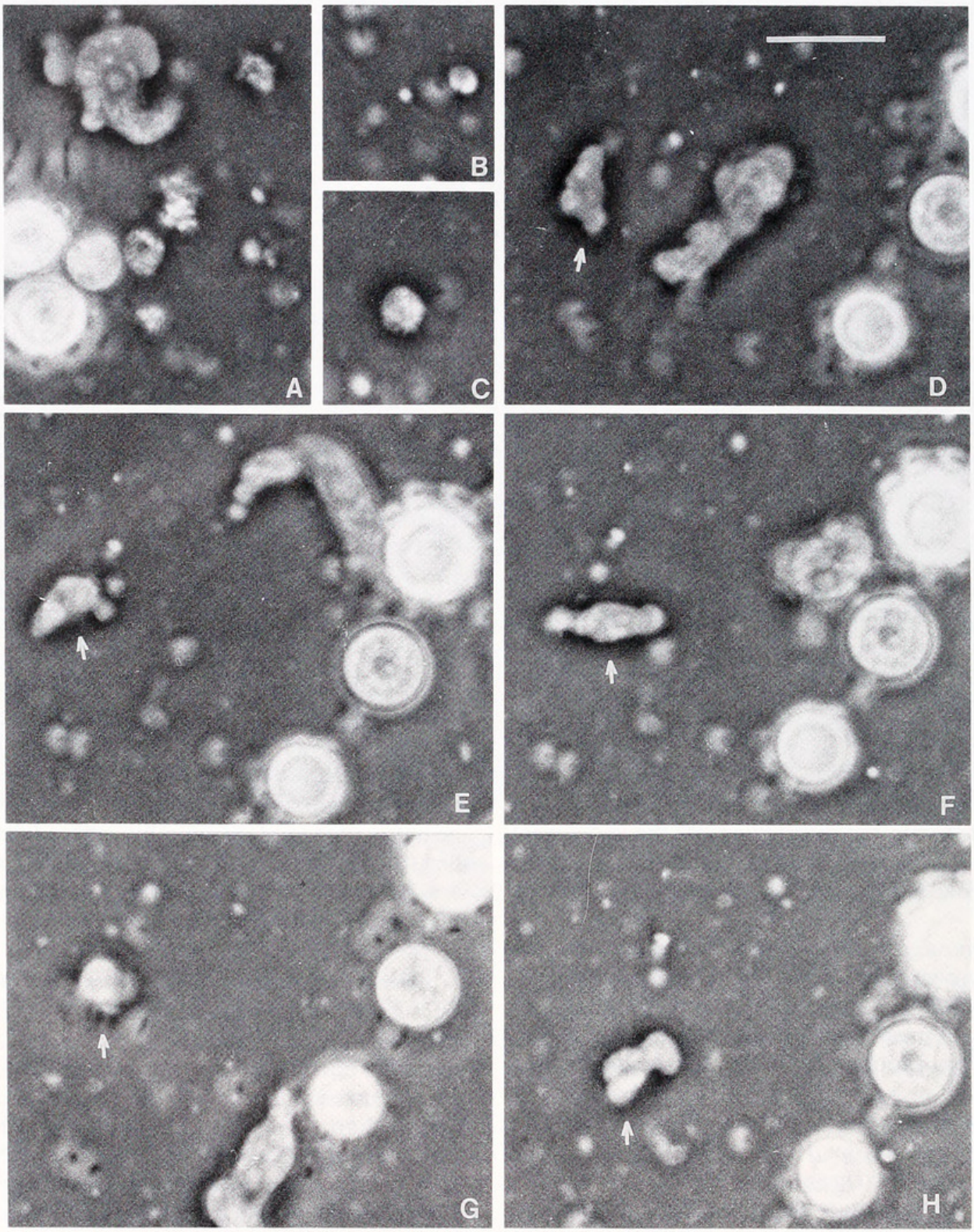

Figure 4. Photomicrographs of live *Paratetramitus jugosus*. A. Amoeba with visible nucleus and several small spherical and irregular-shaped bodies. Cyst in bottom left corner. B,C. Small spherical bodies frequently found in cultures. D–H. Sequential photos showing movement of small amoeba (arrow). The small amoeba closely resembles many other small irregular objects in our cultures. (Compare 4C with 4G) Bar scale = 10 μm.

Coverslips were placed on blotting paper, sample-side up, and flooded with approximately 200 μl of stain solution. A clean glass slide placed over the coverslip was turned over and allowed to stand for one to three hours in the dark. The slide was then blotted dry and sealed around the edges with clear nail polish. Some slide preparations were treated with pancreatic RNase before staining to remove background binding of DAPI to RNA (Coleman *et al.*, 1981). Samples treated with bovine DNase I (Coleman *et al.*, 1981) allowed distinction of stained DNA from

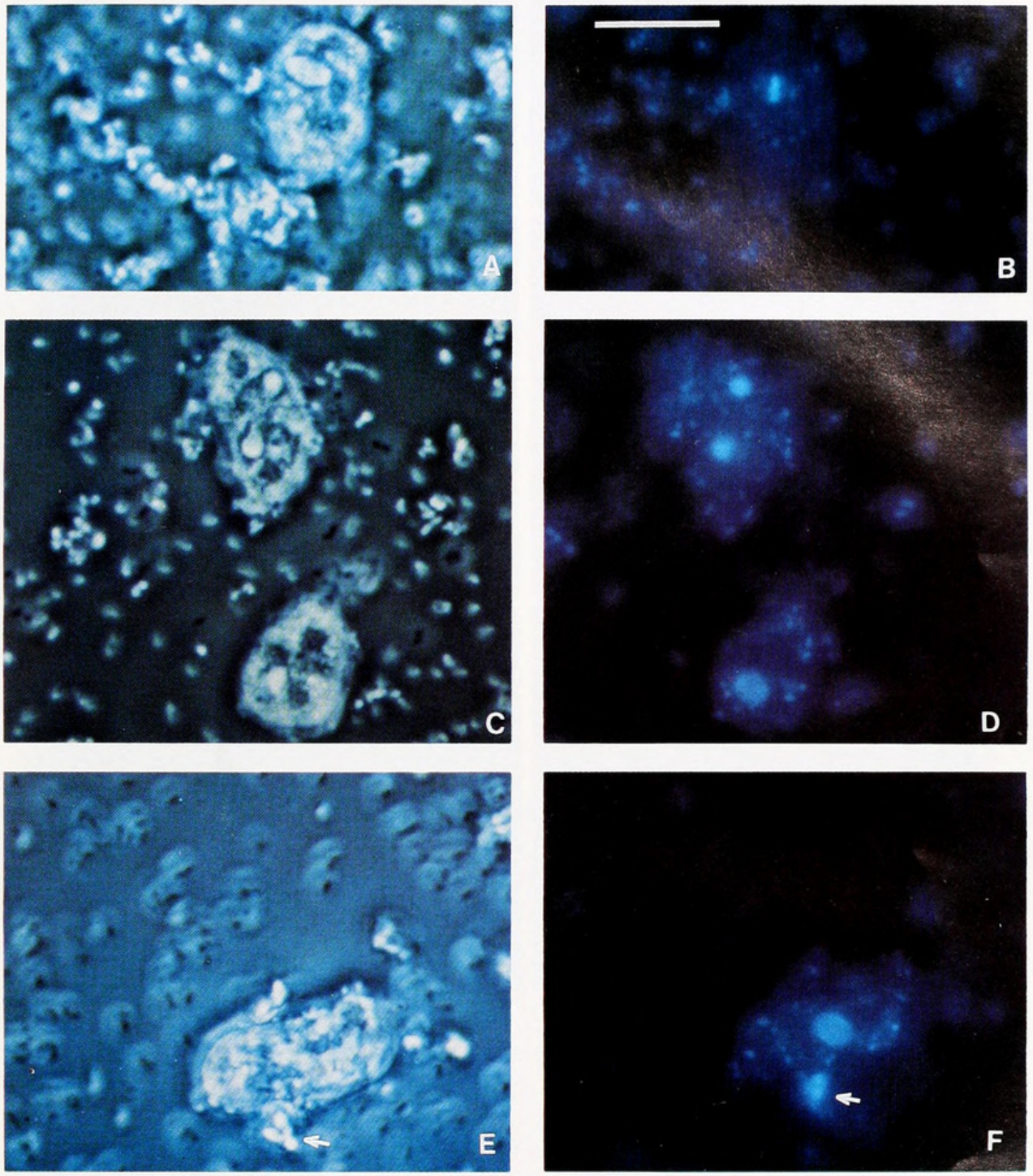

Figure 5. Phase/fluorescence micrographs of DAPI-stained amoebae. A,B. Amoeba with metaphase nucleus. Phase micrograph shows nucleus clearly intact and under fluorescence, DNA is seen condensed in the center. C,D. Amoebae with telophase nucleus. D,F. Cytoplasmic DNA is faintly visible in the amoebae. E,F. Arrow indicates a structure which may be a chromatin body extruded by the amoeba. Its size and fluorescent properties differ from cytoplasmic fluorescence. Bar scale = 10 μm.

nonspecific brightly staining material in the preparations. Unstained amoeba slide preparations were also used as controls to detect and distinguish autofluorescence from authentic DNA staining. Excitation filter sets on the Nikon Fluophot in the UV and blue (approximately 365 nm and 490 nm) were used for epifluorescence observations of DAPI and mithramycin samples, respectively. Photomicrographs were made with a Nikon Microflex AFX photomicrographic attachment. Films used for photomicroscopy were Scotch 640 ASA tungsten film and Kodak Tri-X 400 ASA pushed to 1600.

Electron microscopy

Fixation and embedding of the Cuba strain of P. jugosus. Amoebae inoculated into aqueous suspension (de-

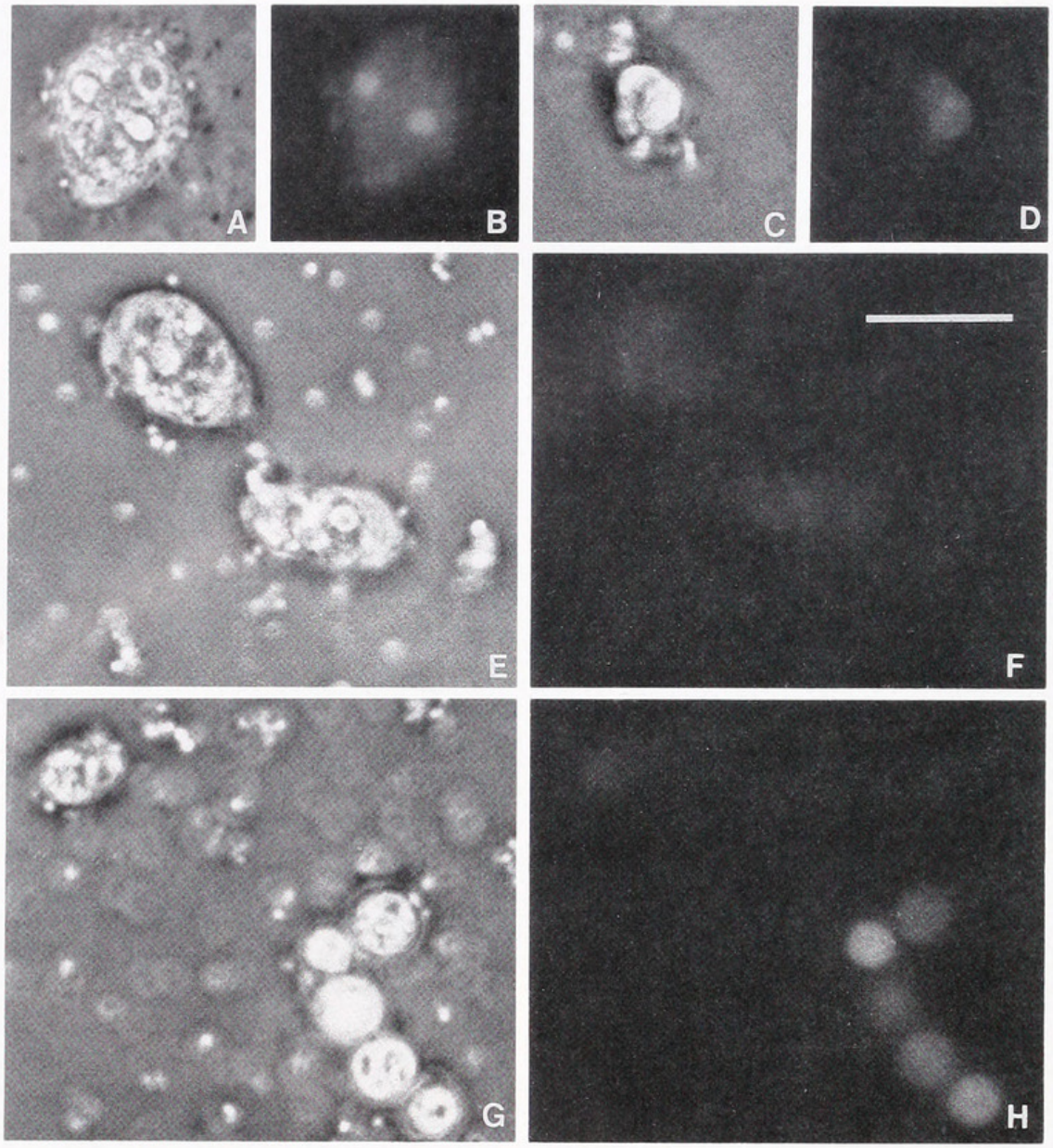

Figure 6. A,B. Phase/fluorescence micrographs of DAPI-stained amoebae with nuclei in telophase. C–H. Phase and fluorescence; Autofluorescence with blue filter (490nm) of small bodies, amoebae, and cysts. Bar scales = 10 μm.

scribed above) were allowed to grow for two days. Several milliliters of distilled water were added to the suspension and 3 ml of this water were transferred to a centrifuge tube to which 0.15 ml of glutaraldehyde was added to make the final concentration of the fixative 5%. After fixation overnight at room temperature and pelleting in a clinical centrifuge, the samples were washed twice in 0.1 *M* cacodylate buffer (10 min each wash). The pellet was post-fixed for 10 min in 2% osmium tetroxide, washed twice with distilled water, and then stained with 0.5% uranyl acetate for 30 min. Samples were then washed with distilled water (10 min) and dehydrated through an ethanol series as follows: 70% (5 min), 80% (5 min), 90% (5 min), 95% (10 min), 100% 3× 10 min. For embedding, the pelleted sample was placed in propylene oxide for 30 min, followed by 30 min in a 1:1 mixture of propylene oxide: Spurr's resin. After transferring to a beam capsule, it was recentrifuged and left in Spurr's for 5 h after which the spent Spurr's was poured off and fresh Spurr's added; the resin was polymerized for 16 h at 70°C.

Mat material from North Pond, Laguna Figueroa, Baja California, was fixed at the site according to the methods of Stolz (1983).

Sectioning and observations. The blocks were sectioned by Floyd Craft (Boston University) on a Porter Blum MT2B ultramicrotome, post-stained with 2% uranyl acetate for 15 min, lead citrate for 5 min, and then observed on a Philips model 410 transmission electron microscope at 20 kV.

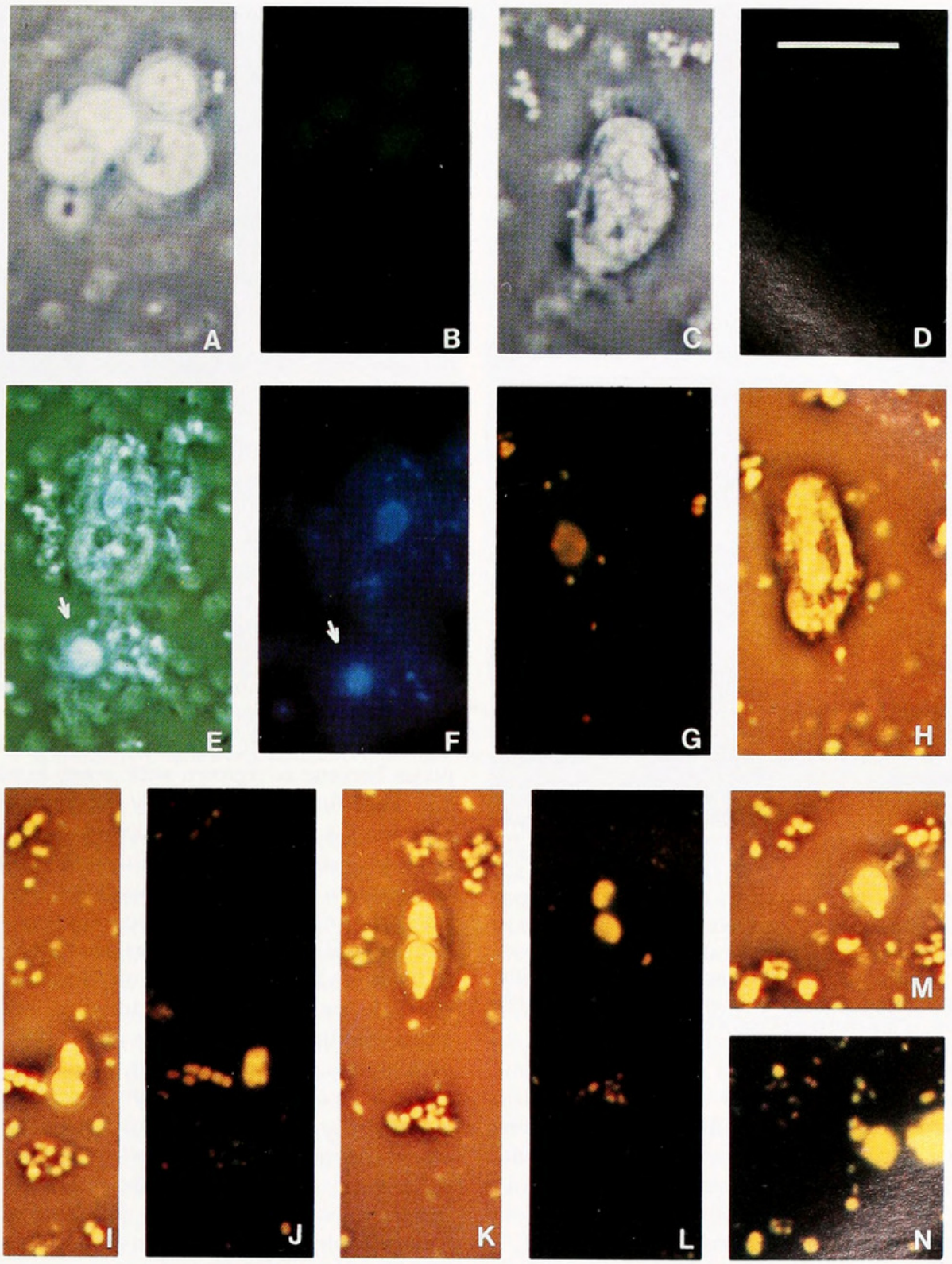

Figure 7. A–D. Phase and fluorescence; DNase treatment of amoebae stained with mithramycin. Nuclear and cytoplasmic fluorescence is completely removed. E,F. Phase/fluorescence; DAPI stained amoeba and associated small spherical body (arrow). Small body has stronger fluorescence than the amoeba nucleus. G,H. Fluorescence and phase; Mithramycin staining clearly showing nucleus of amoeba and possibly other DNA containing organelles inside cell. I–N. Phase and fluorescence; Mithramycin staining of small spherically and irregularly shaped bodies. The fluorescence of these bodies is stronger than the nuclei of the amoebae. Bar scale = 10 μm.

Isoenzyme analysis

Slant cultures of the amoebomastigote isolate from Cuba were sent for isoenzyme analysis to the American Type Culture Collection (ATCC, Rockville, Maryland). Starch gel electrophoretic techniques for isoenzyme patterns were conducted by Tom Nerad (Nerad and Daggett, 1979). The Cuban strain was tested for three isoen-

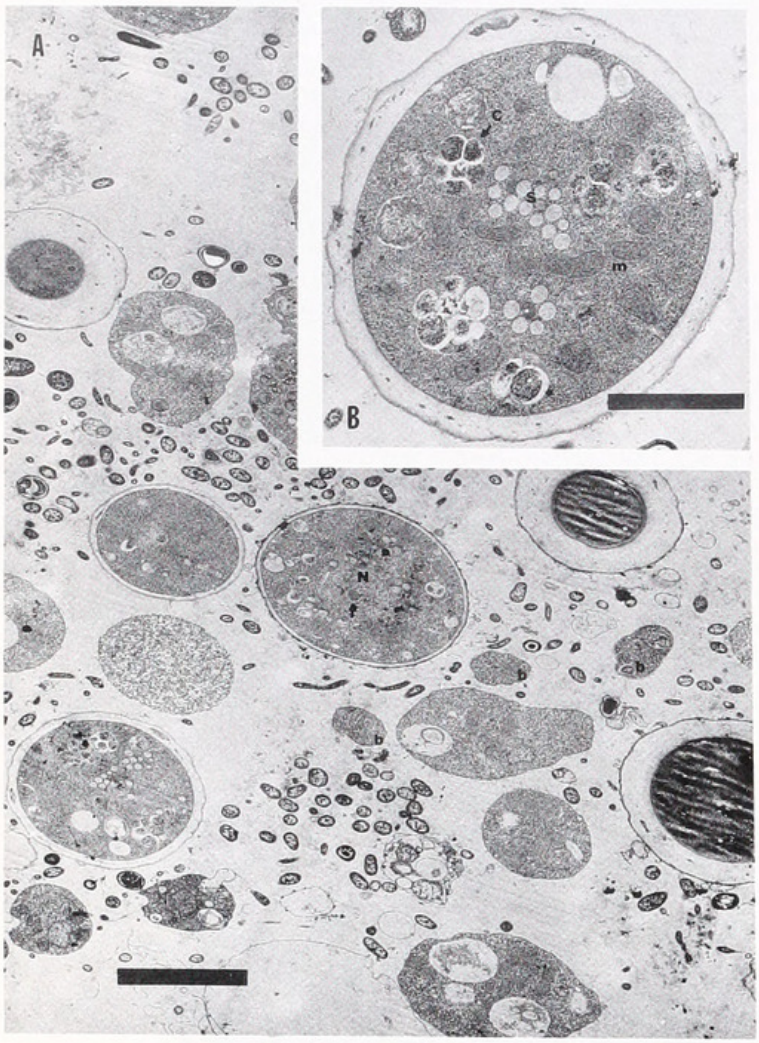

Figure 8. A. Cysts of cultured *Paratetramitus jugosus* in many different stages: from completely desiccated to recently excysting. Arrow indicates a nucleolus in a nucleus (N). Small bodies (b) are also seen which may represent released chromatin bodies or equivalent that develops into small amoebae. Bar scale = 5 μm. B. Cyst showing inner and outer walls, mitochondria (m), storage granules (s), and chromatin body (C) in division (arrow). Bar scale = 2.5 μm. Transmission electron micrograph (TEM) of material from Cuba strain of *P. jugosus*.

zyme systems: proprionyl esterase (PE), leucine aminopeptidase (LA), and acid phosphatase (AP) (Daggett and Nerad, 1983).

Results and Discussion

Field studies

More than eight sets of field studies were undertaken in 1979 and 1980 after the discovery of the extraordinary abundance of small cysts on agar plates designed to detect the presence of algae, cyanobacteria, manganese-oxidizing bacteria, and other organisms. In earlier studies, lack of consistency among samples, presence of nematodes and contamination, especially by an unidentified black manganese-oxidizing fungus, precluded orderly collection of data. The last four sets of observations (May and June 1983; March and August 1988) led to repeatable results (Table II). The appearance of cysts smaller than those of *Acanthamoeba* was taken as putative evidence for *P. jugosus*. Three or four times (by high power light and once by electron microscopy) we verified that the cysts indeed were *P. jugosus*.

Here we summarize the general experience after the eight sets of experiments in which each medium (K, MnAc) was represented by two to four plates per site. *P. jugosus*, or encysting small amoebae indistinguishable morphologically from *P. jugosus*, are invariably present in the laminated sediment when the *Thiocapsa* layer (the red layer, Fig. 2I) is well-developed. *P. jugosus* is usually recoverable from the laminated *Microcoleus* mats such as that depicted in Figures 2E and 2H. *P. jugosus* populations do develop but less frequently in the gypsum mat and black mud samples. They are least frequent in the white gypsum crust.

Our practice now is to collect mats with *Thiocapsa* scums to insure recovery of large populations of healthy *P. jugosus* cysts, amoebae, and in the case of the Cuba samples, amoebae that readily transformed to mastigotes (Fig. 3C).

In order to see abundant populations of *P. jugosus*, plates should be read several times, especially between 10 and 30 days after returning from the field. After this, plates become overgrown with many kinds of bacteria and some fungi as reported by Brown *et al.* (1985). Except for the common presence of *Acanthamoeba*, no other protists have been routinely seen on K plates. It is important to control the quantities of added distilled water, however; if water is too abundant a plethora of encysting ciliates appear that have not been studied. Due to the more limited nutrients on MnAc plates algae (for example the tiny encysting chlorella-like *Mychonastes desiccatus* BROWN, Margulis *et al.*, 1988) may appear on plates incubated in the light.

We have always used two sets of media because more abundant growth of *P. jugosus* (and most other microbes) develops on K plates whereas these amoebae are more easily recognized on the less permissive MnAc plates. We score the presence of *P. jugosus* at a site (e.g., "+" in Table II) only when the small cysts are present on all of the plates of both media.

We assess *P. jugosus* to be a normal component of the microbial mat community whose population develops especially well in the layer dominated by *Thiocapsa*, below *Microcoleus*. Our data are consistent with the interpretation that during the spring rainy season, enormous populations develop and during the hot dry summer when halite and gypsum crystals dominate the mat surface, *P. jugosus* survives by encysting. We have observed excystment in 10–20 min. Upon desiccation, encystment apparently takes place rapidly as well (certainly

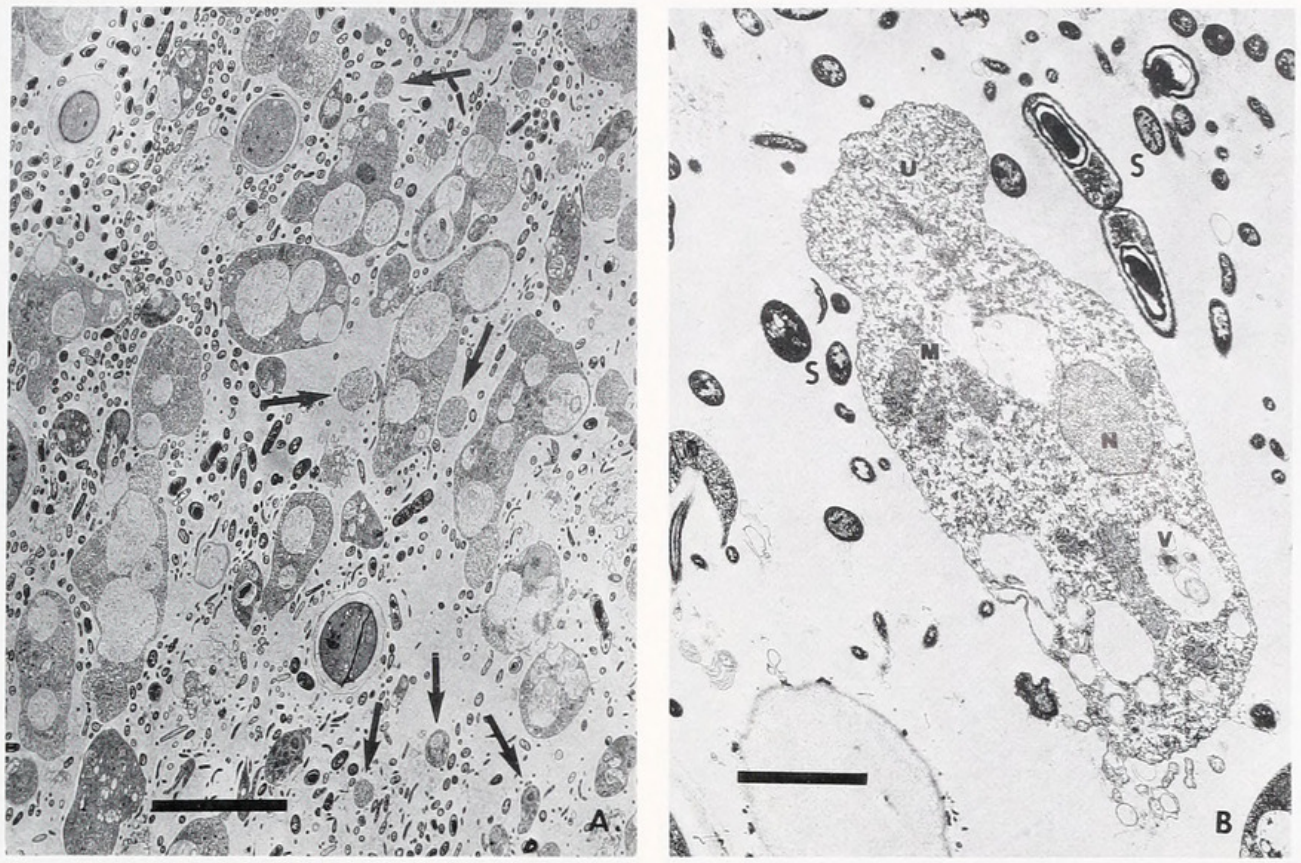

Figure 9. Healthy trophic amoebae, TEMs. A. Highly vacuolated (v) amoebae in a dense population of food bacteria. Small bodies (arrows) could be products of amoebae fission. Bar scale = 10 μm. B. Amoeba with uroid (U), nucleus (N), vacuoles (v) and mitochondria (M); bacterial spores (S). Bar scale = 2.5 μm.

overnight). Because anaerobic bacteria abound in the *Thiocapsa* mat layer, it is also likely that both cysts and amoebae of *P. jugosus* tolerate low oxygen or even totally anoxic conditions. To develop techniques to most reliably detect the greatest number of small cysts on plates by low power microscopic examinations, we compared flooding the agar plates immediately upon collection of the field samples to drying them out entirely before flooding. Because early drying out selects for rapidly growing fungi and spore forming bacteria which may overgrow the amoebae, our best results were with samples flooded with 1 ml distilled water in the field and permitted to dry for the weeks after collection. In conclusion, the optimal collecting conditions for *P. jugosus* involve recognition of small cysts from plated samples of 1-mm cubes of distilled water-flooded *Thiocapsa* layer mat samples. The cysts should be recoverable on both K and MnAc media from 20 to 30 days after return from the field. For further purification to monoprotist cultures see Read *et al.* (1983).

Light microscopy

Culture slides. Observations of live material never revealed amoebae in division. Conditions for normal promitotic amoebic division may not have been favorable under the growth conditions used, yet small bodies were frequently observed (Fig. 4A–H). These bodies were also seen frequently from cultures grown on agar plates with both light and electron microscopy (Fig. 6C, D; 7I–N; 8A; 9A). Many of these small bodies may be pieces of cytoplasm left behind by the amoeba or in EM sections through a small portion of the amoeba, however, on one occasion, a small body was observed budding off a parent amoeba followed by changes in its shape and monopodial movement (Fig. 4D–H) suggesting this body was a small amoeba. We cannot rigorously preclude the possibility that this small body was already present in the culture and that the larger amoeba passed over it, making it look as if it were extruded.

Amoebomastigote transformation. Aqueous suspensions examined 24 h after their preparation were observed to contain both amoebae and cysts. When the distilled water suspension was plated, even six weeks after its preparation, it gave rise to viable *P. jugosus* amoebae and mastigotes; however, whether cysts, mastigotes, or amoebae dominated the suspension was not determined. Amoebae were dominant 24 h after plating, while mastigotes dominated after 48 h. Mastigotes were conspicuous enough in these suspensions to warrant harvesting the cultures for electron microscopic analysis of kinetid structure.

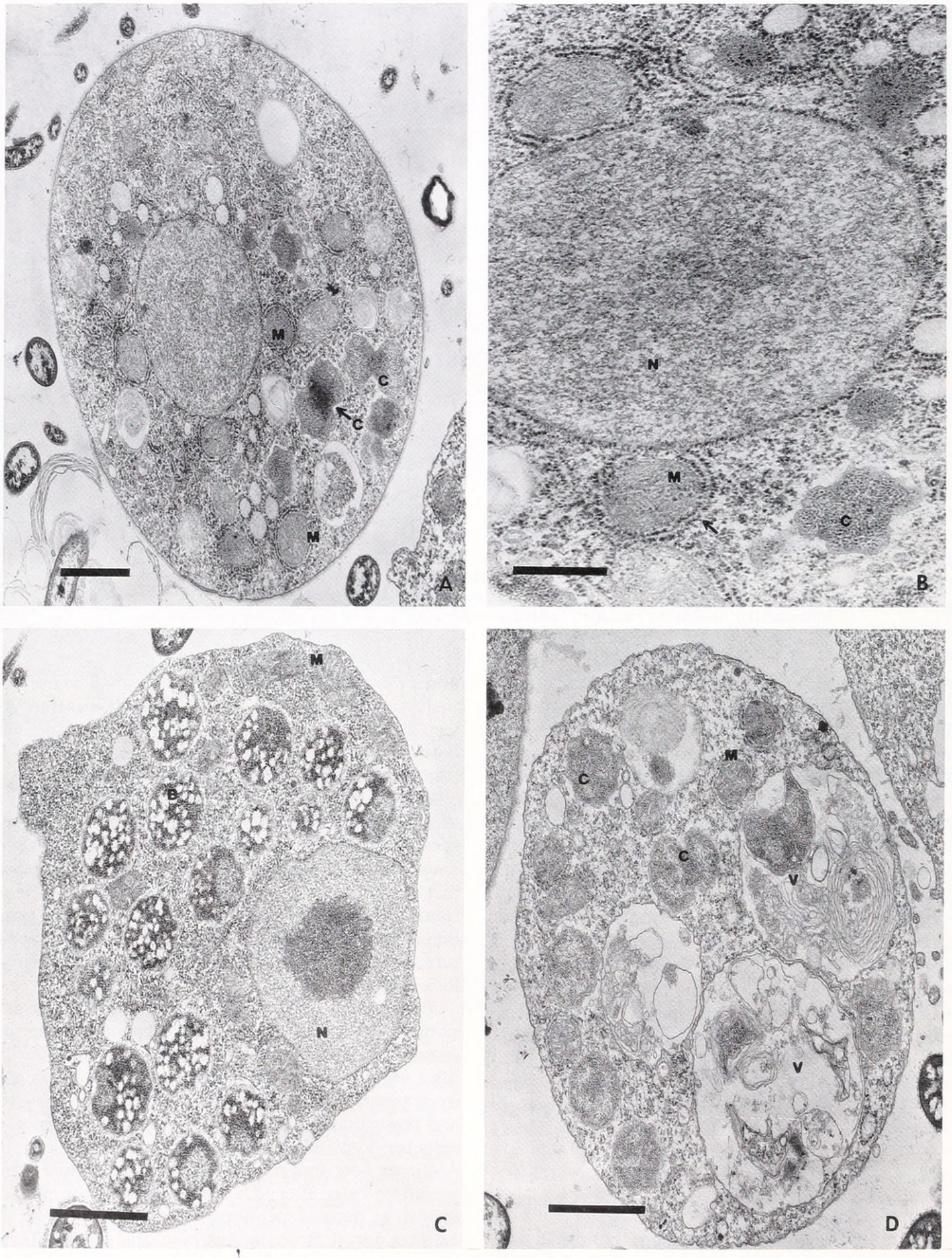

Figure 10. Rounded amoebae, TEMs. A. Dumbbell-shaped chromatin bodies (C) with condensed material (arrow), which are clearly distinguishable from mitochondria (M). Bar scale = 1.0 μm. B. Ribosome-

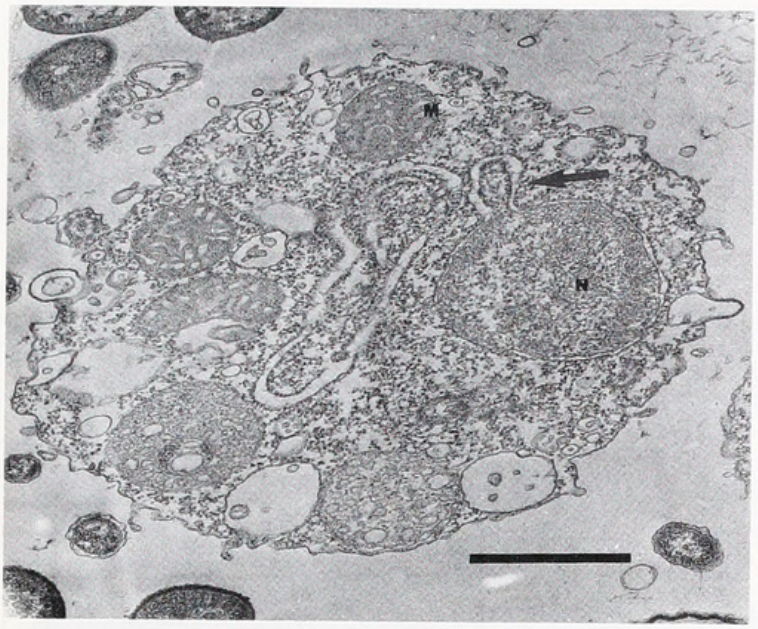

Figure 11. Amoeba with small bleb (arrow) off nucleus (N). Mitochondria (M) are also seen; chromatin bodies are lacking. TEM. Bar scale = 1.0 μm.

Nuclear fluorescent staining. In both DAPI and mithramycin-stained preparations, the nuclei of amoebae were clearly visible with epifluorescence microscopy (Fig. 5; 6A, B; 7E–H). Two stages of nuclear division were revealed: telophase (Fig. 5C, D; 6A, B) and metaphase (Fig. 5A, B). Cytoplasmic DNA was also seen in these preparations although less clearly. DAPI-stained slides tend to show high background fluorescence, making cytoplasmic DNA difficult to see (Fig. 5B, D, F; 7F). This problem has been reported in studies of other protists as well (Coleman *et al.*, 1981). Because mithramycin stains DNA more specifically, preparations made with this stain displayed less background fluorescence (Fig. 7G) and allowed for clear identification of cytoplasmic DNA. Autofluorescence and DNase controls of amoebae showed no nuclear or cytoplasmic fluorescence verifying the presence of DNA (Fig. 6E–H; 7A–D).

Fixed amoeba cultures always contained small irregular and spherically shaped bodies that displayed stronger fluorescence than the nuclei of amoebae, cytoplasmic DNA or the nucleoids of the bacteria upon which the amoebae feed. Strong fluorescence was clearly removed by DNase treatment, indicating that at least some of the small bodies contain DNA. A large number of the small spherical bodies (Fig. 6G, H) and some of the irregularly shaped bodies (Fig. 6C, D) were autofluorescent. Small spherical bodies may be nuclei of ruptured cells or dried-out collapsed cysts. However the amount of autofluorescence in some of the irregularly shaped bodies was insufficient to account for the strong fluorescence seen in stained material (Fig. 7I–N). Several small bodies seen in close proximity to normal-size amoebae (Fig. 5E, F; 7E, F) differ in both extent and intensity from nuclear and cytoplasmic fluorescence: the intensity of fluorescence is greater and the fluorescent structures are intermediate in size between large nuclei and cytoplasmic DNA. Cytoplasmic DNA in DAPI and mithramycin stained preparations is most easily interpreted to be due, at least in part, to mitochondrial nucleoids. Chromatin bodies (described below) detected in electron micrographs are approximately the same size as mitochondria therefore DNA in these structures might also be represented by cytoplasmic fluorescence. However, the more diffuse cytoplasmic DNA (regardless of its origin) stains less brightly and its fluorescence emanates from smaller structures relative to whatever DNA is causing the fluorescence of the five small bodies (as seen e.g., in Fig. 7I–N). Specific correlation between cytoplasmic and the sources of extracellular (*i.e.*, small body) fluorescence could not be made.

The relative abundance of small bodies containing DNA was not measured; attempts to quantify were thwarted by small numbers of irregularly shaped and spherical bodies together in the same field, brightness of the preparation, and variations in population densities of both the amoebae and the bacterial lawns. Quantification would require synchronously grown amoebae with fixation at the same stages of development.

Although their small size precludes definitive identification of these DNA-containing bodies, they may represent the fate of chromatin bodies, *i.e.*, the released "chromidia" reported in the early amoeba literature (Hogue, 1914). We hypothesize that these irregularly shaped bodies represent highly condensed packages of parental DNA that presumably contain information required for the full development of the organism. Although we routinely see irregular bodies in our cultures of *P. jugosus*, including those purchased from the American Type Culture Collection, we have never observed in a single specimen the entire cycle of budding off, followed by development into small and then standard-sized trophic amoebae. Yet such bodies, uncannily like the "buds" Hogue

studded mitochondria (M) adjacent to nucleus (N) with its outer membrane also studded with ribosomes. Chromatin bodies (c) shown at higher magnification. Bar scale = 0.5 μm. C. Amoeba with highly vacuolated bodies which may be a form of chromatin bodies. Mitochondria (M), nucleus (N). Bar scale = 1.0 μm. D. Amoeba with large food vacuoles (v) containing membranous material. Both mitochondria (M) and chromatin bodies (C) are also present. Bar scale = 1.0 μm.

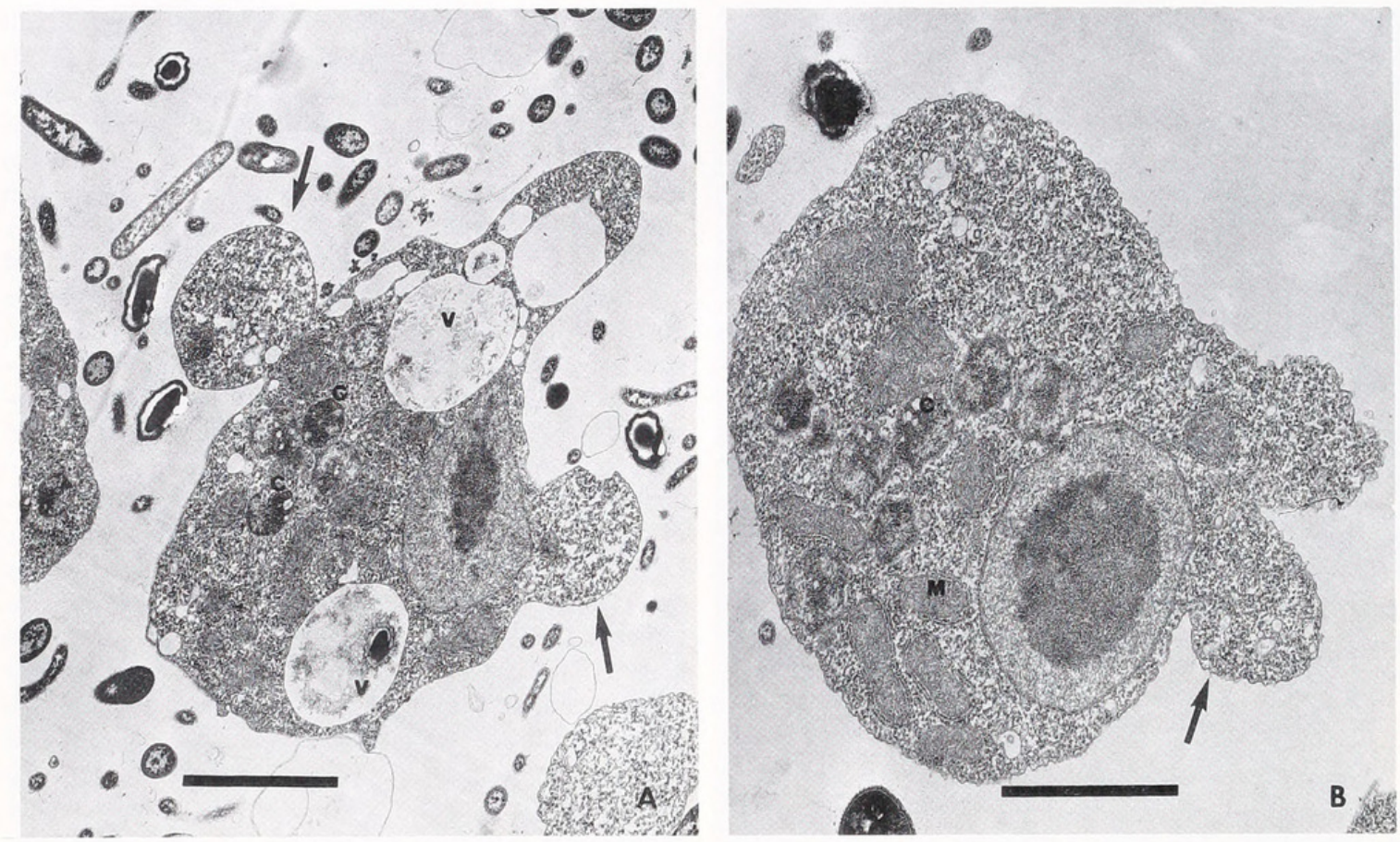

Figure 12. TEMs of amoebae. A,B. Arrows pointing to cytoplasmic buds. Chromatin bodies (C), vacuoles (V) and mitochondria (m) are also visible. Bar scale = A. 2.5 μm, B. 2.0 μm.

(1914) described in larger amoebae parasitic in oysters, are always found if sought in cultures of *P. jugosus*. Promitotic division of amoeba nuclei has also clearly been seen in our preparations (but never in live material) (Figs. 5A–D; 6A, B) suggesting more than one form of reproduction may be responsible for the growth of this organism.

The definitive solution of the problem of multiple fission in this tiny amoeba requires cloning in pure culture of a single isolated 2–4 μm diameter "bud" after the observation of its prior presence in the cytoplasm of the adult amoeba as a chromatin body. Cloning studies coupled with DAPI or mithramycin as a vital stain may eventually resolve the question of nucleus-derived cytoplasmic chromatin bodies which are released as propagules to the medium under conditions of maximal growth on dense, well-fed cultures. Extrusion of these tiny bodies from the nucleus and then from the cell would have to be directly observed to confirm this scenario. Fluorescent DNA stains would facilitate these observations by tracking nuclear DNA.

Electron microscopy

Cell structure and reproduction. Electron microscopy of cultures from Cuban mat samples revealed at least three different forms: cysts, amoebae, and mastigotes. Cysts were roughly spherical with a single, smooth outer layer, 500 nm thick (Fig. 8A,B). Cysts contained a nucleus with a prominent nucleolus, numerous dense mitochondria with tubular cristae surrounded by ribosome-studded endoplasmic reticulum, vacuoles containing various materials (*e.g.*, degrading spore-forming bacteria, stacks or whorls of membranes and unidentifiable structures, most likely degraded remnants of food). They also contain small electron-lucent spheres that may contain storage material (*e.g.*, at least 18 can be seen in Fig. 8B). Other cytoplasmic structures, distinguishable from mitochondria by their lack of cristae, darker staining, amorphous shape, and lack of surrounding endoplasmic reticulum, are here termed chromatin bodies. As indicated by their dumbbell shape, some of these bodies appear to be in division. The combination of these structures is indicative of a metabolically active rather than a dormant cell.

Trophic amoebae characteristic of this species are monopodial ("limax") (Fig. 9). The posterior region of the cell possesses an uroid (Fig. 9B) similar to other isolates of this organism. A more-or-less centrally located nucleus with a prominent nucleolus is seen in many thin sections. The nuclear membrane is surrounded by ribo-

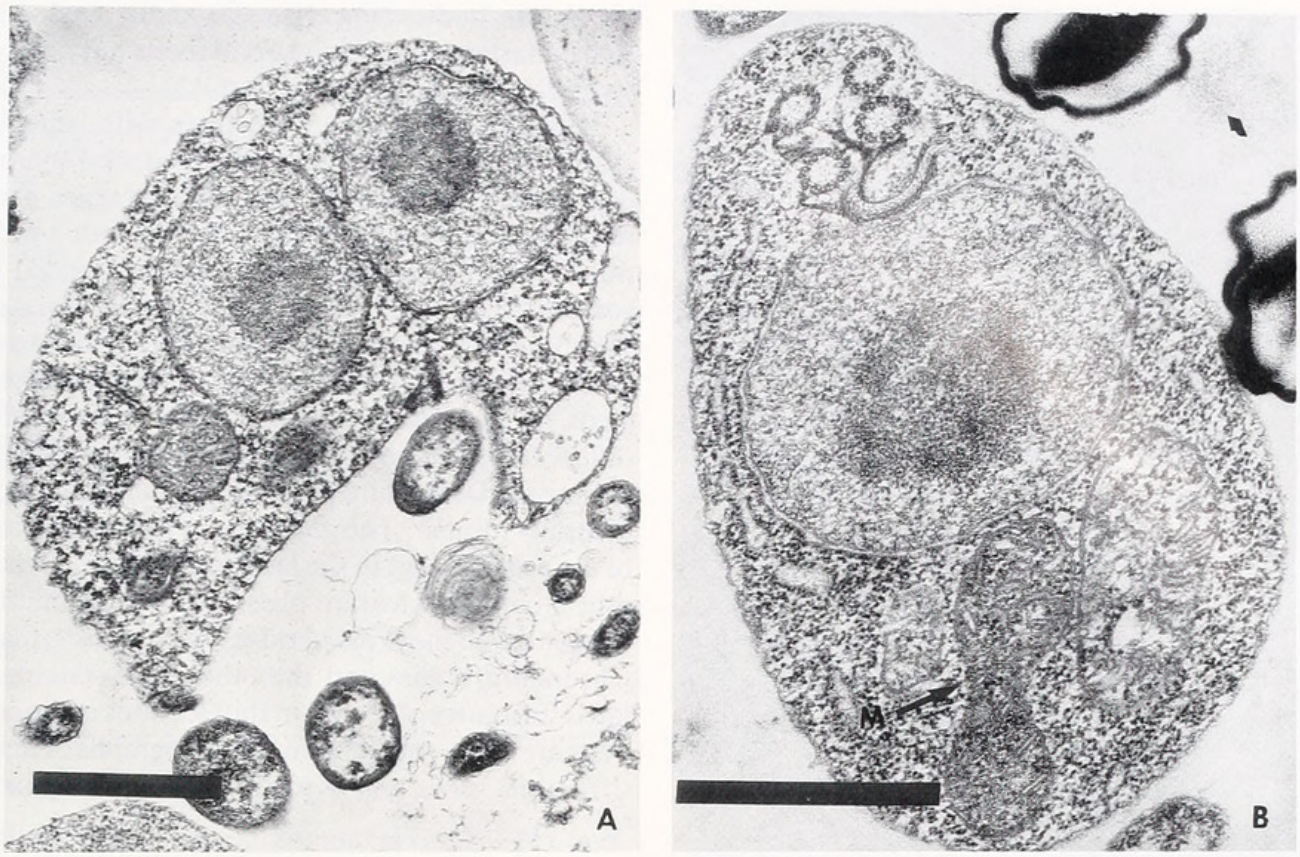

Figure 13. A. Binucleated mastigote with tubules, probably derived from rhizoplast transverse microtubules that extend subcortically (see Fig. 16). B. Mastigote with four [9(2) + 0] kinetosomes showing approach of connecting rhizoplast to the nucleus. Mitochondria (M) interpreted to be in division. A,B. Bar scale = 1.0 μm.

somes (Fig. 10B). Densely staining mitochondria with tubular cristae are often surrounded by ribosome-studded endoplasmic reticulum (Fig. 10A, B, D). Large vacuoles containing bacteria and membranous whorls are often present (Figs. 9; 10D; 17). In some amoebae, these vacuoles occupy a large portion of the cytoplasm (Figs. 9A; 10D; 12A; 17).

Chromatin bodies are almost always found in rounded amoeba, but rarely in amoebae that clearly appear trophic. Rounded amoebae may represent an early stage of encystment in which degeneration of mitochondria to chromatin bodies is caused by the onset of metabolic dormancy. We think this is unlikely because encysted and rounded pre-cyst amoeba always contain both mitochondria and chromatin bodies. Mitochondria including dumbbell-shaped forms (Fig. 11B, 13) are clearly distinguishable from chromatin bodies. Their presence in amoebae, mastigotes, and cysts suggests *P. jugosus* is fundamentally aerobic even though it apparently tolerates high sulfide and anaerobic conditions. Some chromatin bodies contain centrally located, dark-staining masses resembling nucleolar material (Fig. 10A); others are arguably in the process of division (Figs. 8B; 10A, D). Some amoebae contain dark-staining structures that differ from chromatin bodies, *e.g.*, the vacuolated structures of Figure 10C. These vacuolated structures, because they are similar in stain density, size, and number per cell, may represent some developmental form of chromatin bodies. We suggest that these bodies represent "blebs" of nuclear origin containing DNA. Nuclear blebbing was reported by Stevens *et al.* (1980) in *Naegleria*. We also observed nuclear blebs in *P. jugosus* (Fig. 11), although because they are smaller than the chromatin bodies, they may be early developmental stages that then provide the means for their formation. We have not been able to determine at the light microscopic level if any cytoplasmic fluorescent staining comes from these bodies, nor have we done ultrastructural autoradiography for DNA to distinguish these from mitochondria, yet the electron micrographic appearance of the interior of these bodies is that of chromatin (Fig. 10B, D). Our interpretation is entirely consistent with that of the chromatin bodies in oyster parasitic amoebae called "chromidia" (Hogue, 1914).

Cytoplasmic "budding," of interest because of the presence in cultures of numerous small bodies detectable by light microscopy (Figs. 4A–C; 5 E, F; 6C, D; 7E, F, I–M), is found in many sections (Fig. 12). Corresponding small bodies are difficult to identify at the ultrastructural level because the orientation of the amoebae cannot be determined with certainty; however, bodies that may

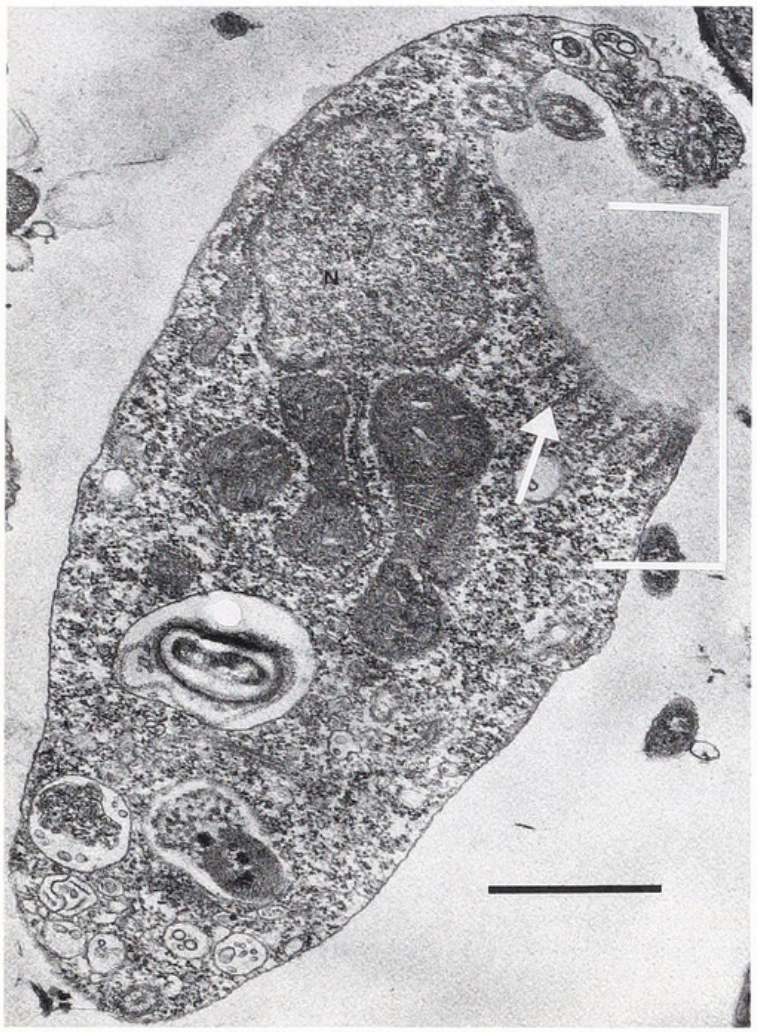

Figure 14. Cortical tubules (arrow) presumably originating from rtm of Figure 16, closely associated with the anteriorly positioned nucleus (N) of mastigote. Bracket indicates "rostral ridge" (rr). TEM. Bar scale = 1.0 μm.

correspond to those observed with light microscopy are depicted (Figs. 8A; 9A). The "buds," at least in Figure 12, are continuous with the cell cytoplasm and probably not an artifact resulting from overlapping amoebae. The "buds" lack both mitochondria and chromatin bodies in this particular micrograph, but this may simply be a function of orientation of this thin section. Because the details of mitotic division remain elusive, the case for multiple fission in this organism is incomplete and we still have no comprehensive view of how the luxuriant, rapid growth of this organism is achieved. The "buds" represent frequently observed oddities; they may or may not represent part of the amoeba reproduction process. If chromatin bodies originate from the nucleus, which is not yet clear, then budding could represent a means of their dispersion.

Although mitosis has never been observed in ultrastructural studies of *Paratetramitus jugosus* amoebae or mastigotes, some events associated with mitosis are evident in thin sections. We have seen dumbbell-shaped chromatin bodies (Fig. 10A, D) and a single binuclear cell: a mastigote in which karyokinesis but not cytokinesis apparently has occurred (Fig. 13).

Mastigotes share many features with amoebae and cysts, including mitochondria surrounded by rough endoplasmic reticulum (RER), large vacuoles, strands of rough endoplasmic reticulum, nuclei with a prominent nucleolus, and nuclear membrane surrounded by ribosomes (Figs. 13, 14). However, the three forms do differ significantly. The nucleus, anteriorly located in mastigotes and attached by rhizoplast microtubules to the nucleus, is in close proximity to the kinetosomes. This differs from previous descriptions of mastigotes in this organism (Darbyshire *et al.*, 1976) in which nuclei were centrally located. The groove seen in the anterior region of the cell adjacent to the kinetid (Fig. 14, 15) may represent a common feature observed in light micrographic studies called the "rostral ridge" of *P. jugosus* mastigotes. Chromatin bodies and the other dark-staining, vacuolated structures observed in thin sections of amoebae and cysts are apparently absent in micrographs of mastigotes.

Kinetid structure. Kinetid structure, reconstructed

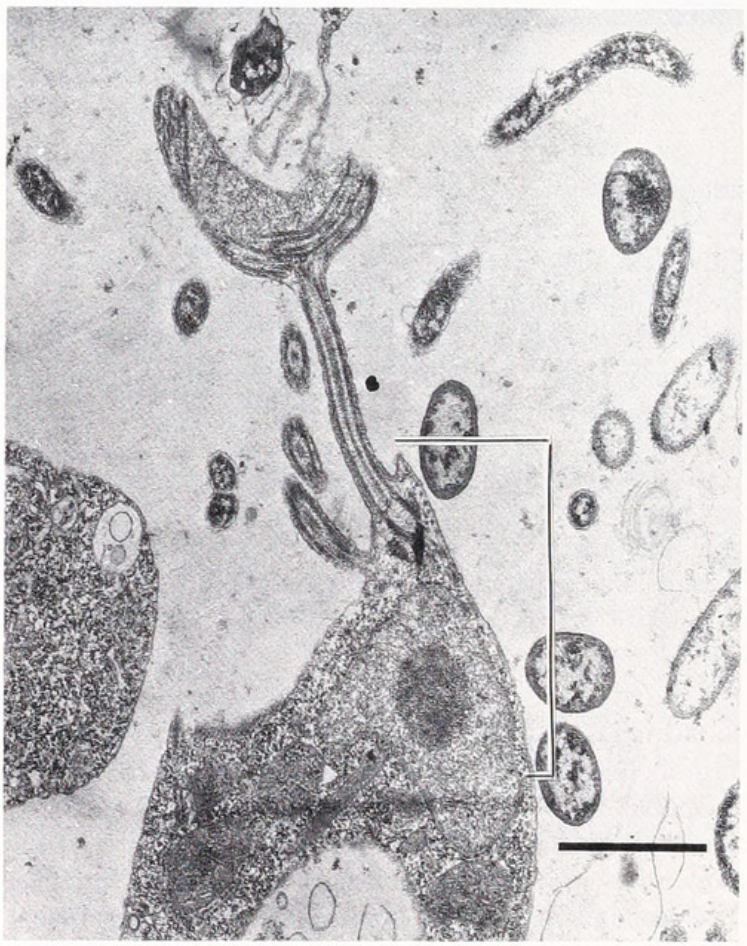

Figure 15. Microtubules of axoneme are seen here in a tangential section, the distal portion of the axoneme is probably resorbing. The cortical tubules from the kinetid are in close association with the nucleus. Bracket indicates "rostral ridge" (rr). TEM of mastigote. Bar scale = 1.0 μm.

Table III

Kinetid structure

Structure or orientation	Abbreviation
Axoneme 1	ax1
Axoneme 2	ax2
Kinetosome 1	K1
Kinetosome 2	K2
Basal sphere	bs
Lateral sphere	ls
Connecting fiber	cf
Nuclear fiber	nf
Rhizoplast	RZ
Rhizoplast microtubules: transverse	rtm
Rhizoplast microtubules: parallel	rpm
Right	R
Left	L
Anterior	A
Posterior	P
Ventral	V
Dorsal	D
Nucleus	n

from numerous electron micrographs (Figs. 13–15), and labeled according to Table III is summarized diagrammatically in Figure 16. The standard [9(2) + 0] kinetosomes (Fig. 13B), and [9(2) + 2] axonemes (Fig. 14) are present in this dikinetid that shows mirror image symmetry. A microtubular rhizoplast containing both parallel and transverse tubules arises between the kinetosomes and runs posteriorly toward the nucleus (Figs. 13A, 14, 15). The parallel kinetosomes are underlain by a basal sphere connected to the nucleus; each possesses a protruding lateral sphere also connected to the nucleus by rhizoplast nuclear fibers (K1/lsnf, K2/lsnf). The mastigote in Figure 15 may either be extending or retracting its undulipodium. Because a large portion of degraded axonemal material is visible with cytoplasm at the distal extremity, resorption of the axoneme is more likely. A row of single microtubules (seen in cross section in Fig. 14 and tangential section in Fig. 15) originates from between the kinetosomes and runs anteriorly and cortically along and past the nucleus. The sheet of microtubules presumably originating from rhizoplast transverse microtubules (rtm, Fig. 16) and supporting the anterior end of the mastigote give the cell the conical appearance that prompted Darbyshire *et al.* (1976) to identify the "rostral ridge." The number of undulipodia, usually two as determined by light microscopy, may vary greatly. While difficult to assess their precise positioning by transmission electron microscopy, four [9(2) + 0] kinetosomes can be seen in a single cell (Fig. 13B).

Amoebae in situ *in a microbial mat.* Although *P. jugosus* is infrequently observed in live mat during dry conditions, it was prevalent throughout the period of freshwa-

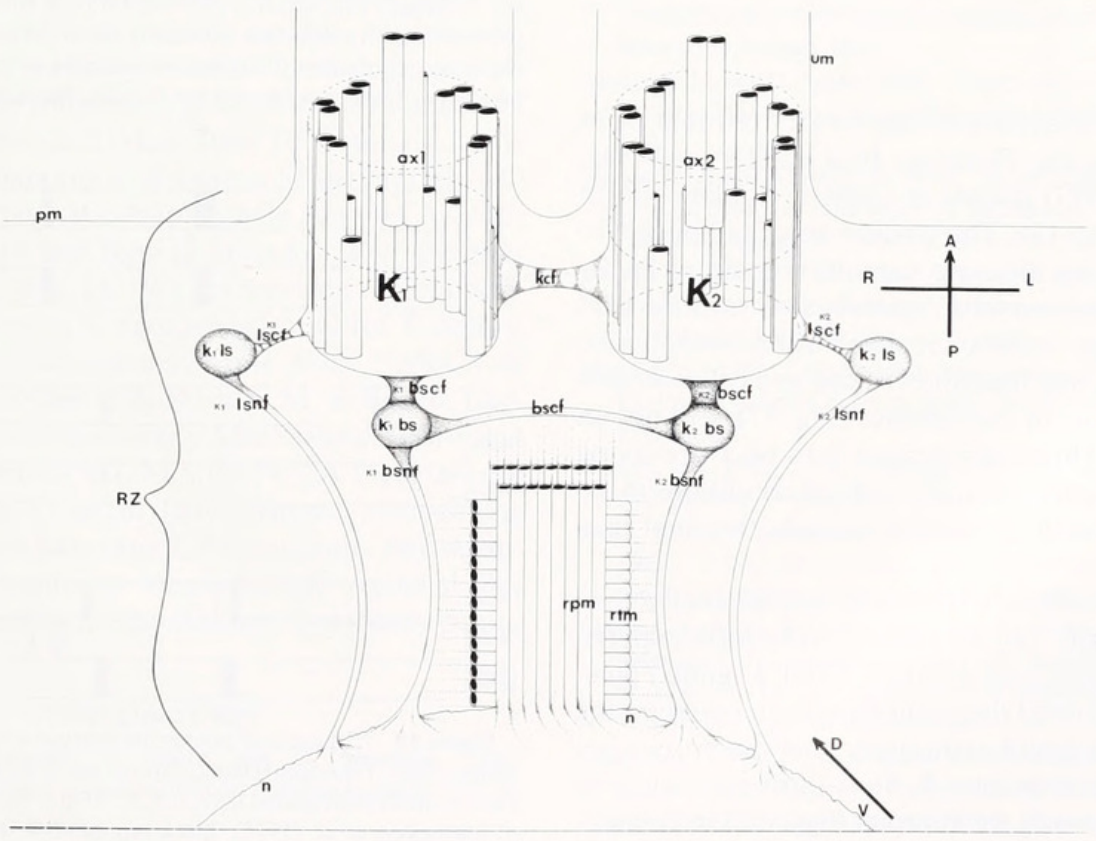

Figure 16. Kinetid structure, diagrammatic representation. See Table III for description of labels. (Drawing by Sheila Manion Artz).

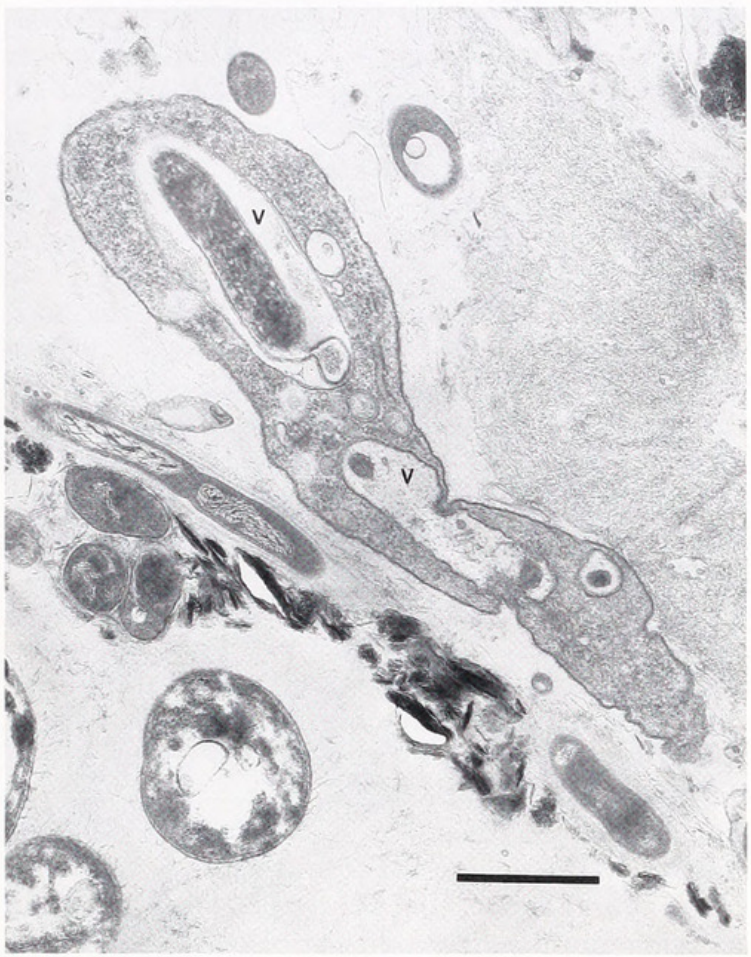

Figure 17. Amoeba from embedded microbial mat with large food vacuoles (v), taken directly from a microbial mat sample at North Pond, Laguna Figueroa, Baja California, Mexico. TEM. Bar scale = 1.0 μm.

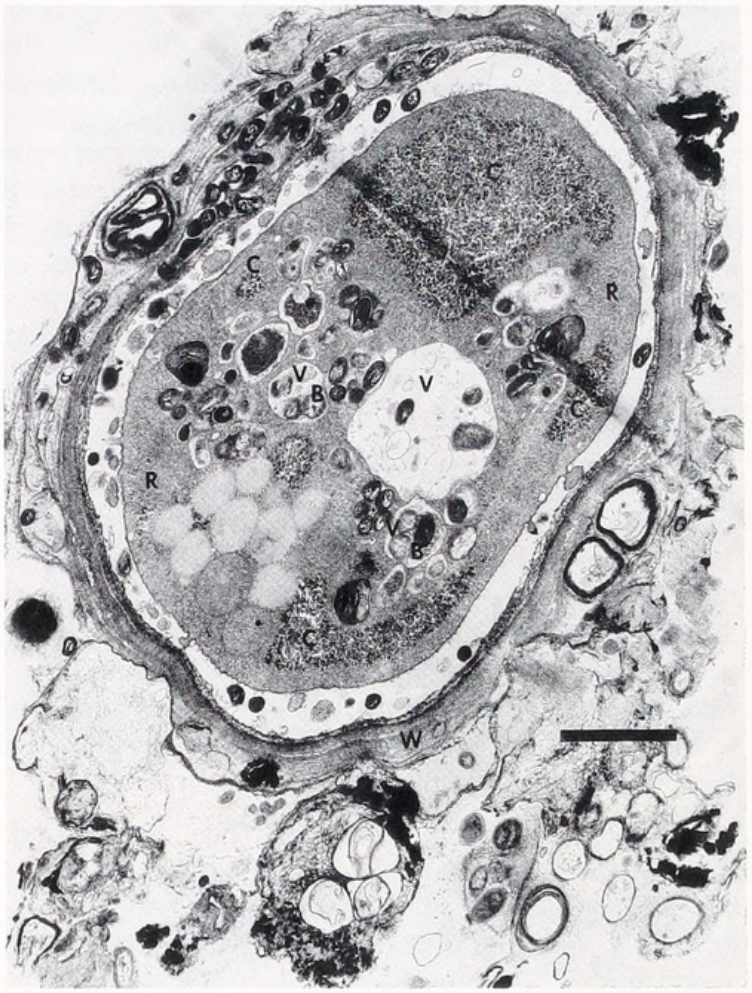

Figure 18. Organism from the *Thiocapsa sp.* layer of the microbial mat from North Pond interpreted to be an ectoplasmic cyst of *P. jugosus*. Although enclosed in a cyst wall (W), the ribosome-studded cytoplasm (R), active digestion of bacteria (B) in vacuoles (V) and the well-developed chromatin (C) indicate metabolic activity in this amoebomastigote. TEM courtesy of John F. Stolz. Bar scale = 2.0 μm.

ter inundation. Electron microscopy of fresh mat from North Pond (Laguna Figueroa, Baja California Norte, taken October 1987) reveals an amoeba similar in form to *P. jugosus* (Fig. 17). The trophic amoeba appears to have uroid and large digestive vacuoles containing bacteria. The only other amoeba regularly seen in these field samples are larger acanthamoebae with acanthopodia. The organism greatly resembles in size and cell structure the limax amoebae in our cultures (Fig. 17); it is not an acanthamoeba. Thus, we interpret it to be a free-living *P. jugosus* photographed under natural conditions in association with purple phototrophic and other mat bacteria.

An encysted amoeba, with at least five areas of active-looking chromatin, can be seen in embedded material from the *Thiocapsa* layer at North Pond, Laguna Figueroa (Fig. 18). Bacterial digestion is evident, emphasizing the rapidity with which temporary encystment can occur. Photographed by John F. Stolz during his study of phototrophic bacteria, we interpret Figure 18 to be a second example of *P. jugosus in situ* in a stratified microbial community. The electron micrograph corresponds to the

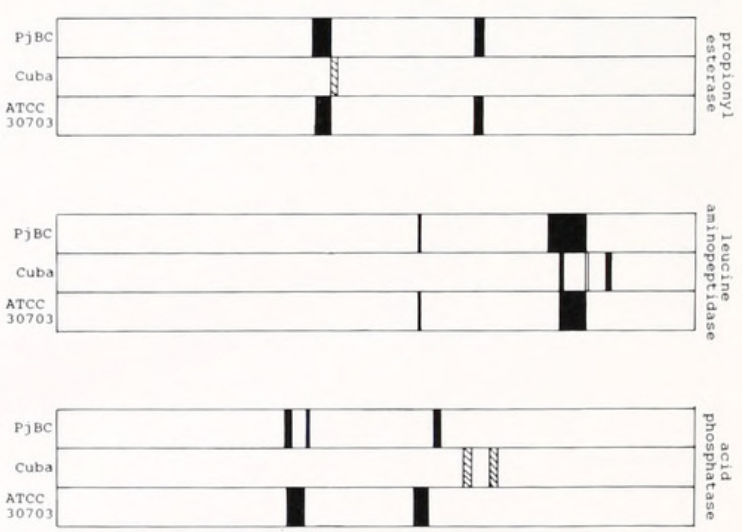

Figure 19. Diagram of isoenzyme analysis of three different isoenzymes. PjBC: the original bajacaliforniensis of Read *et al.* (1983) Cuba: the Cuban strain reported here, ATCC 30703: The standard soil isolate of Darbyshire *et al.* (1976). Black represents a strong band on starch gel electrophoresis, cross-hatching a less conspicuous band, and white represents a very faint band.

"ectoplasmic cysts" reported in light micrographs in Read *et al.* (1983; Figs. 7B–H, 13, and 14H).

Isoenzyme analysis

The results of the stock gel isoenzyme analysis run on amoebae and cysts of the Cuban *P. jugosus* strain compared to the ATCC-type strain (ATCC #30703) and *P. jugosus bajacaliforniensis* are shown in Figure 19. The pattern is unique for any amoebae in the ATCC collection (T. Nerad, ATCC, pers. comm.). The propionyl esterase (PE) and acid phosphatase (AP) each display bands resembling those enzyme patterns present in the Baja California and ATCC *P. jugosus* strains. The three narrow bands of the pattern of leucine aminopeptidase (LAP) of the Cuban strain seem to be unique.

The most abundant protist in the purple phototrophic bacterial layer of microbial mat sediments, *Paratetramitus jugosus* displays a complex life history that correlates with rapid environmental changes in water abundance and salinity. Our evidence for this life history is consistent with Hogue's (1914) assertion that some amoebae reproduce by multiple fission and disperse by "chromidial" propagules.

Acknowledgments

We are grateful to Rene Fester, Gail Fleischaker, Wendy Lazar, Dorion Sagan, and Rae Wallhausser for assistance in manuscript preparation, including cartography. We are especially grateful to Annette Coleman, Floyd Craft, Tom Nerad, and Jane Gifford for their technical assistance with fluorescence staining, electron microscopy, isoenzyme analysis, and field photography, respectively. We are immensely grateful to the members of Earthwatch team 1 & 2 (May, June 1983) for aid in the surveying and mapping of Pentapus Salina and the collection of field data. We thank Sheila Manion Artz for drawing Figure 16 and John F. Stolz for use of his electron micrograph (Fig. 18). We acknowledge helpful suggestions for observing live amoebae from Prof. F. Schuster. Support for this research came from NASA Life Sciences Offices (NGR-004-042 to L.M. at Boston University), the Boston University MacDonald award and The Evolution Fund (to L.M.), the NASA Planetary Biology Internship Program (administered through the Marine Biological Laboratory, Woods Hole, Massachusetts), the University of Massachusetts Amherst Research Trust Funds, and the Richard Lounsbery Foundation.

Literature Cited

Brown, S., L. Margulis, S. Ibarra, and D. Siqueiros. 1985. Desiccation resistance and contamination as mechanisms of Gaia. *Biosystems* **17:** 337–360.

Coleman, A. W., M. J. Maguire, and J. R. Coleman. 1981. Mithramycin- and 4′-6-diamidino-2-phenylindole (DAPI)-DNA staining for fluorescence microspectrophotometric measurement of DNA in nuclei, plastids and virus particles. *J. Histochem. Cytochem.* **29:** 959–968.

Daggett, P. M., and T. A. Nerad. 1983. The biomedical identification of Vahlkampfid amoebae. *J. Protozool.* **30:** 126–128.

Darbyshire, J. F., F. C. Page, and L. P. Goodfellow. 1976. *Paratetramitus jugosus,* an amoebo-flagellate of soils and fresh water, type-species of *Paratetramitus nov. gen. Protistologica* **12:** 375–387.

Gong-Collins, E. 1986. A euryhalic, manganese- and iron-oxidizing *Bacillus megaterium* from a microbial mat at Laguna Figueroa, Baja California Mexico. *Microbios* **48:** 109–126.

Hogue, M. J. 1914. Studies of the life history of an amoeba of the Limax group. *Arch. Protistenkd.* **35:** 154–163.

Margulis, L. 1980. Undulipodia, flagella, and cilia. *Biosystems* **12:** 105–108.

Margulis, L. 1988. Systematics: the view from the origin and early evolution of life. Secession of the Protoctista from the animal and plant kingdoms. Pp. 430–443 in *Prospects in Systematics,* D. L. Hawksworth, ed. Clarendon Press, Oxford.

Margulis, L., E. S. Barghoorn, D. Ashendorf, S. Banerjee, D. Chase, S. Francis, S. Giovannoni, and J. Stolz. 1980. The microbial community in the layered sediments at Laguna Figueroa, Baja California, Mexico: does it have PreCambrian analogues? *Precamb. Res.* **11:** 93–123.

Margulis, L., S. W. Brown, G. Hinkle, H. McKhann, and B. Moynihan. 1988. *Mychonastes desiccatus* BROWN *sp. nova* (Chlorococcales, Chlorophyte)—an intertidal alga forming achlorophyllous dessication-resistant cysts. *Arch. Hydrobiol. Suppl.* **78:** 425–446.

Margulis, L., J. O. Corliss, M. Melknonian, and D. J. Chapman. 1990. *Handbook of Protoctista:* the structure, cultivation, habitats and life cycles of the eurkaryotic microorganisms and their descendants exclusive of animals, plants and fungi. Jones and Bartlett Publishers, Inc. Boston, MA.

Margulis, L., and D. Sagan. 1985. Order amidst animalcules: the Protoctista kingdom and its undulipodiated cells. *Biosystems* **18:** 141–147.

Nerad, T., and P. M. Daggett. 1979. Starch electrophoresis; an effective method for separation of pathogenic and non-pathogenic *Naegleria* strains. *J. Protozool.* **26:** 613–615.

Page, F. C. 1967. Taxonomic criteria for limax amoebae, with descriptions of 3 new species of 3 *Hartmannella* and of 3 *Vahlkampfia. J. Protozool.* **14:** 499–521.

Page, F. C. 1983. *Marine Gymnoamoebae.* Institute of Terrestrial Ecology, Culture Centre of Algae and Protozoa, Cambridge, England. 54 pp.

Raikov, I. B. 1982. The Protozoan Nucleus: morphology and evolution. Springer-Verlag, New York. Pp. 73, 112.

Read, L. K., L. Margulis, J. Stolz, R. Obar, and T. K. Sawyer. 1983. A new strain of *Paratetramitus jugosus* from Laguna Figueroa, Baja California, Mexico. *Biol. Bull.* **165:** 241–264.

Stevens, A. R., J. De Jonckheere, and E. Willaert. 1980. *Naegleria lovaniensis* new species: isolation and identification of six thermophilic strains of a new species found in association with *Naegleria fowleri. Int. J. Parasitol.* **10:** 51–64.

Stolz, J. F. 1983. Fine structure of the stratified microbial community at Laguna Figueroa, Baja California Mexico. I. Methods of *in situ* study of the laminated sediments. *Precamb. Res.* **20:** 479–492.