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Growth of chromidia-forming vahlkampfiid amoebae from Laguna Figueroa, Baja California del Norte, Mexico and Eel Pond, Woods Hole, Massachusetts, U.S.A. under limited oxygen gas conditions

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GROWTH OF CHROMIDIA-FORMING VAHLKAMPFIID AMOEBAE FROM LAGUNA FIGUEROA, BAJA CALIFORNIA DEL NORTE, MEXICO AND EEL POND, WOODS HOLE, MASSACHUSETTS, U.S.A. UNDER LIMITED OXYGEN GAS CONDITIONS

A Thesis Presented

by

MELISHIA I. SANTIAGO-RAMIREZ

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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GROWTH OF CHROMIDIA-FORMING VAHLKAMPFIID AMOEBAE FROM LAGUNA FIGUEROA, BAJA CALIFORNIA DEL NORTE, MEXICO AND EEL POND, WOODS HOLE, MASSACHUSETTS, U.S.A. UNDER LIMITED OXYGEN GAS CONDITIONS

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To my parents for providing all their love and support.
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This research would not have been possible without Professor Lynn Margulis. I thank Professor Margulis for all her useful criticism and her unconditionally support of my work. I also want to thank my committee members, Professor Richard W. Wilkie, Professor Dennis G. Searcy, and our chairman, Professor Lynn Margulis. Thanks also to Professor Piper R. Gaubatz, Dr. Michael Dolan, Christina Arieta, James MacAllister, Celeste Asikainen, Sean Faulkner, Kendra Clark, Alex Salhany, Max DeLong, and other members of the Margulis Laboratory. I gratefully acknowledge the University of Massachusetts, Amherst, Graduate School, Department of Geosciences, the NASA Planetary Biology Internship Program and The Tauber Fund for financial support.
ABSTRACT

GROWTH OF CHROMIDIA-FORMING VAHLKAMPFIID AMOEBAE FROM LAGUNA FIGUEROA, BAJA CALIFORNIA DEL NORTE, MEXICO AND EEL POND, WOODS HOLE, MASSACHUSETTS, U.S.A. UNDER LIMITED OXYGEN GAS CONDITIONS

FEBRUARY 2011

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Directed by: Professor Lynn Margulis

Paratetramitus jugosus, a vahlkampfiid amoebomastigote, was isolated into monoprotist/monobacterial (Bacillus sp.), cultures from laminated microbial mats (Laguna Figueroa, Baja California Norte, Mexico) and muds (Eel Pond, Woods Hole, Massachusetts). Chromidia, roughly spherical (2-4 µm in diameter) were released from both walled spherical cysts (10-12 µm) and phagocytotic amoebic forms. Desiccation-resistant walled chromidia, at first spherical, resorb their walls and develop into small pleiomorphic phagocytotic amoeba. Small amoebae feed and mature into typical monopodial vahlkampfiid adults confirming previous work (Dobell, 1913, and especially the superb analysis of a larger encysting vahlkampfiid amoeba associated with Long Island oyster disease studied at Woods Hole by Mary Jane Hogue, 1914). I show here that P. jugosus reproduces and develops through its life history stages of chromidia, mature monopodial amoebae, and cysts at least as rapidly and abundantly under low oxygen levels as at ambient atmospheric oxygen concentrations. Anoxia was achieved in the laboratory by incubation of entirely desiccated inocula from old mat or mud samples in Brewer jars with or without gas packs to control atmospheric conditions. Three sets of
experiments yielded the same results: vigorous growth on bacillus food occurred on manganese acetate media by two weeks on the surface of agar plates under ambient oxic or hypoxic to anoxic conditions in GasPaks™ with H₂-CO₂ and Anaerobe Container System with Indicator. Preliminary investigations of similar amoeba from geographically distinct field sites in Europe (e.g., Alicante, Delta del Ebro, Spain), in North America (e.g., Cape Cod, Massachusetts, Baja California del Norte, Mexico), and in the Caribbean (e.g., Cabo Rojo, Puerto Rico) were made. From them, I suggest it is likely these coastal amoebomastigotes that propagate by small desiccation resistant, oxygen-independent, manganese tolerant chromidia are genuinely cosmopolitan in its distribution.
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CHAPTER 1
GROWTH OF CHROMIDIA-FORMING VAHLKAMPFIID AMOEBAE FROM LAGUNA FIGUEROA, BAJA CALIFORNIA DEL NORTE, MEXICO AND EEL POND, WOODS HOLE, MASSACHUSETTS, U.S.A. UNDER LIMITED OXYGEN GAS CONDITIONS

Introduction

Many prokaryotes (i.e., bacteria) can survive unfavorable conditions for growth, such as desiccation and extreme temperatures independent of their geographic location. Molecular biological field studies reveal that life on Earth is far more diverse and widespread than previously realized. *Paratetramitus jugosus*, a naked vahlkampfiid amoebomastigote, was isolated into monoprotist cultures. *P. jugosus* is from laminated microbial mats at Laguna Figueroa, Baja California Norte, Mexico and muds from Eel Pond, Woods Hole, Massachusetts.

My biogeographical study of *P. jugosus*, from two different coastal settings was designed to determine the importance of oxygen to growth and survival of these protoctists assumed to be obligate aerobes. The first isolated was taken from microbial mats from a tidal basin at Laguna Figueroa, Baja California Norte, Mexico, where *P. jugosus* exposed to sub-aerial condition was subject to periodic desiccation in 1983. Approximately, half of the time the tide is out, and the evaporite flat covers them while they are suspended in muddy water. The second setting is among mud sediments from Eel Pond, Woods Hole, Massachusetts, U.S.A., which are permanently wet and beneath the surface.

Biogeographers have studied the growth patterns of testate amoebae. Wilkinson (2001) compared testate amoebae assemblages from the Arctic and Antarctic. Species
restricted to the Arctic and Antarctic exhibited sizes up to 230 µm while the largest cosmopolitan species was 135 µm in size (Wilkinson, 2001). Smith and Wilkinson (2007) studied the cosmopolitan distribution of Nebela (Apodera) vas Certes, and concluded that Nebela vas is an example that does not have a cosmopolitan distribution.

Preliminary investigations of similar naked amoeba from geographically distinct field sites in Europe, such as Alicante, Delta del Ebro, Spain and in North America (e.g., Cape Cod, Massachusetts, Baja California del Norte, Mexico), and in the Caribbean (e.g., Cabo Rojo, Puerto Rico) were made. It is likely these coastal amoebomastigotes at propagate by small desiccation resistant, oxygen-independent, manganese tolerant chromidia are genuinely cosmopolitan in its distribution.

**P. jugosus Background**

The analysis of *P. jugosus* starts with the early 20th century identification of chromidia by R. Hertwig and R. Goldschmidt. The classification of *P. jugosus* is described in table 1. Hertwig first suggested and Goldschmidt developed the chromidia hypothesis in 1902 (Margulis, *et. al.*, 1990). Clifford C. Dobell (1909) wrote “…by chromidia I understand any fragments of chromatin-irrespective of their shape or function-which lie freely in a cell without being massed together into a definite nucleus” (Dobell, 1909; Wherry, 1913). Dobell studied chromidia in a description of the life history of *Arachnula impatients* four years later (Dobell, 1913). In 1914, Mary J. Hogue reported how oysters around New York were infected with pathogenic amoebae. The amoebae were named *Vahlkampfia calkensii* (nov. spec.) (Hogue, 1914). The level of infections in the oysters from New York varied from many amoebae to only a few. After studying the digestive tract of the dead oysters, Hogue agreed the amoebae she studied
released chromidia. In 1925, E.B. Wilson also concluded chromidia were “small granules or larger irregular clumps of chromatin or a related substance through the protoplasm without forming a single individualized body (nucleus)” (Margulis et. al., 1990).

V.A. Dogiel and other protozoologists rejected the chromidia hypothesis by 1965. Dogiel concluded that the chromidia hypothesis should be abandoned, and in order to avoid any confusion, this term should not be used. The hypothesis was revived in 1990 (Margulis et. al., 1990). Multiple fission of a mature *P. jugosus* resulted in the production of many small, roughly spherical (2-7 um in diameter) amoebae in laminated microbial mats from Laguna Figueroa. Chromidia are “nucleus derived bodies composed of the same material as chromatin (i.e., nucleoprotein complexes comprising eukaryotic chromosomes) and capable of producing new cells – or at least new nuclei” (Margulis et. al., 1990). The original chromidia hypothesis for reproduction of amoebae was to be an explanation for the rapid growth of *P. jugosus* with no documented mitotic stages.

Dobell’s study of amoeba reproduction accurately described *P. jugosus* life history. Margulis and her colleagues were able to compare Dobell’s light micrographs drawings of *A. impatients* with *P. jugosus*. *A. impatients* differed from *P. jugosus* mainly by its larger size (Margulis et. al., 1990).

Chromidia (2-4 µm diameter propagules) are released from both walled spherical cysts (10-12 µm) and amoebic forms (Margulis et. al., 1990). Parent amoebae released offspring membrane-bounded chromidia that become walled quickly upon desiccation. The chromidia, at first spherical, developed into small pleiomorphic phagocytotic amoeba. Small amoebae matured into typical monopodial vahlkampfiid adults
confirming the work of Dobell, 1913, and especially the superb description of the
vahlkampfiid amoeba disease associated with the Long Island oyster studied at Woods
Hole by Mary Jane Hogue (1914). Because of these findings, I tested the hypothesis that
*P. jugosus* reproduced and developed through its life history stages of chromidia and
mature monopodial amoebae and cysts at least as rapidly and abundantly under low
oxygen levels as at ambient atmospheric oxygen concentrations. Anoxia is achieved in
the laboratory by incubation of inocula from mat or mud samples in Brewer jars with or
without gas packs to control atmospheric conditions. The results of the three sets of
experiments were the same: vigorous growth on bacillus food – manganese acetate media
in two or three weeks on the surface of the agar plates under ambient oxic compared to
H$_2$ - CO$_2$ hypoxic to anoxic conditions.

**Biogeography of *P. jugosus* in this Study**

The samples used in this experiment are from the North Pond flat laminated
microbial mat at Laguna Figueroa, Baja California del Norte, Mexico (Bahia San
Quintín) and from the School Street Marsh Street just under the bridge adjacent, Eel
Pond, Woods Hole, Massachusetts (Figure 1). The salt pond in Woods Hole is a 24-acres
of salt pond that receives excess nitrogen inputs from the surrounding places (e.g., septic
systems). On the other hand, the Laguna Figueroa site is a lagoon at Bahia San Quintín
on the Pacific Fog Coast 200 km south of the Mexican-United States border (Stolz,
1985). This hypersaline lagoon is 16 km long and 2–3 km wide with a salt marsh and the
evaporite flat separated from the ocean by a barrier dune, salt marshes, and beach (Stolz,
1985). Recent flooding events have had a dramatic effect on the composition of the mats.
The first study site, Laguna Figueroa, is at latitude 30º 40' N, longitude 116º 00' W (Enzien et. al., 1989). At this latitude, precipitation is greater than evaporation (Rohli and Vega, 2008). The weather of Laguna Figueroa is warm all year around, but this area has a surplus of water from the months of December to April. The annual average of precipitation in this area is 1667.4 millimeters (mm) (Matsuura et. al., 2009). In contrast, the second study site, Eel Pond, Massachusetts, is at latitude 41º 31’ 32” N, longitude 70º 23’ 16” W, and here evaporation is greater than precipitation (Teal, 1996; Rohli and Vega, 2008). This area has strong winter seasons and warm summers. A surplus of precipitation occurs every year from the months of March to May. The annual average precipitation in this area is 453.0 mm (Matsuura et. al., 2009).

In the case of Laguna Figueroa, during the winters of 1978–79 and 1979–80, severe floods submerged the stratified communities of Laguna Figueroa mats beneath 1–3 meters of meteoric water and buried the laminated sediment under 5–10 cm of siliclastic and clay sediment (Enzien et. al., 1989). These flooding events dramatically changed, although temporarily, the composition of the mats in many ways. The intertidal region where the mats are abundant experienced rapid changes in salinity – (from meteoric water < 0.001 ppt thru 3.4%; sea water to dry crystalline sea salts; from fresh water to complete desiccation; from bright sunlight to darkness; from exposure to coastal winds and fog to no-wind calm waters). Below the mat surface, anoxic organic black muds are found. The mat communities easily survived such dramatically changing variables.
**Materials and Methods**

**Field Sites**

Microbial mat samples used for this experiment were collected from Laguna Figueroa (LF) by Dr. Lynn Margulis and her colleagues in 1983 (see Figure 1). The mud samples from Eel Pond (EP) were collected in the summer of 1991 by Thomas Teal, a graduate student of Dr. Margulis.

The (LF) microbial mat samples were collected using the bottom half of a Petri plate to core out a 2-3 cm deep circle of mat including all stratified layers. Samples were sealed with Parafilm® and labeled to transport back to the lab. Total dry (LF) microbial mat samples have been in the lab since 1983. The (EP) mud samples have been in a Winogradsky column in the lab since they were collected in 1991. The column is maintained by adding distilled water and kept on the lab bench next to the window.

**Enrichments**

The inoculation procedure for the two samples was done in the sterilizing hood. Approximately, $1 \text{mm}^3$ of mat sediment sample was cut and transferred directly at the center of the thin poured sterile enrichment manganese acetate media plate (Table 2). Immediately, 1 ml of distilled water was added directly on the dry sample. This suspends the organisms and initiates feeding growth reproduction of *P. jugosus* (Read *et. al.*, 1983; Enzien *et. al.*, 1989).

*Bacillus* sp. was the major food source for *P. jugosus* (Read *et. al.*, 1983). The morphological characteristics of these possible bacillus colonies were shiny, moist, circular, smooth, raised, and pink. These physical characteristics are similar to colonies of *Bacillus anthracis* (Leboff and Pierce, 2005). After bacillus is exposed to harsh
environments (e.g., extreme temperatures), bacillus forms an endospore which can be dormant for long periods of time (Priest, 1993).

The medium used to grow *P. jugosus* is MnAc and is described in table 2. The medium used to grow *Bacillus* sp. was modified K medium also described in table 2. The materials include agar plates with thinly poured sterile enrichment manganese acetate media were used for inoculation. The (EP) samples in order not to enrich for the desiccation-resistant amoebae and not have a plethora of uninterpretable contaminates needed to be completely desiccated before they were used for inoculation. A desk fan was used to speed up the process. Bunsen burner, spatula, distilled water, and Parafilm® were also used during the inoculation process.

**Gases**

The goal for this project was to set up one set of manganese acetate plates and inoculate them with samples from (LF) and (EP). Inoculated manganese acetate plates were placed on a lab bench at room temperature (25ºC) while other sets of plates were placed under low oxygen gas conditions and anoxia to limit the exposure to oxygen at 25ºC. Other conditions were constant (e.g., temperature, light during the day and dark at night). Low oxygen gas conditions were maintained by GasPak™ EZ Campy Container System H₂ + CO₂. Anoxia was also maintained by GasPak™ EZ Anaerobe Container System with Indicator. The indicator is a white tablet for anoxic conditions, but it changes to blue under aerobic conditions. The plates exposed to low oxygen gas conditions and anoxia were inoculated immediately following the preparation of the manganese acetate medium. The water that was used to inoculate the plates was also sterilized via autoclave.
**Counting Amoebae, Chromidia, and/or Mastigotes**

The amoebae, chromidia, and/or mastigotes were counted from limited oxygen gas condition and anoxia in BD Falcon Integrid dish with grids 150 x 25 mm. The same procedure was repeated with the same organisms from the plates on the lab bench exposed to ambient oxygen concentrations typical of the atmosphere. This procedure was done to quantify the amoebae, chromidia, and mastigotes, and compare the number of organisms exposed to limited oxygen gas condition with those grown at ambient oxygen conditions (Page, 1983).

**Bacillus sp. as Food for P. jugosus**

Bacillus adheres to the amoeba cysts and/or chromidia. Read *et. al.*, (1983) reported bacillus colonies growing on MnAc media.

Samples of (EP) chromidia plates that had been inoculated previously, and they were divided into four groups. The idea for this procedure is to boil bacilli and kill all organisms, including *P. jugosus* and its chromidia. One part of the medium is placed in a test tube and boiled in a water bath for 18-20 minutes. Immediately, the nearly-melted medium is placed on a fresh manganese acetate plate and placed on the lab bench at room temperature and under low oxygen gas conditions.

**DAPI Stain**

DAPI, a fluorescent stain that binds strongly to DNA, passes through the cell membrane and can be used to stain both live and fixed cells. Previous DAPI stain tests have been done by Margulis *et. al.*, 1990 to determine that chromidia contain DNA. This stain is used to test the organisms observed in low oxygen conditions.
200 µl of distilled water were put in an Eppendorf tube by using an Eppendorf pipette. With a flamed spatula, cysts and chromidia were removed from an inoculated plate and inserted in the Eppendorf tube. 100 µl of DAPI stock solution was added to each Eppendorf tube. Each Eppendorf tube was centrifuged for 1 minute at slow speed. After each tube was centrifuged to separate the cells, 20 minutes passed to allow the cells to form visible pellets before a sample was taken from each tube. 100 µl were put on a slide with a cover slip and observed under the microscope.

**Flow Cytometry**

Flow cytometry is a technique for counting and examining microscopic particles, such as cells, by suspending them in a stream of fluid and passing them by an electronic detection/beam apparatus. The flow cytometer is able to physically sort cells and organisms based on their properties, physical characteristics as size, shape, and density. The idea was to isolate *P. jugosus* after isolating pure bacillus colonies for food. As soon as *P. jugosus* was isolated, samples of the organism were taken to the flow cytometer facility located in Room 68, ISB (Integrated Science Building) Building, at UMass Amherst.

The procedure to grow *P. jugosus* was done in the sterilizing hood. 200 µl of distilled water were put in an Eppendorf tube by using an Eppendorf pipette. A flamed spatula was used to remove *P. jugosus* and *Bacillus* sp. from a plate and put them in an Eppendorf tube. The same procedure was repeated for each sample. The Eppendorf tubes were centrifuged for six minutes, so the cells were suspended and form visible pellets. The Eppendorf tubes were then taken to the flow cytometry facility for analysis.
Light Microscopy Observations

A Nikon Diaphot TMD inverted microscope was used to observe the plates with bright field. A Nikon Labophot light microscope was used to observe samples and organisms placed on slides. A Digital HD Sony Video Digital Camera Recorder Handyman HDR-HC9 was also used to take pictures and films from both microscopes.

Results

Limited Oxygen Conditions

The experimental results show that *P. jugosus* reproduces well under low oxygen gas conditions (Figures 2b and 3b). The data collected indicates that *P. jugosus* grows at a higher rate as at ambient atmospheric conditions (Table 3). Molecular studies are required to confirm any new species.

Anoxic Conditions

MnAc media supported the growth of *P. jugosus*. *P. jugosus* tolerated anoxic conditions for three months or as long as water was available (100% distilled and autoclaved water). No fungi or any other contamination was reported growing under anoxic conditions. *P. jugosus* is an aerobic protist able to tolerate anaerobic conditions. Spherical cysts developed in drying Petri plates cultures (Figures 2c and 3c). Cysts measure 10-15 µm in diameter. The age of the cysts can be determined by looking at the outer layer surrounding the cysts. The thicker the outer layer, the older the cyst is. Whether cysts form as mitotic or meiotic products, it is unknown.
**External Morphology**

*P. jugosus* ranges in size from 25-30 µm in length and 3-5 µm in width. The amoeboid movement of *P. jugosus* in low oxygen gas conditions is the same than at ambient oxygen atmospheric conditions (Figures 2a, 3a, 2b, and 3b).

The size of *P. jugosus* in anoxic conditions is the same (25-30 µm in length and 3-5 µm in width) as in limited oxygen gas conditions (Figures 2b, 3b, 2c, and 3c). However, the amoeboid movement of *P. jugosus* in anoxic conditions is slower than at ambient oxygen atmospheric conditions. This is because *P. jugosus* feeds from bacillus spores in anoxic conditions instead of bacillus colonies that grow in ambient atmospheric oxygen concentrations and low oxygen conditions. *Bacillus* sp. are aerobic. *P. jugosus* was observed in mastigote stage in two occasions under anoxic gas conditions.

**Bacillus sp.**

*Bacillus* sp. grew exceptionally well on K medium (Figure 4). Bacillus colonies grow on K medium in 24 hours. Bacillus colonies were not able to grow on MnAc media as vigorously as on K medium (Figure 4a). This figure shows the left plate with visible bacillus colonies growing on K medium while the right plate shows no visible colonies growing on MnAc. The bacillus colonies were pink, moist, circular, smooth, and raised. These physical characteristics are similar to colonies of *Bacillus anthracis* (Leboff and Pierce, 2005).

A Gram Stain Test was performed, and the bacilli growing in the lab are Gram-Positive (Figure 5). The morphological characteristics of bacilli under the Nikon Labophot light microscope were rod shaped from 3-5 µm in length. After searching the literature, these bacilli are very similar to *Bacillus anthracis* (Priest, 1993).
Bacillus sp. plates were placed in BD GasPak™ EZ Campy Container System (H₂ + CO₂) jar and GasPak™ EZ Anaerobe Container System with Indicator jar. The results show that bacillus colonies grew in the control jar as it was expected (Figure 4b). Also, bacillus colonies grew under low oxygen gas conditions (Figure 4c). The bacillus colonies had the same morphological characteristics as the ones growing in the control jar.

Bacillus sp. did not grow well on K medium under anoxic conditions (Figure 4d). Small white dots were visible under anoxic conditions, but no pink bacillus colonies were observed after 24 hours. This is because only bacillus spores grew under anoxic gas conditions. The plates were taken from the anoxic jar and put on the lab bench for 48 hours, but no pink bacillus colonies were observed.

**DAPI Stain**

This stain was used to test organisms in low oxygen gas and anoxic conditions, and *P. jugosus* contains DNA. Previous DAPI stain tests have been done by Margulis *et al.*, 1990 to determine that chromidia contain DNA. *P. jugosus* was stained from low oxygen and anoxia gas conditions (Figure 6). Cell structures were stained and were visible under the microscope.

**Flow Cytometry**

The flow cytometer was able to sort *P. jugosus* by its physical characteristics. None of the samples tested in the flow cytometer were stained. The flow cytometer results are in figure 7. The x-axis indicates the size of the cell while the y-axis shows the granularity of the cells or how complex the cells are.
The scattered plot graph for (LF) control samples show the parent population (in red with 10,000 cells) concentrated in the middle with similar size (Figure 7a). These are walled cysts and \textit{P. jugosus}. The green population indicates \textit{Bacillus} sp. colonies. The graph for (LF) low oxygen gas conditions samples, show an increase in size in the cells but with a smaller parent population. This is because the population size in this sample was only 4,320 cells. The anoxia (LF) graph shows a smaller population but similar in size than in the previous graph. This was expected since only bacillus spores are available for \textit{P. jugosus}. The y-axis or granularity of the cells is not dramatically different for each graph.

The (EP) scattered plot graph for control samples shows a sparse population (in red) since only 6,070 cells were tested, but they ranged in size (Figure 7b). These cells are walled cysts and \textit{P. jugosus}. The green population shows the \textit{Bacillus} sp. to distinguish them from the rest of the cells. The (EP) low oxygen gas conditions graph shows \textit{P. jugosus} with a bigger parent population of 10,000 cells, and \textit{P. jugosus} has a bigger size than in the previous graph. The anoxia graph shows a smaller population in size since only 6,406 cells were detected, but this was expected since \textit{P. jugosus} is only feeding from bacillus spores. The granularity of the cells does not differ much in each graph. The \textit{Bacillus} sp. graph helps to visualize the bacillus size and granularity in each graph (Figure 7c).

\textbf{Discussion and Conclusion}

In spite of differences in latitudes and climatic conditions, \textit{P. jugosus} is tolerant of low oxygen gas conditions and anoxia from both geographic locations. The climate of Laguna Figueroa is warm all year around, and at this latitude, precipitation is greater than
evaporation. Half of the time, the tide is out, and the evaporite flat covers in while they are suspended in muddy water. On the other hand, the mud sediments from Eel Pond are under water all the time. The climate at this latitude has strong winter seasons and warm summers. Where Eel Pond is located, evaporation is greater than precipitation.

The size of *P. jugosus* was not affected as oxygen decreased from both geographical locations. The amoeboid movement of *P. jugosus* was affected as oxygen decreased from both Laguna Figueroa and Eel Pond samples. Based on these results, I conclude that the amoeboid movement of *P. jugosus* is slower because *P. jugosus* was only feeding from bacillus spores instead of bacillus colonies like it is the case when bacillus colonies are exposed to ambient atmospheric conditions or low oxygen gas conditions.

*P. jugosus* was able to feed from bacillus colonies in low oxygen gas conditions, and from bacillus spores in anoxic conditions. *Bacillus* sp. only grew on K medium and not on MnAc media since this medium was not supportive for bacillus to grow.

It is important to test these results in other geographic locations. Future experiments include growing amoebae found in microbial mat samples from Salinas de Cabo Rojo, Puerto Rico. My pre-experiments show that the amoebae are also tolerant and reproduce under low oxygen and anoxic gas conditions. The amoebae observed are larger (30-40 µm) in size than *P. jugosus* and they move much faster. Another experiment would be to stain *P. jugosus* (from Laguna Figueroa and Eel Pond) and bacillus colonies and test them in the flow cytometer to see more details of the cells.
Table 1 Classification of *P. jugosus* (Margulis and Chapman, 2010)

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<th>Protoctista</th>
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</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Amoebomastigota</td>
</tr>
<tr>
<td>Class</td>
<td>Heterolobosea</td>
</tr>
<tr>
<td>Order</td>
<td>Schizopyrenida</td>
</tr>
<tr>
<td>Family</td>
<td>Vahlkampfiidae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Paratetramitus</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>jugosus</em></td>
</tr>
</tbody>
</table>
Table 2 Manganese acetate (MnAc) 0.2% media for *P. jugosus* growth

0.1 g MnAc or 0.14 g of MnAc x 4H₂O

500 mls *Artificial sea water (ASW)*

7.5 g Agar

*Artificial Sea Water*

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ x 2H₂O</td>
<td>1.45 g</td>
</tr>
<tr>
<td>MgSO₄ x 7H₂O</td>
<td>12.35 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.75 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>17.55 g</td>
</tr>
</tbody>
</table>

**Tris Buffer**

(1.0M, pH 7.5, 50 ml)

Distilled water 950 ml

**Tris Buffer**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl</td>
<td>33.3 ml</td>
</tr>
<tr>
<td>Tris base</td>
<td>60.55 g</td>
</tr>
</tbody>
</table>

Bring to 500 ml with DI water

Make sure the pH is 7.5

Plates: The plates used are Fisher brand plastic plates 100 x 15 mm.

Inoculation: A Bunsen burner spatula, distilled water, and Parafilm are used during the inoculation process.

***Modified K medium for bacillus growth:***

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnAc x 4H₂O</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Bacto-peptone</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Agar</td>
<td>7.5 g</td>
</tr>
<tr>
<td>ASW</td>
<td>500 ml</td>
</tr>
</tbody>
</table>


**Table 3** Growth of *P. jugosus* in limited oxygen gas conditions and anoxia. Samples are from Eel Pond (EP) and Laguna Figueroa (LF) plates. These results indicate the number of mature amoebae per plate.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Days after inoculation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
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<td>8</td>
<td>8</td>
</tr>
<tr>
<td>13</td>
<td>24</td>
<td>32</td>
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</tr>
<tr>
<td>14</td>
<td>24</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>21</td>
<td>24</td>
<td>32</td>
<td>12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>(LF) Control Plates</th>
<th>(LF) Gas Phase (H$_2$+CO$_2$)</th>
<th>(LF) Anoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Days after inoculation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>24</td>
<td>16</td>
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<tr>
<td>7</td>
<td>24</td>
<td>56</td>
<td>4</td>
</tr>
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<td>16</td>
<td>16</td>
<td>8</td>
</tr>
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<td>120</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>21</td>
<td>96</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>
**Figure 1** Maps of Field Sites


b. Laguna Figueroa (LF) and Pentapus Salina from Baja California del Norte, Mexico. Enzien *et. al.*, (1983).
Figure 2 *P. jugosus* from Eel Pond (EP)

  a. *P. jugosus* in control samples, (400x, phase contrast).

  b. *P. jugosus* under low oxygen gas conditions (200x, phase contrast).

  c. *P. jugosus* in anoxia (200x, phase contrast).
Figure 3 *P. jugosus* from Laguna Figueroa (LF)

a. *P. jugosus* control samples (200x, phase contrast).

b. *P. jugosus* samples under low oxygen gas conditions (200x, phase contrast).

c. *P. jugosus* samples in anoxic conditions (200x, phase contrast).
**Figure 4** *Bacillus* sp.

a. Bacillus growth on K medium vs. MnAc media after 24-hours.

b. Bacillus colonies in control jar after 72 hours.

c. Bacillus colonies in low oxygen gas conditions (BD GasPak™ EZ Campy Container System $H_2 + CO_2$) after 72 hours.

 d. No visible pink bacillus in plates with K medium under anoxic conditions 72 hours later.
**Figure 5** *Bacillus* sp. Gram Stained

a. *Bacillus* sp. under the microscope (1000x, phase contrast) in oil-immersion.

b. *Bacillus* sp. rod-shaped (1000x).

c. Gram-Positive *Bacillus* sp. (1000x).
**Figure 6 DAPI Stain**

a. Eel Pond (EP) limited oxygen gas conditions (H$_2$+CO$_2$)
b. Eel Pond (EP) anoxia
c. Laguna Figueroa (LF) control limited oxygen gas conditions (H$_2$+CO$_2$).
d. Laguna Figueroa (LF) anoxia.
Figure 7 Flow Cytometry

a. Laguna Figueroa control, limited oxygen gas conditions (H₂+CO₂), and anoxia
b. Eel Pond control, limited oxygen gas conditions (H₂+CO₂), and anoxia
c. Bacillus sp.
a.
b.

Specimen 001-EP Control

Specimen 001-EP H2 Thresh 200

Specimen 001-EP Anoxia
BIBLIOGRAPHY


