DEVELOPMENT OF A PROTEIN-FREE CHEMICALLY DEFINED CULTURE MEDIUM FOR THE PROPAGATION OF THE OYSTER PATHOGEN *PERKINSUS MARINUS*

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Summary :

In the present study we describe a protein-free, chemically defined culture medium, designated JL-ODRP-3, which supports the propagation of *Perkinsus marinus*, a parasite of the eastern oyster, *Crassostrea virginica*. *P. marinus* adapted rapidly to the defined medium and the growth rate of the protozoan increased significantly following a few subcultures. Two isolates of *P. marinus*, one from the Chesapeake Bay (Virginia) and the other from the Gulf of Mexico (Texas) were cultured for at least ten passes. The doubling times for the isolates from Virginia and Texas, in log phase, were 18 ± 1.2 and 28.6 ± 3.2 hours respectively, after ten passes in JL-ODRP-3. Moreover, *P. marinus* cells cultured in the defined medium were infective to eastern oysters. Finally, the defined medium was used successfully to initiate continuous cultures of *P. marinus* from heart fragments of infected oysters. The absence of proteins and peptides from this chemically defined medium demarcates JL-ODRP-3 as the most suitable medium to study *P. marinus* proteins, to produce antigens for antibody production, and to screen chemotherapeutic agents.

KEY WORDS : Perkinsus marinus, protozoan, *in vitro*, protein-free chemically defined culture medium, oyster parasite, *Crassostrea virginica*.

MOTS CLÉS : Perkinsus marinus, protozoaire, in vitro, milieu de culture défini et sans protéine, parasite d'huître, Crassostrea virginica.

Résumé : Développement d'un milieu de culture sans protéine et de composition définie pour la propagation d'un pathogène d'huitre, *Perkinsus marinus* (Apicomplexa)

Dans cette étude, nous avons décrit un milieu de culture, désigné JL-ODRP-3, sans protéine et dont la composition est définie, qui permet la propagation de Perkinsus marinus, un parasite de l'huître américaine, Crassostrea virginica. Perkinsus marinus s'est adapté rapidement à ce milieu de culture défini et le taux de prolifération de ce protozoaire a augmenté d'une manière significative suite à quelques sous-cultures. Deux isolats de P. marinus, l'un de la baie de Chesapeake (Virginie) et l'autre du golfe du Mexique (Texas) ont été cultivés et repiqués au moins dix fois en sous-culture. Les temps de générations pour les isolats de Virginie et du Texas, en croissance logarithmique, étaient de 18 ± 1,2 et de 28,6 ± 3,2 heures respectivement, après dix passages dans le milieu de culture JL-ODRP-3. Des cellules de P. marinus cultivées dans le milieu défini étaient capable d'infecter des huîtres américaines. Finalement, le milieu défini a été utilisé pour l'initiation de cultures continues de P. marinus à partir de fragments de cœurs d'huîtres infectées. L'absence de protéines et de peptides dans ce milieu défini présente JL-ODRP-3 comme le milieu le plus propice à l'étude des protéines de P. marinus, à la production d'antigènes pour la création d'anticorps, et à la détection d'effets d'agents chémothérapeutiques.

INTRODUCTION

The protozoan *Perkinsus marinus* causes heavy losses in oyster populations of the Atlantic and Gulf coasts of the United States (reviewed in Burreson and Calvo, 1996; Ford, 1996; Soniat, 1996). This oyster pathogen was discovered in the Gulf of Mexico in 1948 (Mackin *et al.*, 1950) and named *Dermocystidium marinum* as it was thought to be a fungus. Since Perkins (1976) showed that the motile stage of *P. marinus*, the zoospore, contained an apical complex, the parasite was renamed and included in the phylum Apicomplexa (Levine, 1978).

Following four decades of trials to culture *P. marinus*, it was recently demonstrated that *P. marinus* could be propagated *in vitro* (reviewed in La Peyre, 1996). The culture medium used for the first isolation of *P. marinus*, JL-ODRP-1, consisted of more than 60 ingredients combined to resemble the known composition, osmolality, and pH of plasma of bivalve mollusks (La Peyre *et al.*, 1993). This medium was supplemented with purified fraction V of bovine serum albumin (BSA), cod liver oil, and yeastolate ultrafiltrate (10 kDa). The use of animal serum was avoided to eliminate the effects of undefined complex mixtures on *P. marinus* (Barnes and Sato, 1980; Maurer, 1992). In order to identify and purify proteins of *P. marinus*, BSA was eliminated from JL-ODRP-1 and culture conditions were optimized (La Peyre and Faisal, 1996). Using this protein deficient medium, multiple parasite extracellular proteins including serine proteases were identified (La Peyre *et al.*, 1995).

The BSA-free JL-ODRP-1 medium, however, contains the undefined ingredients yeastolate and cod liver oil,

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both of which could interfere with a variety of experiments. For example, peptides in the yeastolate ultrafiltrate can potentially inhibit the uptake of certain chemotherapeutic agents (Kerridge and Vanden Bossche, 1990). The presence of yeastolate ultrafiltrate in the culture medium has also induced polyclonal mitogenesis in mouse splenocytes, thus interfering with the development of monoclonal antibodies against *P. marinus* extracellular proteins (Faisal *et al.*, 1996). In this study we report the composition of the protein-free, chemically defined culture medium, JL-ORDP-3. We also provide evidence that JL-ORDP-3 supports the propagation of established *P. marinus* cell lines, is suitable for the initiation of primary cultures of *P. marinus* from naturally infected oysters, and that following many subcultures in this medium, *P. marinus* remains infective to eastern oysters.

MATERIALS AND METHODS

MODIFICATIONS
OF THE CULTURE MEDIUM JL-ORDP-1

The protein-free chemically defined medium was modified from JL-ORDP-1 medium (La Peyre *et al.*, 1993) by elimination of BSA, yeastolate ultrafiltrate and cod liver oil, by increasing the amino acid and vitamin concentrations and by adding a chemically defined lipid solution and vitamin B_{12}. A detailed procedure to prepare this chemically defined medium, designated JL-ORDP-3, is provided below and its final composition is given in Table I. The pH of the chemically defined medium was 7.4 and osmolality was 754 (± 4) mOsm/kg as measured with a 5,500 vapor pressure osmometer (Wescor Inc., Logan, UT). No adjustment in the pH or osmolality of JL-ORDP-3 was necessary.

Ingredients	mg/L	Source*
Major inorganic salts and buffers		
Calcium Chloride Anhydrous	199.8	S
Magnesium sulfate Anhydrous	3,371.3	S
Magnesium Chloride Anhydrous	2,031.3	S
Potassium Chloride	574.6	S
Sodium Chloride	15,973.3	S
Sodium Bicarbonate	2,000.0	S
HEPES	5,957.5	S
Trace elements		
Boric acid	0.4	S
Sodium Bromide	0.686	S
Sodium Fluoride	0.2799	S
Strontium Chloride Hexahydra	17.77	S
Cupric Sulfate 7H$_2$O	0.00249	S
Ferrous Sulfate 5H$_2$O	0.834	S
Zinc Sulfate 7H$_2$O	0.1438	S
Manganese Sulfate H$_2$O	0.0000338	G
Sodium Metasilicate 9H$_2$O	0.0284	G
Molybdic Acid 4H$_2$O (Ammonium)	0.0000248	G
Ammonium Metavanadate	0.000117	G
Nickel Chloride 6H$_2$O	0.0000238	G
Stannous Chloride 9H$_2$O	0.0000226	G
Amino Acids		
L-Arginine .HCl	63.2	G
L-Cystine	12.0	G
L-Glutamine	50.0	G
L-Histidine HCl H$_2$O	21.0	G
L-Leucine	26.2	G
L-Isoleucine	26.26	G
L-Lysine HCl	36.26	G
L-Methionine	7.56	G
L-Phenylalanine	16.5	G
L-Threonine	23.8	G
L-Tryptophan	3.1	G
L-Tyrosine	18.0	G
L-Valine	23.4	G
L-Alamine	108.9	G
L-Asparagine H$_2$O	15.0	G
L-Asparctic Acid	13.3	G
L-Glutamic Acid	14.7	G
L-Glycine	57.5	G
L-Proline	11.5	G
L-Serine	60.5	G
Taurine	150.0	G
Carbohydrates		
Glucose	500.0	S
Galactose	100.0	S
Trehalose	100.0	S
Nucleic Acid Precursors		
Adenosine 5'-Monophosphate	1.0	S
Cytidine 5'-Monophosphate	1.0	S
Uridine 5'-Triphosphate	1.0	S
Vitamins		
D-Ca Pantothenate	0.4	S
Choline Chloride	3.0	S
Folic Acid	2.0	S
i-Inositol	35.0	S
Niacinamide	2.0	S
P-AminoBenzoic Acid	2.0	S
D-Pantothenic Acid	0.5	S
Pyridoxin HCl	2.0	S
Riboflavin	0.4	S
Thiamine HCl	2.0	S
Vitamin B-12	0.01	S
Lipids		
Arachidonic Acid	0.07	G
Cholesterol	2.20	G
DL-a-Tocopherol-Acetate	0.7	G
Linoleic Acid	0.1	G
Linolenic Acid	0.1	G
Myristic Acid	0.1	G
Oleic Acid	0.1	G
Palmitric Acid	0.1	G
Palmitic Acid	0.1	G
Stearic Acid	0.1	G
Miscellaneous		
Coenzyme A	1.0	S
Phenol Red	1.0	S
Chloramphenicol	5.0	S
Ethyl alcohol	1,000.0	G
Pluronic® F-68	1,000.0	G
Tween 80	2,000.0	G

* Source of ingredients: G = Gibco laboratories, Grand Island, NY; S = Sigma Chemical Co., St Louis, MO.

Table I. — Composition of the medium JL-ORDP-3. Osmolality 756 mOsm/Kg, pH 7.4.

PROCEDURE TO CONSTITUTE THE PROTEIN-FREE CHEMICALLY DEFINED MEDIUM JL-ORDP-3

All chemicals used were tissue culture grade reagents purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. A basal salt solution (BSS) was first prepared by dissolving 22 g of Seawater Synthetic Basal Salt Mixture and 2 g sodium bicarbonate in a final volume of 912 ml of culture grade water (Milli-Q UF water purification system, Millipore Co. Bedford, MA). Potassium chloride (0.1772 g) was added to maintain a Na^+/K^+ ratio of 23. The BSS solution was then filtered (0.22 µm) sterilized. HEPÈS (25 ml of a 1 M solution) was added for buffering at a 5 % CO_2 atmosphere. The following solutions were then added to BSS under sterile conditions:

1) 2 ml of Trace Element Mix (Gibco BRL, Grand Island, NY),

2) 1 ml of a solution containing 0.834 mg/ml of ferrous sulfate and 0.143 mg/ml of zinc sulfate,

3) 1 ml of a solution containing 0.249 mg/ml of cupric sulfate,

4) 10 ml of MEM Amino Acids Solution without glutamine (Gibco BRL),

5) 10 ml of MEM Non-Essential Amino Acids Solution (Gibco BRL),

6) 10 ml of a solution containing 10 mg/ml of alanine, 5 mg/ml of glycine, 5 mg/ml serine, 15 mg/ml taurine and 5 mg/L glutamine,

7) 6 ml of RPMI 1640 Vitamin Solution,

8) 10 ml of a carbohydrate solution containing 50 mg/ml of glucose, 10 mg/ml of trehalose, and 10 mg/ml of galactose,

9) 10 ml of Chemically Defined Lipid Concentrate (Gibco BRL),

10) 2 ml of a solution containing 0.5 mg/ml each of adenosine 5′-monophosphate, cytidine 5′-monophosphate, uridine 5′-triphosphate and coenzyme A,

11) 10 ml of a solution containing 0.5 mg/ml of chloramphenicol,

12) 1 ml of Phenol red solution (Gibco BRL).

DETERMINATION OF THE CAPABILITY OF JL-ORDP-3 TO SUPPORT THE PROPAGATION OF ESTABLISHED P. MARINUS CELL LINES

Two isolates of *Perkinsus marinus*, *Perkinsus*-1 and LMTX-1, were used to demonstrate the capability of JL-ORDP-3 to support their propagation *in vitro*. *Perkinsus*-1 is the first isolate of *P. marinus* to be succesfully propagated *in vitro*. This cloned and fully characterized isolate of *P. marinus* was obtained from the heart of an infected oyster from the lower Chesapeake Bay, in February of 1992, and has been subcultured every two to four weeks in JL-ORDP-1 medium (La Peyre *et al.*, 1993). The medium JL-ORDP-1 was slightly modified and contained 4 mg/ml instead of 12 mg/ml of BSA after June 1993. The isolate LMTX-1 was kindly provided by Dr. Dave Bushek (Belle W. Baruch Institute of Marine Biology and Coastal Research, University of South Carolina) in June 1994. Culture of LMTX-1 was initiated in July 1993 from hypnospores of an infected oyster collected in Laguna Madre, Texas using a technique described by La Peyre and Faisal (1995), Bushek (1994). This isolate has also been propagated in JL-ORDP-1. Division of both isolates propagated in JL-ORDP-1 medium is by schizogony.

Stock cultures of *Perkinsus*-1 and LMTX-1 were propagated in 75 cm^2 flasks (Corning Glassworks, Corning, NY) using the JL-ORDP-1 medium (50 ml/flask) at a seeding density of 10^6/ml, and subcultured every four weeks. All culture flasks were maintained at 28 °C in a humidified chamber in the presence of 5 % CO_2 tension. *Perkinsus marinus* cells were harvested from stock cultures in JL-ORDP-1 and rinsed three times in either JL-ORDP-1 or JL-ORDP-3 medium. Cells were then resuspended in 50 ml of either medium in triplicate 75 cm^2 culture flasks at a seeding density of 2×10^5 cells/ml. Samples (1.5 ml/flask) of cultured media were collected on days one, three, five, seven, nine, twelve and fifteen for cell counts with a Bright-line hemacytometer (Reichert, Buffalo, NY). Cell clumps arising from division of the schizonts were disaggregated by three passages through a 25-gauge needle prior to counting. The viability of the cells was determined by staining with 0.005 % of neutral red.

The growth of *P. marinus* cells in JL-ORDP-3 medium during the first subculture was compared to growth in JL-ORDP-1. *Perkinsus marinus* cells in each medium were then subcultured every three weeks and their growth was followed for the first three consecutive subcultures (i.e., 2nd to 4th subculture) and at the eleventh subculture as previously described.

INFECTION OF OYSTERS WITH P. MARINUS CELLS PROPAGATED IN JL-ORDP-3

To determine whether *P. marinus* would retain its infectivity to eastern oysters following several subcultures in the chemically defined medium, *P. marinus*-free oysters (Pemaquid Oyster Co., Waldoboro, Maine) were experimentally infected with cultured cells. *Perkinsus*-1 cells propagated in JL-ORDP-3 (18th subculture) were harvested, resuspendend at a density of 10^7 cells/ml of an iso-osmotic salt solution (27 ppt, ~ 750 mOsm, Forty Fathoms marine mix, Marine Enterprises Inc., Baltimore, MD) and 100 µl of this suspension injected into the mantle cavity of 25 notched oysters. After 14 days, oysters received a second dose of *Perkinsus*-1 (10^6 cells/oyster). Control oysters (25) received the same regimen of a salt solution. Each oyster group was maintained in an aerated 80 liter tank

at 28 °C. The water was changed weekly with 1 μm filtered estuarine water (York River, Gloucester point, VA) adjusted to 27 ppt with Forty Fathoms marine mix salts. The oysters were not fed to avoid the introduction of extraneous *P. marinus* cells *via* algae.

Forty-two days post-infection, the presence of *P. marinus* cells in both oyster groups was determined in the rectal, gill and mantle tissue of each individual oyster using the Ray's Test (Ray, 1952). The intensity of infection in each oyster was rated according to the categories of Ray (1954) by estimating the percentage of tissue occupied by the parasite.

SUITABILITY OF JL-ORDP-3
TO SUPPORT PRIMARY CULTURES OF *P. MARINUS* FROM NATURALLY INFECTED OYSTERS

Perkinsus marinus-infected eastern oysters were collected from Wreck Shoal, James River (VA). Cultures of *P. marinus* were initiated from the heart fragments of ten infected oysters according to the procedure described in La Peyre *et al.* (1993). Briefly, the heart from each oyster was removed aseptically, rinsed in a concentrated antibiotic solution and finely minced with a razor blade. The heart fragments were then washed five times in a sterile filtered (0.2 μm) salt solution, resuspended in JL-ORDP-3 medium, and placed in 25 cm² culture flasks. The cultures were incubated at 28 °C in the presence of 5 % CO_2 atmosphere. Propagation of *P. marinus* was monitored using an Olympus (CK-2) inverted light microscope with phase contrast optics at a magnification of 200 ×. After three weeks of incubation, subculturing was attempted.

STATISTICAL ANALYSIS

Growth rate was expressed as doubling time (i.e., the time for a population to double in number) during log phase. In this study, log phase was defined as the period of maximum increase in cell number between two sampling time. Doubling time data was log_{10} transformed and compared by one factor analysis of variance followed by SNK's multiple comparisons of means when significant differences ($p < 0.05$) were found.

RESULTS

PROPAGATION OF *P. MARINUS* ESTABLISHED
CELL LINES IN JL-ORDP-3

*P*erkinsus-1 cells proliferated in JL-ORDP-3 following their transfer from JL-ORDP-1 medium, however, the growth rate in JL-ORDP-3 was significantly lower than that of the same stock of cells grown in JL-ORDP-1 (Fig. 1). The doubling time
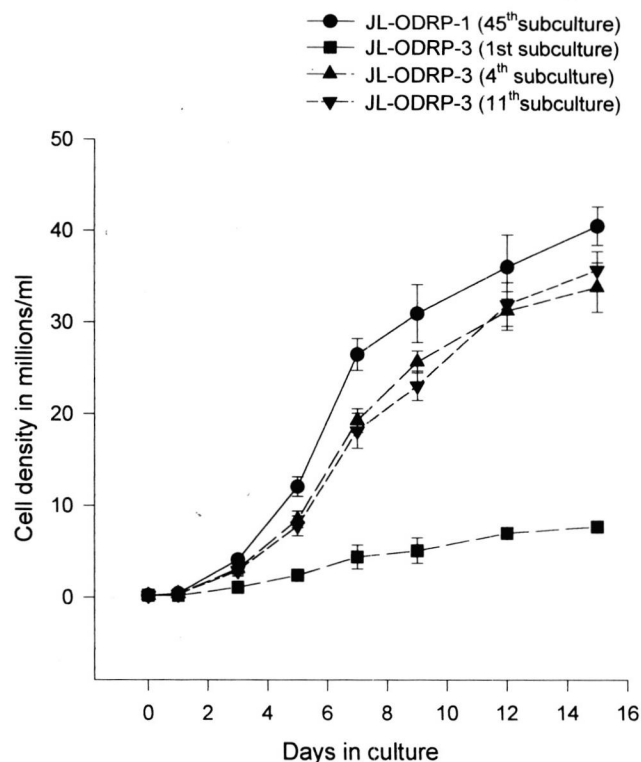

Fig. 1. — Propagation of *Perkinsus*-1 in JL-ORDP-1 (48th subculture) and in JL-ORDP-3 media (1st, 4th and 11th subcultures following transfer from JL-ORDP-1). Culture flasks (N = 3) were seeded with 2×10^5 cells/ml and were incubated at 28° C under 5 % CO_2 tension.

for *Perkinsus*-1 cells in JL-ORDP-3 was about 22 ± 1.4 hours which is significantly greater than that of cells cultured in JL-ORDP-1 (*i.e.*, 15 ± 0.8 hours, Table II). Viability of *Perkinsus*-1 cells in defined medium after the initial transfer from JL-ORDP-1 was always > 95 %. The growth rate of *Perkinsus*-1 increased between the first and second subculture and did not significantly change after 11 subcultures (Fig. 1, Table II).

The medium JL-ORDP-3 also sustained the propagation of LMTX-1 cells after a period of adaptation. Cells of LMTX-1 did not initially multiply following their transfer and their viability was reduced to 78 %. LMTX-1 required a longer time to adapt to JL-ORDP-3. The growth rate increased progressively from the second to the eleventh subculture (Fig. 2, Table II). The viability of cultured LMTX-1 cells after the 11th subculture rose to > 95 %.

MORPHOLOGY OF *P. MARINUS* IN JL-ORDP-3

Microscopical examination of *Perkinsus*-1 and LMTX-1 cells cultured in JL-ORDP-3 medium revealed that their morphology was alike the reported morphology of *P. marinus* observed either *in vivo* or freshly isolated

Mémoire

Isolate	Medium	Subculture	Doubling time* (h)
Perkinsus-1	JL-ODRP-1	45th	15.0 ± 0.8
	JL-ORDP-3	1st	22.3 ± 1.4
		2nd	17.1 ± 2.4
		3rd	16.5 ± 1.3
		4th	16.9 ± 1.6
		11th	18.0 ± 1.2
LMTX-1	JL-ORDP-1	16th	23.8 ± 3.2
	JL-ODRP-3	1st	np**
		2nd	50.4 ± 3.6
		3rd	35.3 ± 7.4
		4th	37.1 ± 4.1
		11th	28.6 ± 3.2

* Calculated during log phase.
** np indicates no propagation.

Table II. — Doubling times of *Perkinsus*-1 and LMTX-1 cells subcultured in JL-ORDP-1 and in the protein-free defined medium JL-ORDP-3. Cells were seeded into 50 cm² flasks at a density of 2×10^5 cells/ml.
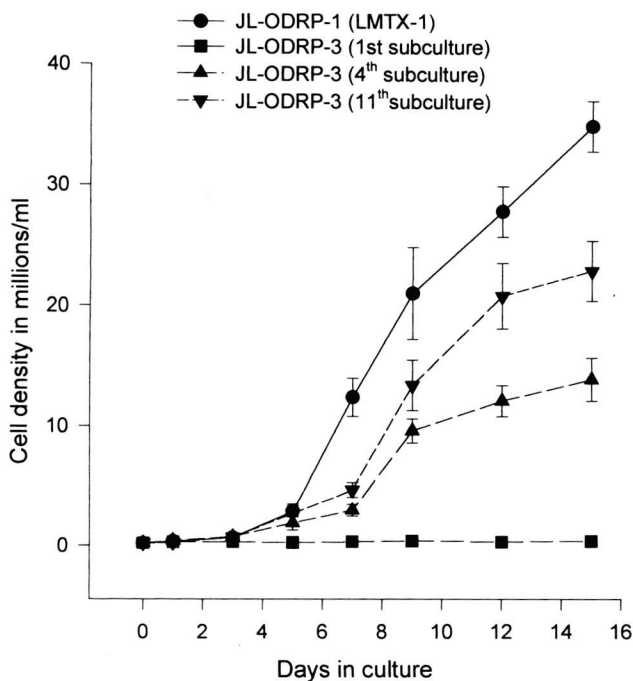

Fig. 2 – Propagation of LMTX-1 in JL-ORDP-1 and in JL-ORDP-3 media (1st, 4th and 11th subcultures following transfer from JL-ORDP-1). Culture flasks (N = 3) were seeded with 2×10^5 cells/ml and were incubated at 28° C under 5 % CO_2 tension.

from infected oysters (Mackin *et al.*, 1950; Perkins, 1969, 1976; La Peyre and Chu, 1994). The smaller cells were about 2-5 µm in diameter and exhibited the typical prominent refractile bodies of merozoites (Fig. 3A). Some of the cells enlarged to about 15 to 30 µm (Fig. 3B), acquired a vacuole, and divided by schizogony releasing the smaller cells (Fig. 3C). Other cells in the same culture flask appeared to divide by binary fission (Fig. 4A & B). Since examination of doublet cells revealed that the cell wall of the original mother cell frequently ringed the daughter cells (Fig. 4C), the apparent binary fission was in fact schizogony.

INFECTION OF OYSTERS WITH *P. MARINUS* CELLS PROPAGATED IN JL-ORDP-3

The prevalence of *P. marinus* in oysters challenged with cells cultured in JL-ORDP-3 was 100 %. Twenty one oysters developed light infection while four oysters developed moderate infection as defined by Ray (1954). No *P. marinus* infection was detected in control oysters.

INITIATION OF PRIMARY CULTURES OF *P. MARINUS* FROM INFECTED OYSTERS USING JL-ORDP-3

Perkinsus marinus isolated from infected heart fragments propagated in the defined medium. During the first three weeks of primary culture, protozoal cells showed the typical morphology of *P. marinus*, enlarged, and divided by schizogony. Subculturing was attempted at a density of 10^6 cells/ml and continuous growth were obtained from eight out of ten oysters. The remaining heart cells did not divide and were diluted out after a few subcultures.

DISCUSSION

*P*erkinsus marinus could be propagated in the protein-free culture medium JL-ORDP-3. It was important to subculture *P. marinus* cells for at least eleven passages in JL-ORDP-3 medium in order to eliminate low concentrations of chemical ingredients that may have been taken up by the cells while growing in JL-ORDP-1 and, to acclimate protozoal cells to the new medium. As our results demonstrate, JL-ORDP-3 supported the continuous growth of two *P. marinus* isolated through at least ten subcultures. The growth rates measured for both *P. marinus* isolates, *Perkinsus*-1 and LMTX-1, in the defined medium were lower than in the original medium JL-ORDP-1, however their doubling times were within the range reported for *P. marinus* grown in protein supplemented media (Gauthier and Vasta, 1995; Dungan and Hamilton, 1995). The decreased growth rates can be attributed to the absence of proteins, peptides, and possibly growth factors that may have been present in the original medium undefined components. Our previous studies have demonstrated that proteins present in JLORDP-1 medium were consumed by *P. marinus* (La Peyre and Faisal, 1995) that produces multiple serine proteases (La Peyre *et al.*, 1995).
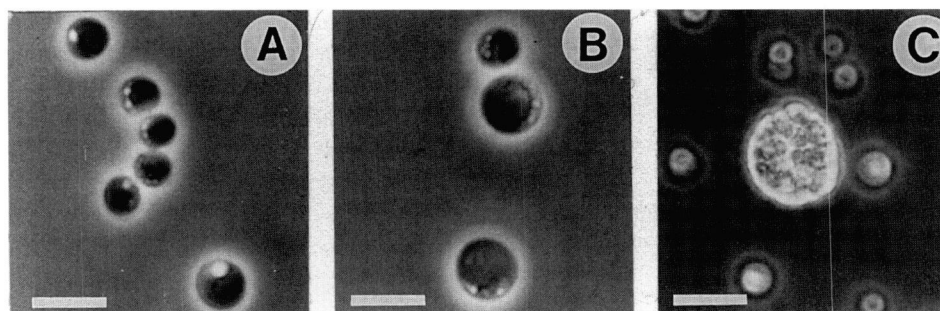
Fig. 3. – Light micrographs of *Perkinsus*-1 and LMTX-1 cells after 11th subculture in JL-ORDP-3 medium. Flasks were seeded with 2×10^5 cells/ml and maintained at 28° C under 5 % CO_2. A) small-sized *Perkinsus*-1 cells : notice the refractile lipid bodies (bar = 10 µm); B) larger *Perkinsus*-1 cells with clear vacuole (bar = 10 µm); C) enlarged LMTX-1 cell dividing by schizogony (bar = 25 µm).
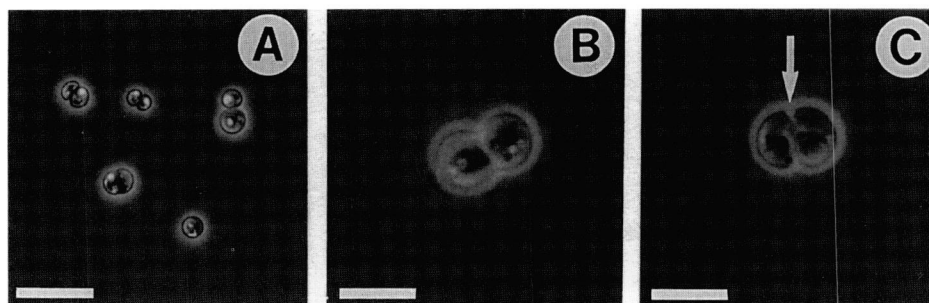

Fig. 4. – Light micrographs of *Perkinsus*–1 cells after 17th subculture in JL-ORDP-3 medium. Flasks were seeded with 2×10^5 cells/ml and maintained at 28° C in 5 % CO_2 for one week prior to photographing. A) *Perkinsus*-1 mother cells yielding two daughter cells (bar = 25 µm); B) *Perkinsus*-1 mother cell dividing (bar = 10 µm); C) cell wall (arrow) of a mother cell following division by schizogony which yielded two daughter cells (bar = 10 µm).

The morphology of *Perkinsus*-1 and LMTX-1 cells in JL-ORDP-3 was similar to the morphology of *P. marinus* observed either *in vivo* or freshly isolated from infected oysters (Mackin *et al.*, 1950; Perkins, 1969, 1976; La Peyre and Chu, 1994). The size of the smaller cells (*i.e.*, 3-6 µm) was identical to the size of merozoites isolated from infected oysters by La Peyre and Chu (1994). The small cells had prominent refractile bodies, presumably lipid droplets, like those observed in isolated merozoites. Moreover, the size of the largest dividing cells never exceeded about 30 µm which is the size of *P. marinus* schizonts *in vivo* (Perkins, 1969). On the other hand, cells cultured in JL-ORDP-1 had a much larger size that sometimes exceeded 45 µm in diameter (La Peyre *et al.*, 1993). The high protein concentration (12 mg/ml BSA) in the original medium JL-ORDP-1 may have caused greater enlargement of cultured cells. Division of *P. marinus* cells of both isolates in JL-ORDP-3 was by schizogony. Mother cells yielded from two to many daughter cells (presumably 4-32).

A defined medium has recently been developed by Gauthier *et al.* (1995). This medium consists of Dubelcco modified Eagle's medium/Ham's F12 nutrient mixture (1:1) supplemented with 1.7 mg/ml of fetuin. The major advantage of this medium is its ease of preparation since each component is commercially available. However, fetuin and fetuin breakdown products could interfere with studies on *P. marinus*-derived proteins and peptides. All other commercial media that have been used to culture *P. marinus* were supplemented with fetal bovine serum (FBS, 5-20 %) alone or with oyster plasma (5-20 %) (Gauthier and Vasta, 1993; Kleinshuster and Swink, 1993; Dungan and Hamilton, 1995).

Results of this study clearly demonstrated that despite the absence of proteins, JL-ORDP-3 supported the propagation of *P. marinus in vitro* and the initiation of primary cultures. The propagated cells retained their infectivity and were identical in their morphology to *P. marinus in vivo*. The absence of proteinaceous materials from this chemically defined medium demarcates JL-ORDP-3 as the most suitable medium to study *P. marinus* proteins, to produce antigens for antibody production, and to screen chemotherapeutic agents. Moreover this medium will greatly simplify biochemical, physiological and nutritional studies of *P. marinus*.

ACKNOWLEDGEMENTS

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Mémoire