Characterization and immunolocalization of a main proteinaceous component of the cell wall of the protozoan parasite *Perkinsus atlanticus*

J. F. MONTES1,2*, M. DURFORT1,2, A. LLADÓ1 and J. GARCÍA-VALERO1,2

1 Departament de Biologia Cel·lular, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, E-08028 Barcelona, Spain
2 Centre de Referència i Desenvolupament en Aquicultura de la Generalitat de Catalunya, CRAC

(Received 17 July 2001; revised 7 December 2001; accepted 12 December 2001)

**Summary**

Described in the present study is a major component of the cell wall of 2 of the most pathogenic parasites of molluscs, *Perkinsus atlanticus* and *P. marinus*. The component is a high molecular weight protein (233 kDa), which we have named PWP-1 (for Perkinsus wall protein-1). Western blots, using a polyclonal serum generated against purified PWP-1 from *P. atlanticus*, revealed that this protein is expressed by all walled developmental stages of this protozoon. By means of immunogold electron microscopy, labelling for PWP-1 was strong and specifically associated with the cell wall. The label density and distribution pattern was quite different between trophozoites and prezoosporangia. With regard to the structural organization of this protein, PWP-1 is disulphide-linked to other cell wall components and released from the cell wall only following treatment with a sulphhydryl agent. We also report that PWP-1 is a trypsin-resistant protein, both in its native and heat-denatured conformation. In addition, results from the N-terminal microsequence of this protein allow us to define PWP-1 as a novel cell wall protein. Overall, our findings strongly suggest that PWP-1 plays a key role in the organization of the cell wall of these protozoa, promoting their survival.

Key words: cell wall, *Perkinsus atlanticus*, *Perkinsus marinus*, *Tapes semidecussatus*, trypsin-resistant protein.

**Introduction**

Protozoa belonging to the genus *Perkinsus* are major disease agents in marine molluscs and have been associated with extensive mortality in bivalves and gastropods worldwide (reviewed by Bower, McGlad-dery & Price, 1994; Perkins, 1996). Six *Perkinsus* species have been described so far: *P. marinus* (Mackin, Owen & Collier, 1950) in the oyster *Crassostrea virginica*, *P. olsenii* (Lester & Davis, 1981) in the abalone *Haliotis ruber*, *P. atlanticus* (Azevedo, 1989) in the clam *Tapes decussatus*, *P. aquwadi* (Blackbourn, Bower & Meyer, 1998) in the scallop *Patinopecten yessoensis*, *P. chesapeake* (McLaughlin *et al.* 2000) in the clam *Mya arenaria* and, recently, *P. andrewsi* (Coss *et al.* 2001) in the clam *Macoma balthica*. At present, *Perkinsus* species are placed within the recently established phylum Perkinsozoa (Nören, Moestrup & Rehnstam-Holm, 1999), bridging the groups Apicomplexa and Dinoflagellata, in which *Perkinsus* was previously included (Levine, 1978; Siddall *et al.* 1997).

On the Atlantic and Mediterranean coasts of Europe, parasitism by *P. atlanticus* has been associated with epizootic outbreaks involving large-scale mortalities of commercially valuable venerid clams of the genus *Tapes* (= *Ruditapes = Venerupis*), such as the indigenous species *T. decussatus* (Da Ros & Canzonier, 1985; Comps & Chagot, 1987; Azevedo, 1989; Figueras, Robledo & Novoa, 1992; Montes, Durfort & García-Valero, 1996) and the introduced species *T. semidecussatus* (= *T. philippinarum = T. japonica*) (Montes, Durfort & García-Valero, 1995a; Sagristà, Durfort & Azevedo, 1995). In addition, we have recently reported that *P. atlanticus*-infected clams *T. semidecussatus* from the northern Mediterranean coast of Spain develop viral and bacterial opportunistic infections, which have detrimental effects on this clam population (Montes, Durfort & García-Valero, 2001).

The life-cycle of *P. atlanticus* consists of 3 main stages: trophozoite, prezoosporangium and zoospore (Azevedo, 1989; Sagristà, Durfort & Azevedo, 1996). Trophozoites are the vegetative form that proliferates in the connective tissue of the host. During incubation in fluid thioglycollate medium, and probably in dying hosts (Auzoux-Bordenave *et al.* 1995), they enlarge substantially and differentiate into prezoosporangia. When this life-cycle form is placed into seawater, it releases biflagellate zoospores, which are the main infective stage of this parasite. Electron microscopic examination of *P.
atlanticus life-cycle stages reveals that both trophozoites and prezoosporangia are characterized by the presence of a well-developed cell wall, which considerably increases in thickness during differentiation of the trophozoite to prezoosporangium (Azevedo, 1989; Sagristá et al. 1996). The thickened wall of the prezoosporangia stains blue-black with Lugol’s iodine solution, without acid hydrolysis (Ray, 1952).

Little is known, however, about the composition, structure and organization of the cell wall of Perkinsus species. Earlier studies by Stein & Mackin (1957), based on cytochemical analyses, suggested that cellulose and hemicellulose constitute the polysaccharidic matrix of the cell wall of P. marinus. Recently, we have reported, using lectin histochemistry, that the trophozoite cell wall of P. atlanticus shows a low degree of glycosylation (Montes et al. 1995a). Progress towards understanding the epidemiology and pathogenesis of Perkinsus infections requires further knowledge of the surface molecules of this parasite. In this regard, we report the isolation and partial characterization of a main proteinaceous component of the cell wall of P. atlanticus. Polyclonal antibodies have been raised against this protein, allowing us to determine that it is expressed by all walled stages of this parasite. The cross-reactivity of this serum with other Perkinsus species is also reported.

MATERIALS AND METHODS

Animals

Market-sized specimens of non-parasitized and P. atlanticus-infected clams, T. semidecussatus, were obtained from different clam beds of the Delta of the River Ebro, Tarragona (N.E. Spain), Mediterranean Sea, an area endemic for P. atlanticus. Specimens were collected during July–September 1999.

Isolation and purification of P. atlanticus prezoosporangia

The prezoosporangia of P. atlanticus were purified from isolated parasitized gills following essentially the procedure described by Chu & Greene (1989). Briefly, 425 g of gills (from approx. 2500 clams) were incubated in fluid thioglycollate medium (FTM; Difco Laboratories; Detroit, MI), rehydrated with 0.22 µm filtered seawater of 25%o salinity and supplemented with 400 U/ml penicillin G (Sigma Chemical Co.; St Louis, MO), 500 µg/ml streptomycin sulphate (Sigma) and 200 U/ml nystatin (Sigma). Culture was carried out in the dark under anaerobiosis at room temperature. After 72 h of incubation, tissues were chopped and treated with 0.25% trypsin (Sigma) in seawater for 15 min at 37 °C. The resultant tissue suspension was sequentially filtered through nylon meshes of 300 and 100 µm to remove gill tissue fragments. Finally, the prezoosporangia fraction was enriched by sequential centrifugation at 825, 200 and 50 g for 5 min each at 4 °C. Each centrifugation step was repeated 3 times and the supernatant was always discarded. The purity of the isolation was determined by differential interference contrast microscopy and Lugol’s iodine stain.

Prezoosporangium processing and isolation of antigenic poly peptides

The enriched fraction of prezoosporangia was resuspended in 4 volumes of lysis buffer (15 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl₂, 1% Triton X-100, 0.2% SDS, 1 mM EDTA) containing a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 0.3 µM aprotinin, 28 µM E-64, 1 µM pepstatin) and then incubated for 15 min on ice. Thereafter, prezoosporangia were treated by repeated cycles of freezing–thawing and homogenized with a motorized glass-Teflon homogenizer. Prezoosporangium lysates were subsequently sonicated (Vibracell; Sonics & Materials; Danbury, CT) on ice 4 times for 10 s at 50 w at 15 s intervals.

A total of 136 mg of protein from prezoosporangium homogenates was electrophoretically resolved on a Protean II vertical slab gel unit (Bio-Rad; Hercules, CA) with 9% polyacrylamide separating gels and 4% stacking gels under reducing conditions. Prezoosporangium homogenates were mixed 1:2 with SDS–PAGE sample buffer (62.5 ml Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol), heated at 95 °C for 5 min and clarified by centrifugation. Apparent molecular masses of polypeptides were determined from their relative mobilities compared with a broad molecular weight standard (Bio-Rad). The 5 major polypeptides of highest molecular weight were selected for rabbit immunization. After electrophoresis, the selected bands were removed from the gels following the side-strip method (Harlow & Lane, 1988) and kept at −80 °C until injection.

Immunization and production of antisera

Specific polyclonal antisera were obtained by direct immunization with SDS–PAGE slices containing the selected polypeptides essentially as described elsewhere (Boulard & Lecroisey, 1982). In brief, young white male New Zealand rabbits, weighing 2–3 kg, were immunized 3 times by intramuscular injections at 2 week intervals with a total of 450 µg of the purified polypeptides. Immunizations were performed by emulsion of the immunogen in Freund’s adjuvant (Sigma), complete for the first injection and
incomplete thereafter. The rabbits were bled before the immunizations, and the sera obtained were used for control studies.

Each antiserum was tested following immunoblotting and immunocytochemical techniques by electron microscopy. According to the results, the serum against a polypeptide of an apparent molecular weight of 233 kDa (PWP-1; for *Perkinsus marinus* protein-1) was selected for analysis.

**Sample processing and immunoblotting**

The specificity of the serum against PWP-1 was tested by the Western blot technique in samples from *P. atlanticus* prezoosporangia, and gills from both parasitized and non-parasitized clams, *T. semidecussatus*. Non-parasitized specimens were determined after thioglycollate assay of the two hemibranchs from one side. Gills were homogenized at 4 °C in lysis buffer by 20 strokes in a glass Dounce homogenizer followed by sonication. The reactivity of the serum was also assayed in samples of *P. marinus* isolate LICT-1 acquired from the American Type Culture Collection (ATCC 50508). *Perkinsus marinus* homogenates were obtained as described above for *P. atlanticus* prezoosporangia.

For immunoblotting, denatured samples were separated by either reducing or non-reducing SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell; Dassel, Germany) following the method of Towbin, Staehelin & Gordon (1979). Transfer was achieved at 100 mA overnight at 4 °C in a Mini Trans-Blot apparatus (Bio-Rad). Thereafter, the membranes were pre-soaked in TBST (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20) and treated with blocking buffer (5% non-fat dried milk in TBST) for at least 30 min. Membranes were incubated with serum against PWP-1 diluted 1:2000 in blocking buffer for 3 h at room temperature. After 3 washes in TBST, peroxidase-conjugated swine anti-rabbit Ig (Dako; Glostrup, Denmark) was applied for 2 h. Immunoreactive bands were detected using the enhanced chemiluminescence system, and the luminol excitation was imaged on X-OMAT UV films (Kodak; Hemel Hempstead, UK). For the controls, the specific antiserum was replaced by the matched pre-immune serum. In additional controls, incubation with the primary antibody was omitted.

**Trypsin digestion**

To assess the stability of PWP-1 against proteolysis, gill homogenates from heavily *P. atlanticus*-infected clams were treated with trypsin. Gill homogenates were obtained as mentioned before, but the lysis buffer did not contain detergents or protease inhibitors. The assay was carried out as follows: both non-denatured and denatured (95 °C, 5 min) homogenate samples were incubated at 37 °C for 30 min with either trypsin or heat-inactivated trypsin at an enzyme:substrate ratio of 1:1. The final concentration of trypsin was 4 µg/ml. As a digestion control, the homogenate samples were supplemented with purified rabbit IgGs before the incubation. The reactions were stopped by addition of SDS–PAGE sample buffer and heating at 95 °C for 5 min. Digestion was analysed by immunoblotting with the serum against PWP-1 as described.

**N-terminal amino acid microsequencing of PWP-1**

For Edman microsequencing, prezoosporangium proteins were fractionated by SDS–PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane (Schleicher & Schuell) using a Trans blot cell (Bio-Rad). After transfer, the membrane was stained with Coomassie Blue and the band corresponding to PWP-1 was excised. The N-terminal microsequence was determined by automated Edman degradation on a Beckman LF3000 protein sequencer, equipped with a phenylthiohydantoin (PTH)-amino acid analyser System Gold (Beckman Instruments; Fullerton, CA).

**Immunogold labelling**

Abscesses from *P. atlanticus*-infected gills and isolated prezoosporangia were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) for 2 h at 4 °C. Isolated prezoosporangia were then embedded in 30% ovalbumin in PBS and polymerized in the same fixative. After washing, all samples were processed for Lowicryl K4M infiltration at low temperature following the manufacturer’s guidelines (Polysciences LTD; Northampton, UK). Polymerization was induced by UV irradiation at −35 °C for 2 days. Immunogold labelling was performed as described elsewhere (Montes, Durfort & García-Valero, 1995b). After washing and blocking, the grids were incubated with the serum against PWP-1(1:500) and then bound polyclonal antibodies were visualized following incubation with 10 nm protein A gold (Dr Slot, Utrecht, The Netherlands). Finally, the grids were washed thoroughly in PBS and double-distilled water, and stained with uranyl acetate and Reynold’s lead citrate. Controls were performed by replacing the specific antiserum with matched pre-immune serum. Additional controls were carried out by omitting the specific antiserum from the immunolabelling procedure. Observations were carried out with a Hitachi H-600 AB transmission electron microscope.

**Quantitative evaluation**

Label density (LD) was estimated as the number of gold particles per sectioned area of trophozoite and
prezoosporangium cell wall. The area parameter was estimated by stereological methods (Weibel, 1979). Values were expressed as mean $\pm$ s.e.m. from at least 30 measurements performed in various parasites and significance of mean differences was tested by Student’s $t$-test.

**RESULTS**

**SDS–PAGE and immunoblotting**

Under denaturing and reducing conditions, the electrophoretic profile of isolated *P. atlanticus* prezoosporangia consisted of 12 major bands with mobilities ranging from 233 to 42 kDa (Fig. 1A). To obtain a polyclonal serum against a cell wall protein of *P. atlanticus*, the 5 major bands of highest molecular weight (233, 198, 172, 149 and 102 kDa) were isolated for rabbit immunization. The serum obtained against 233 kDa polypeptide (PWP-1; for *Perkinsus wall protein-l*) showed the highest specificity and was selected for the study.

The immunoreactivity of the serum against PWP-1 was determined by Western blotting. A single band of an expected apparent molecular mass of 233 kDa was detected in the homogenate samples from both *P. atlanticus* prezoosporangia (Fig. 1B, lane 1) and *P. atlanticus* trophozoites-containing gills (Fig. 1B, lane 2). There were no positive signals when this antiserum was assayed in non-parasitized samples (Fig. 1B, lane 3). In addition, using this polyclonal serum, PWP-1 was identified in immunoblots of *P. marinus* isolate LICT-1 (Fig. 1B, lane 4). No apparent differences were observed between these two *Perkinsus* species in molecular weight or in antibody recognition of the polypeptide. In contrast, no protein band was detected on these blots when the pre-immune rabbit serum was used as the specific control (data not shown).

To determine whether PWP-1 is linked by disulphide bonds to other cell wall components, denatured (hot SDS) homogenate samples from both *P. atlanticus* prezoosporangia and *P. atlanticus* trophozoites-containing gills were analysed by immunoblotting under non-reducing conditions (Fig. 2). After incubation with the specific serum, no reactive bands were detected at 233 kDa or at any other mobility (Fig. 2, lanes 2 and 4), indicating that PWP-1 is only released after treatment with a sulphhydril agent, such as 2-mercaptoethanol (Fig. 2, lanes 1 and 3).

**Trypsin digestion**

The stability of non-denatured and heat-denatured PWP-1 against trypsin proteolysis was probed in *P. atlanticus* trophozoites-containing homogenates as described in the Materials and Methods section. Western blotting using the specific serum showed that trypsin treatment had no detectable effect on the molecular weight or on the recognition of PWP-1, regardless of its conformation (Fig. 3). On the other hand, the degradation of the rabbit IgGs, added to the reaction mixture as internal control, supported the validity of the experimental conditions.

**N-terminal amino acid microsequencing of PWP-1**

N-terminal sequencing of PWP-1 revealed that its first 8 amino acid residues were MEDEGAGG (SWISS-PROT accession number P83195; PWP-1). The comparison of this sequence with the Swiss Prot database did not show homology with any other known protein.

**Immunolocalization**

The immunoreactivity of the serum against PWP-1 was also assayed by immunogold electron microscopy with gill abscesses from *P. atlanticus*-infected clams, *T. semidecussatus*. The resultant labelling was exclusively associated with the trophozoites of *P. atlanticus* (Fig. 4A and B). Neither the capsule nor other host tissues showed affinity for this antiserum. In addition, the lack of labelling after incubation with the pre-immune serum demonstrated the specificity of this antiserum (data not shown). Labelling for PWP-1 was entirely confined to the cell wall of the trophozoites (Fig. 4A and B). No significant reactivity was detected in the other trophozoite structures, such as cisternae of endoplasmic reticulum or vacuolar compartment (Fig. 4A).

Labelling of the serum against PWP-1 was strong and distributed throughout the trophozoite cell wall without any regional predominance. The evaluation of the labelling pattern revealed that PWP-1 showed an ordered distribution parallel to the plasmalemma (Fig. 4A). No apparent differences were observed between the maturation stages of the parasite. This ordered distribution was most obvious when the immunogold staining of PWP-1 was analysed in the remnants of mother cell walls originated during parasite proliferation (Fig. 4B).

When immunolocalization of PWP-1 was carried out in the isolated prezoosporangia, the resultant pattern of immunoreactivity closely resembled that obtained in trophozoites, since the prezoosporangium cell wall was the only compartment stained (Fig. 4C and D). However, differences were observed when the labelling pattern for PWP-1 was compared between these two life-cycle forms. Thus, in the thicker prezoosporangium cell wall, formed during FTM incubation, the immunogold staining was patchy, revealing regional variations in the distribution of this protein. Labelling was usually stronger in the distal than in the proximal areas of
High molecular weight proteins (≥ 200 kDa) are characteristic constituents of the cell wall of unicellular eukaryotes, including apicomplexans (Tilley et al. 1990; Bonnin, Dubremetz & Camerlynck, 1991), dinoflagellates (Markell, Trench & Iglesias-Prieto, 1992), diatoms (Krogner, Bergsdorf & Sumper, 1994; Krogner et al. 1997) and yeasts (Casanova et al. 1992; Mrsa et al. 1997). In this regard, the current study shows that a major proteinaceous component of the cell wall of the protozoan parasite *P. atlanticus* is a novel cell wall protein with an apparent molecular weight of 233 kDa, which we have named PWP-1 (for *Perkinsus* w*all*protein-1).

In this study, we report that these two *P. atlanticus* life-cycle forms differ in both the label density and pattern of labelling for PWP-1. These findings allow us to demonstrate, for the first time, that *Perkinsus* trophozoite to prezoosporangium differentiation not
only involves a change in the cytochemical and immunological properties of the cell wall, as mentioned above, but also a substantial modification in its organization.

As to the structural organization of PWP-1, immunoblotting assays showed that PWP-1 is released from the cell wall only following treatment with a sulphydryl agent, such as 2-mercaptoethanol. This finding indicates, therefore, that PWP-1 is linked by disulphide bonds to other cell wall constituents of *P. atlanticus*. Likewise, numerous disulphide-bound protein complexes have been described in the cell wall of other unicellular organisms, including prokaryotes (Newhall et al.).
1980) and eukaryotes (Chaffin et al. 1998; De Stefano et al. 1998; Lipke & Ovalle, 1998). It has been suggested that these protein complexes play a structural role, contributing to the stability of the cell wall of these organisms (Chaffin et al. 1998; De Stefano et al. 1998). Therefore, the presence of disulphide protein complexes in the cell wall of Perkinsus may explain the extreme resistance of these protozoa to chemical and mechanical disruption (Saunders, Powell & Lewis, 1993; Krantz, 1994).

On the other hand, there is growing evidence that the trophozoites of Perkinsus species survive exposure to lysosomal enzymatic activities after phagocytosis by mollusc haemocytes (La Peyre, Chu & Vollbein, 1995; Sagristá et al. 1995; Perkins, 1996; Bushek et al. 1997), so much so that a characteristic feature of mature trophozoites is the presence of host lysosomal membranes embedded in the cell wall (Perkins, 1988, 1996). Given that the wall of Perkinsus is the cell compartment responsible for direct interaction with host cells, our finding that a major proteinaceous component of this structure is resistant to proteolysis, may partially explain why molluscan phagocytes are not very effective in combatting this disease agent.

In conclusion, we have demonstrated that a high molecular weight protein, PWP-1, is a major component of the cell wall of two of the most pathogenic parasites of molluscs, P. atlanticus and P. marinus. Our findings strongly suggest that PWP-1 plays a key role in the organization of the cell wall of these protozoa favouring, additionally, their survival.

The authors wish to thank Ms Almudena García and the staff of Serveis Científico-Técnics (Universitat de Barcelona) for their technical assistance. We also thank Dr Dolores López Tejero for her support and Mr Robin Rycroft for linguistic advice. This study was supported by a research grant from the CIRIT (Comisión Interdepartamental de Recerca i Innovació Tecnològica), Generalitat de Catalunya.

REFERENCES

Auzoux-Bordeneuve, S., Vigario, A. M., Ruano, F., Domart-Coulon, I. & Doumenç, D. (1995). In vitro sporulation of the clam pathogen Perkinsus atlanticus (Apicomplexa, Perkinsidae) under various environmental conditions. Journal of Shellfish Research 14, 469–475.

Azevedo, C. (1989). Fine structure of Perkinsus atlanticus n. sp. (Apicomplexa, Perkinsidae) parasite of the clam Ruditapes decussatus from Portugal. Journal of Parasitology 75, 627–635.

Blackbourn, J., Bower, S. M. & Meyer, G. R. (1998). Perkinsus quagga sp. nov. (incertae sedis), a pathogenic protozoan parasite of Japanese scallops, Patinopecten yessoensis, cultured in British Columbia, Canada. Canadian Journal of Zoology 76, 942–953.

Bonnin, A., Dubremetz, J. F. & Camerlynck, P. (1991). Characterization and immunolocalization of an oocyst wall antigen of Cryptosporidium parvum (Protozoa: Apicomplexa). Parasitology 103, 171–177.

Bouard, C. & Lecroisey, A. (1982). Specific antiserum produced by direct immunization with slices of polyacrylamide gel containing small amounts of protein. Journal of Immunological Methods 50, 221–226.

Bower, S. M., McGladdery, S. E. & Price, I. O. (1994). Synopsis of infectious diseases and parasites of commercially exploited shellfish. Annual Review of Fish Diseases 4, 1–199.

Bushek, D., Allen, S. K., Alcox, K. A., Gustafson, R. G. & Ford, S. E. (1997). Response of Crassostrea virginica to in vitro cultured Perkinsus marinus: preliminary comparisons of three inoculation methods. Journal of Shellfish Research 16, 479–485.

Casanova, M., López-Ribot, J. L., Martínez, J. P. & Sentandreu, R. (1992). Characterization of cell wall proteins from yeast and mycelial cells of Candida albicans by labelling with biotin: comparison with other techniques. Infection and Immunity 60, 4898–4906.

Chaffin, W. L., López-Ribot, J. L., Casanova, M., Gozalbo, D. & Martínez, J. P. (1998). Cell wall and secreted proteins of Candida albicans: identification, function, and expression. Microbiology and Molecular Biology Reviews 62, 130–180.

Choi, K.-S., Lewis, D. H., Powell, E. N., Frelier, P. F. & Ray, S. M. (1991). A polyclonal antibody developed from Perkinsus marinus hypnospores fails to cross react with other life stages of P. marinus in oyster (Crassostrea virginica) tissues. Journal of Shellfish Research 10, 411–415.

Chu, F.-L. E. & Greene, K. H. (1989). Effect of temperature and salinity on in vitro culture of the oyster pathogen, Perkinsus marinus (Apicomplexa: Perkinsidae). Journal of Invertebrate Pathology 53, 260–268.

Comps, M. & Chagot, D. (1987). Une parasitose nouvelle chez la Palourde Ruditapes decussatus L. Comptes Rendus de l’Académie des Sciences 304, 41–44.

Coss, C. A., Robledo, J. A. F., Ruiz, G. M. & Vasta, G. R. (2001). Description of Perkinsus andrewsi n. sp. isolated from the baltic clam (Macoma balthica) by characterization of the ribosomal RNA locus, and development of a species-specific PCR-based diagnostic assay. Journal of Eukaryotic Microbiology 48, 52–61.

Da Ros, L. & Canzonier, W. J. (1985). Perkinsus, a protistan threat to bivalve culture in the Mediterranean basin. Bulletin of the European Association of Fish Pathologists 5, 23–27.

De Stefano, J. A., Myer, J. D., Du Pont, D., FoY, J. M., Theus, S. A. & Walzer, P. D. (1998). Cell wall antigens of Pneumocystis carinii trophozoites and cysts: purification and carbohydrate analysis of these glycoproteins. Journal of Eukaryotic Microbiology 45, 334–343.

Figueras, A., Robledo, J. A. F. & Novoa, B. (1992). Occurrence of haplosporidian and Perkinsus-like infections in carpet-shell clams, Ruditapes decussatus (Linnaeus, 1758), of the Ría de Vigo (Galicia, NW Spain). Journal of Shellfish Research 11, 377–382.
Harlow, E. & Lane, D. (1988). *Antibodies: a Laboratory Manual*. Cold Spring Harbor Laboratory, New York.

Krantz, G. E. (1994). Chemical inhibition of *Perkinsus marinus* in two *in vitro* culture systems. *Journal of Shellfish Research 13*, 131–136.

Kröger, N., Bergsdoerf, C. & Sumper, M. (1994). A new calcium binding glycoprotein family constitutes a major diatom cell wall component. *The EMBO Journal 13*, 4676–4683.

Kröger, N., Lehmann, G., Rachel, R. & Sumper, M. (1997). Characterization of a 200-kDa diatom protein that is specifically associated with a silica-based substructure of the cell wall. *European Journal of Biochemistry 250*, 99–105.

La Peyre, J. F., Chu, F.-L. E. & Vogelrein, W. K. (1995). *In vitro* interaction of *Perkinsus marinus* merozoites with eastern and Pacific oyster hemocytes. *Developmental and Comparative Immunology 19*, 291–304.

Lester, R. J. G. & Davis, G. H. G. (1981). A new *Perkinsus* species (*Apicomplexa*, Perkinsinea) from the abalone *Halilis ruber*. *Journal of Invertebrate Pathology 37*, 181–187.

Levine, N. D. (1978). *Perkinsus* gen. n. and other new taxa in the protozoan phylum *Apicomplexa*. *Journal of Parasitology 64*, 549.

Lipke, P. N. & Ovalle, R. (1998). Cell wall architecture in yeast: new structure and new challenges. *Journal of Bacteriology 180*, 3735–3740.

Mackin, J. G., Owen, H. M. & Collier, A. (1950). Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). *Science 111*, 328–329.

Markell, D. A., Trench, R. K. & Iglesias-Prieto, R. (1992). Macromolecules associated with the cell walls of symbiotic dinoflagellates. *Symbiosis 12*, 19–32.

McLaughlin, S. M., Tall, B. D., Shaheen, A., Elsayed, E. E. & Faisal, M. (2000). Zoosporulation of a new *Perkinsus* species isolated from the gills of the softshell clam *Mya arenaria*. *Parasite 7*, 115–122.

Montes, J. F., Durfort, M. & García-Valeco, J. (1995a). Cellular defence mechanism of the clam *Tapes semidecussatus* against infection by the protozoan *Perkinsus* sp. *Cell and Tissue Research 279*, 529–538.

Montes, J. F., Durfort, M. & García-Valeco, J. (1995b). Characterization and localization of an Mr 225 kDa polypeptide specifically involved in the defence mechanisms of the clam *Tapes semidecussatus*. *Cell and Tissue Research 280*, 27–37.

Montes, J. F., Durfort, M. & García-Valeco, J. (1996). When the venetid clam *Tapes decussatus* is parasitized by the protozoan *Perkinsus* sp. it synthesizes a defensive polypeptide that is closely related to p225. *Diseases of Aquatic Organisms 26*, 149–157.

Montes, J. F., Durfort, M. & García-Valeco, J. (2001). Parasitism by the protozoan *Perkinsus atlanticus* favours the development of opportunistic infections. *Diseases of Aquatic Organisms 46*, 57–66.

MRSA, V., Seidl, T., Gentzsch, M. & Tanner, W. (1997). Specific labelling of cell wall proteins by biotinylation. Identification of four covalently linked O-mannosylated proteins of *Saccharomyces cerevisiae*. *Yeast 13*, 1145–1154.

Newhall, W. J., Wilde, C. E., Sawyer, W. D. & Haak, R. A. (1980). High-molecular-weight antigenic protein complex in the outer membrane of *Neisseria gonorrhoeae*. *Infection and Immunity 27*, 475–482.

Noren, F., Moestrup, O. & Reinhart-Holm, A.-S. (1999). *Parvilucifera infectans* Noren et Moestrup gen. et sp. nov. (Perkinsozoa phylum nov.): a parasitic flagellate capable of killing toxic microalgae. *European Journal of Protistology 35*, 233–254.

Perkins, F. O. (1988). Structure of protistan parasites found in bivalve molluscs. *American Fisheries Society Special Publication 18*, 93–111.

Perkins, F. O. (1996). The structure of *Perkinsus marinus* (Mackin, Owen and Collier, 1950) Levine, 1978 with comments on taxonomy and phylogeny of *Perkinsus* spp. *Journal of Shellfish Research 15*, 67–87.

Ray, S. M. (1952). A culture technique for the diagnosis of infections with *Dermocystidium marinus* Mackin, Owen, and Collier in oysters. *Science 116*, 360–361.

Sagrasta, E., Durfort, M. & Azevedo, C. (1995). *Perkinsus* sp. (Phylum Apicomplexa) in Mediterranean clam *Ruditapes decussatus*: ultrastructural observations of the cellular response of the host. *Aquaculture 132*, 153–160.

Sagrasta, E., Durfort, M. & Azevedo, C. (1996). Ultrastructural data on the life cycle of the parasite, *Perkinsus atlanticus* (Apicomplexa), on the clam, *Ruditapes philippinarum*, in the Mediterranean. *Scientia Marina 60*, 283–288.

Saunders, G. L., Powell, E. M. & Lewis, D. H. (1993). A determination of *in vivo* growth rates for *Perkinsus marinus*, a parasite of the eastern oyster *Crassostrea virginica* (Gmelin, 1791). *Journal of Shellfish Research 12*, 229–240.

Siddall, M. E., Reece, K. S., Graves, J. E. & Burreson, E. M. (1997). ‘Total evidence’ refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa. *Parasitology 115*, 165–176.

Stein, J. E. & Mackin, J. G. (1957). Some cytochemical studies of the cell wall of *Dermocystidium marinus*. *Texas A&M Research Foundation 24*, 1–15.

Tilley, M., Upton, S. J., Blagburn, B. L. & Anderson, B. C. (1990). Identification of outer oocyst wall proteins of three *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) species by 125I surface labeling. *Infection and Immunity 58*, 252–253.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, USA 76*, 4350–4354.

Weibel, E. R. (1979). *Stereological Methods*. Academic Press, New York.