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Purification of Rickettsiales-like organisms associated with *Pecten maximus* (Mollusca: Bivalvia): serological and biochemical characterization

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ABSTRACT: Significant mortalities have been observed in populations of Saint-Jacques scallops *Pecten maximus*, in association with numerous intracellular colonies of Rickettsiales-like organisms (RLO) in the gills. Isolation of the procaryotes from infected tissues and their subsequent purification were achieved by a specific protocol based on differential and isopycnic centrifugations on sucrose and renografin density gradients. The purity and integrity of purified procaryotes were verified by electron microscopy. Specific polyclonal antibodies were prepared for serological characterization. Fifteen major constituent proteins were electrophoretically identified, their molecular weights ranging between 148 and 16 kD. Ten enzyme activities were biochemically identified, including acid phosphatase and catalase, which could be related to procaryote pathogenicity.

INTRODUCTION

Intracellular procaryotes have been reported in many invertebrate groups, but only a few have been extensively investigated, namely, those that are related to human, animal or plant pathology and transmitted by hematophagous or phytophagous insects and arachnids (Weiss 1981, 1982, Weiss & Dasch 1981). Studies on biological control of pests have also contributed to knowledge about some insect-specific procaryotes (Leu et al. 1989). The association of intracellular procaryotes with arthropod hosts appeared in some cases to be truly symbiotic. Because of the impact of infectious diseases on mollusc production in recent years, new interest has been devoted to intracellular procaryotes (Lauckner 1983). Morphologically, these procaryotes were related to the Chlamydiales (Moulder 1984) or the Rickettsiales (Weiss & Moulder 1984). In the majority of cases associated with mass mortalities, they were identified as Rickettsiales-like organisms (RLO). Among the molluscan species concerned, some cases were described in the Pectinidae family (Gulka et al. 1983, Elston 1986). Populations of *Pecten maximus* suffered heavy losses during recent winters in France, and the only detected microorganism that was a potential pathogen was a gill Rickettsiales-like organism (Le Gall et al. 1988). An epizootiological survey has subsequently been used to argue the pathogenic status of this organism; however, environmental factors may also be synergistically involved (Le Gall et al. 1991b). In order to better understand the significance of mollusc RLO in terms of pathogenicity, epidemiology and taxonomy, it is first necessary to isolate and characterize them.

In this paper, an original protocol is described for purifying the RLO from infected gills of *Pecten maximus*. Some protocols have been established for vertebrate Rickettsiales which can be cultivated in the yolk sac of chicken embryos (Dasch & Weiss 1977, Williams et al. 1981) or grown in tissue cultures (Weiss et al. 1975), but no prior methodology exists for purification directly from naturally infected invertebrate tissues.

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Because of the lack of molluscan cell lines for *in vitro* culture of specific intracellular procaryotes, the most obvious method for purifying RLO was to apply centrifugation techniques that have been successfully used to purify intracellular protozoans from infected oyster tissues (Mialhe et al. 1985, 1988).

The availability of highly purified procaryote suspensions permitted the preparation of specific polyclonal antibodies, the electrophoretic characterization of major proteins and the biochemical identification of enzymes, some possibly related to pathogenicity.

MATERIALS AND METHODS

Scallops. Infected 2 to 4 yr old Saint-Jacques scallops *Pecten maximus* were collected by dredging in the Saint-Brieuc Bay (Brittany, France), where Rickettsiales-like procaryote prevalences are maximal (Le Gall et al. 1991b). Healthy scallops were obtained from Norway (S. Mortensen).

Light and electron microscopy. For light microscopy, infected gill smears, homogenates or procaryote suspensions, at different steps of purification, were air-dried on glass slides and then fixed and stained with a Hemacolor kit (Merck). For electron microscopy, pellets of purified RLO were fixed in sodium cacodylate/HCl buffer (0.1 M), glutaraldehyde (1.25 %) and paraformaldehyde (2 %), pH 7.4. The osmolarity was raised to 1100 mOsm with sucrose and measured with an automatic cryoosmometer (Knauer). The specimens were then prepared and embedded in resin (LX 112) using an LKB ultraprocessor automat. Ultrathin sections were contrasted with lead citrate and aqueous uranyl acetate by means of an LKB ultrastainer automat. Grids were examined with a Jeol 1200 CX electron microscope.

Purification protocol. The scallop gills were thoroughly and vigorously washed with filtered (0.22 µm) seawater with Tween 80 added (0.1 %) (FSWT). The gills were then dissected and homogenized with an Ultra-Turrax homogenizer (Janke & Kunkel GmbH) in FSWT (100 ml per scallop). The homogenates were sieved successively through 300, 60 and 25 µm nylon meshes to remove large fibrous fragments and resulting suspensions were centrifuged (3500 × *g*, 30 min, 6 °C). The pellets were resuspended in FSWT with Ultra-Turrax and again centrifuged. The pellets were resuspended as described previously, and centrifuged through a 15 % (w/w) sucrose solution in FSWT (3200 × *g*, 30 min, 6 °C). The pellets were removed and centrifuged again on 50 % (w/w) sucrose cushion in FSWT (3200 × *g*, 30 min, 6 °C). The interface fractions were collected, diluted 5-fold very slowly in FSWT and pelleted (3500 × *g*, 30 min, 6 °C).

The resulting pellets were resuspended in HN buffer [HEPES (N-2-hydroxyethyl piperazine N'-2-ethane sulfonic acid) 20 mM, NaCl 0.5 M, pH 7.5, 1000 mOsm] and layered on 25 ml Renografin discontinuous gradient (14 % to 21 % to 28 %) (v/v) prepared from the commercial product Radioselectan 76 % (Schering Laboratories). For this purpose, 1 vol of Radioselectan 76 % (osmotic pressure 1800 mOsm) was diluted with 0.8 vol of distilled water to obtain a Renografin 42 % solution which was iso-osmotic with scallop hemolymph. The different concentrations were then easily prepared by diluting the Renografin 42 % with suitable volumes of HN buffer. After isopycnic centrifugation in a 50.2 Ti Beckman rotor (300 000 × *g*, 30 min, 6 °C), the major band at the 21 %–28 % interface was collected with a long needle and the fractions from all of the tubes were regrouped, diluted 5-fold with HN buffer to reduce the density of the Renografin solution and centrifuged (50 000 × *g*, 30 min, 6 °C) in order to pellet the purified procaryotes. The number of procaryotes was routinely determined by observation using a Malassez cell at 400 × magnification.

Specific polyclonal antibody preparation. Mice were immunized over a 4 wk period by intravenous and intraperitoneal injections of purified procaryote suspensions made at weekly intervals. Immunosera were collected 15 d after the third booster injection and clarified by centrifugation.

Indirect immunofluorescence (IIF). Gill smears of healthy and diseased scallops were air-dried and fixed in acetone (10 min). They were then overlaid with the immunoserum diluted from 1/10 to 1/5000 in IIF buffer (Institut Pasteur Diagnostic). After a 20 min incubation period at room temperature in a moist chamber, the slides were washed with IIF buffer and then overlaid with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin antiserum (Institut Pasteur Diagnostic) diluted (1/100) in IIF buffer with Evans Blue (0.01 %). The slides were again incubated in a moist chamber for 20 min and washed in IIF buffer. The slides were examined for bright green fluorescent RLO cells. The control antibodies consisted of sera from non-immunized mice.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed using the Phast-System (Pharmacia LKB). Purified procaryote suspensions (10^8 procaryotes ml^{-1}) were frozen, thawed and boiled (5 min) in sample buffer (4 % sodium-dodecylsulfate (SDS); 5 % β-mercaptoethanol). The procaryote samples and the molecular-weight markers (Pharmacia) were deposited (1 µl) on a polyacrylamide gradient gel ready to use (10 %–15 %) and current was then applied (10 mA, 15 °C). The gels were treated according to the protocol of Pharmacia LKB for silver staining.

Enzymatic activity characterization. Using the API

ZYM system (API SYSTEM, La Balme les Grottes, France), 19 enzymatic activities were examined from purified procaryote suspensions (10^8 procaryotes ml^{-1}) previously frozen, thawed and homogenized by ultrasound (100 W, 60 s). The wells of the API ZYM plate were filled with 65 µl of different dilutions of the procaryote stock homogenate. After incubation (37 °C, 4 h), the plates were treated according to the standard protocol of the supplier (API SYSTEM).

Acid phosphatase was also estimated by colorimetry using the degradation of phenylphosphate to phenol and phosphate at pH 4.9 (Kit Biomerieux). The reactions were performed with 100 µl of procaryote homogenate, equivalent to 10^7 cells. Moreover, the L-tartrate resistant acid phosphatases were estimated in parallel. The activities were expressed in nmol min^{-1} per 10^6 procaryotes by reference to a control equivalent to 20 Kind and King units (1 unit is equivalent to the enzyme quantity producing 1 mg h^{-1} at 37 °C).

Catalase was demonstrated by the production of oxygen resulting from the degradation of hydrogen peroxide. A drop of purified procaryotes (about 5×10^4) was mixed with hydrogen peroxide (3 %) and the production of bubbles was observed under binocular magnification.

RESULTS

Purification protocol

Histological study of this Rickettsiales-like organism has shown that the infection is limited to the gills (Le Gall et al. 1988). Thus, the purification protocol (Fig. 1) was performed only with gill tissues that had been thoroughly and vigorously washed with filtered seawater and 0.1 % Tween 80 detergent to eliminate microorganisms and inert material that contaminate the external surfaces of gills. Tween 80 detergent was selected because it had previously proved very efficient for limiting aggregation without any deleterious effect on protozoans like *Bonamia ostreae* (Mialhe et al. 1988). The gills were dissected and homogenized in FSWT with an Ultra Turrax tissue homogenizer, which is well suited for dissociation of scallop cells without damage to the RLO. Relatively large volumes of FSWT were used for the initial homogenization step (100 ml for the gills of one scallop) in order to obtain very fluid homogenates. The subsequent straining of the homogenates through different nylon meshes was a simple and efficient way to remove large amounts of fibrous materials. The resulting homogenates, as shown by light microscopic examination, contained many intact nuclei, amorphous material and numerous RLO. The first centrifugation eliminated soluble material and concentrated RLO. The second centrifugation produced pellets containing essentially particulate matter. Centrifugation of the resuspended pellets through a 15 % sucrose solution eliminated thinner fragments. At the 50 % sucrose stage the pellet contained primarily nuclei and the supernatant appeared opalescent. The RLO were located at the interface with nuclei. These fractions were collected and diluted with FSWT very slowly to avoid submitting the procaryotes to osmotic shock, and then centrifuged to pellet them.

During isopycnic centrifugation, the RLO formed a minor band at the 14 %–21 % interface containing primarily nuclei and formed a major band at the 21 %–28 % interface with only a few nuclei present. This last band was collected.

During the different steps of this purification pro-

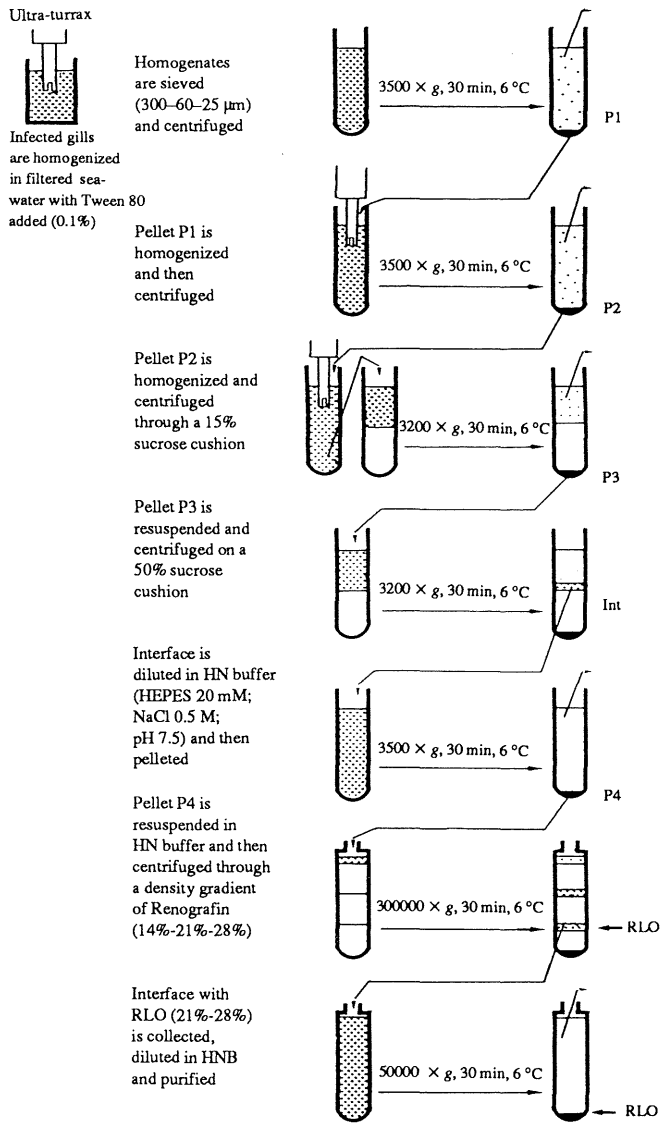

Fig. 1. Protocol for purification of Rickettsiales-like organisms (RLO) from infected gills of St-Jacques scallop *Pecten maximus*

tocol, the RLO were located in the different fractions by light microscopic examination of stained preparations, since this Rickettsiales-like procaryote can be easily recognized by its size and globular morphology. In these studies, the number of purified procaryotes per scallop varied, but was as high as 10^8.

Examination of purified products

Electron microscopy. Ultrastructural examination of the purified microorganisms allowed identification of characteristics, particularly the wall structure, which demonstrated that they were the gill Rickettsiales-like procaryotes of *Pecten maximus* (Le Gall et al. 1988). Moreover, the micrographs revealed the morphological integrity of purified procaryotes and the homogeneity of procaryote suspensions (Fig. 2).

Indirect immunofluorescence. The sera of immunized and control mice were tested against gill smears of healthy Norwegian and diseased French scallops. Specific recognition of procaryote cells without background noise was obtained for the dilution range between 1/100 and 1/1000. The optimal concentration was 1/500. No fluorescence was observed from healthy Norwegian scallops or with control sera.

Biochemical characterization of purified procaryotes

Electrophoretic pattern of proteins. Fifteen major proteins (P1 to P15) were identified by SDS-PAGE, the initial sample deposit being equivalent to about 10^5 procaryotes. Their molecular weights, estimated by comparison with the electrophoretic mobilities of reference proteins, ranged between 148 and 16 kD: P1, 148 kD; P2, 143 kD; P3, 100 kD; P4, 88 kD; P5, 74 kD; P6, 70 kD; P7, 52 kD; P8, 48 kD; P9, 39.5 kD; P10 34.5 kD; P11, 31 kD; P12, 24 kD; P13, 22.5 kD; P14, 20 kD; P15, 16 kD.

Table 1. Enzymatic activity characterization, for suspensions of Rickettsiales-like organisms purified from *Pecten maximus*, using the API ZYM system; index values are indicated

Enzymes	Abbreviation	Index
Alkaline phosphatase	ALP	+
Esterase (C4)	EST(C4)	++
Esterase lipase (C8)	ELIP	+
Lipase (C14)	LIP	0
Leucine arylamidase	LAA	+
Valine arylamidase	VAA	0
Cystine arylamidase	CAA	0
Trypsine	TR	0
α-Chemotrypsin	CTR	0
Acid phosphatase	ACP	+
Phosphoaminidase	PH	++
α-Galactosidase	αGAL	0
β-Galactosidase	βGAL	+
β-Glucuronidase	βGLU	0
α-Glucosidase	αGLC	0
β-Glucosidase	βGLC	0
N-acetyl-β-glucosaminidase	AGLA	+
α-Mannosidase	αMAN	0
α-Fucosidase	αFUC	+

Enzymatic activities. Only 9 enzymatic activities were detected from ca 6.5×10^6 isolated procaryotes. They were alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, phosphoaminidase, β-galactosidase, N-acetyl-β-glucosaminidase and α-fucosidase (Table 1). Among these

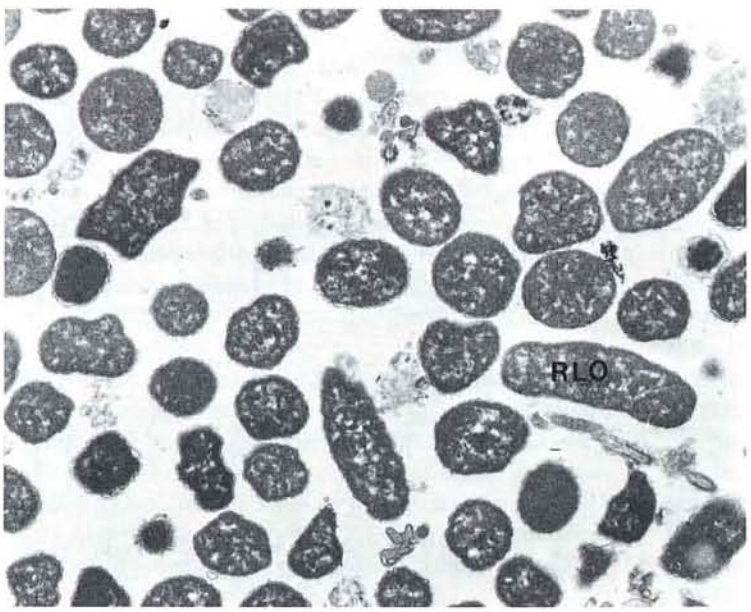

Fig. 2. Ultrastructure of purified Rickettsiales-like organisms (RLO) from *Pecten maximus*. Electron microscopy, × 10 000

enzymes, esterase and phosphoaminidase had the greatest activity.

The presence of acid phosphatase was confirmed using another commercially available microassay (Biomérieux), which allows better quantification of the enzymatic activities and an estimation of their sensitivity to L-tartrate. On the basis of 4 different analyses, the acid phosphatase activity was estimated at 17.7 (± 8.8) nmol min^{-1} per 10^6 procaryotes. Inhibition by L-tartrate was estimated at 26 % since the values were decreased to 13.2 (± 10.4) nmol min^{-1} per 10^6 procaryotes in the presence of this chemical. Additionally, catalase was detected on the basis of a quick degradation of hydrogen peroxide by procaryotes.

DISCUSSION

Electron microscope examination and specific immunofluorescence techniques demonstrated that the products of the purification protocol were the same procaryotes as the gill RLO associated with *Pecten maximus*. This is an original protocol in the field of mollusc pathology, for which several Rickettsiales-like procaryotes have been reported. It is unique because it demonstrates the purification of intracellular procaryotes directly from infected marine invertebrate tissue without previous *in vitro* culture, either into the yolk sac of chicken embryo or on cell lines as is usually necessary with vertebrate and insect rickettsias (Welburn et al. 1987). This may be advantageous for isolating RLO from different invertebrate groups for which the lack of cell lines continues to be a barrier to the study of intracellular pathogens. Two other Rickettsiales-like organisms found in 2 pectinids, *Chlamys opercularis* and *Patinopecten yessoensis*, have been purified in this manner (authors' unpubl.).

In view of the complex initial tissue homogenates, this protocol was very effective. This was confirmed by the specificity of immunosera, which did not need to be purified with acetone powder from healthy scallop tissues for eliminating antibodies directed to contaminant or scallop antigens.

The ability to obtain relatively large quantities of purified suspensions permitted biochemical characterization of the RLO. First, a protein pattern was established leading to identification of 15 major proteins whose molecular weights range between 148 and 16 kD. One of them, P9 (39.5 kD), may be related to the matrix protein from the outer membrane of gram negative bacteria (Lugtenberg et al. 1977). The use of the miniaturized system was very suitable for simple protein pattern recognition, as results were obtained with only 1 µl from a suspension of 10^8 procaryotes ml^{-1}. The use of Renografin was well suited for the study of rickettsial enzymatic activity here and in a previous study (Weiss et al. 1975) and appeared to have no effect on parasite metabolic and antigenic properties.

The use of the miniaturized API ZYM system was previously applied to biochemical identification of bacteria (Humble et al. 1977, Tharagonnet et al. 1977), *Trypanosoma* sp. protozoans (Kirkpatrick et al. 1985) or *Penicillium* fungi (Bridge & Hawksworth 1984). In this study several enzymatic activities were identified, 2 of which may be related to intracellular procaryote pathogenicity. The first one is catalase, which is involved in detoxification of hydrogen peroxide produced during phagocytosis (Roos 1980). This enzyme was previously demonstrated in *Coxiella burnetii*, an intravacuolar Rickettsiales of vertebrates (Akporiaye & Baca 1983). The second one is acid phosphatase, whose activity was quantified and sensitivity to L-tartrate was demonstrated. Several acid phosphatases have been characterized in intracellular protozoans belonging to *Leishmania* species (Gottlieb & Dwyer 1981), one of them being L-tartrate resistant. Its ability to reduce phagocytic cell production of toxic oxygen metabolites suggests that this enzyme may be important for intracellular survival of the parasite (Remaley et al. 1984). This hypothesis was recently confirmed for the *Pecten maximus* RLO in experiments to study the *in vitro* interactions between hemocytes and purified procaryotes (Le Gall et al. 1991a). These investigations of mollusc response to RLO illustrate one application of the purification protocol for studying the microbiocidal mechanisms of hemocytes and pathogen adaptations for intracellular survival (Moulder 1985).

The Rickettsiales-like procaryotes of *Pecten maximus* have been characterized biochemically and serologically and it may be assumed that these methodologies can be extrapolated to the microorganisms associated with other molluscan species. It would then become possible to better define the host range and the taxonomic positions of the molluscan Rickettsiales-like organisms within this class of procaryotes (Williams et al. 1981, Weiss 1982, Urakami et al. 1986, Weiss et al. 1987, Leu et al. 1989).

Finally, the ability to purify Rickettsiales-like procaryotes from molluscs makes possible experimental investigation of the mechanism of infection in terms of vertical and/or horizontal transmission.

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