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Characterization of abalone *Haliotis tuberculata–Vibrio harveyi* interactions in gill primary cultures

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Abstract:

The decline of European abalone *Haliotis tuberculata* populations has been associated with various pathogens including bacteria of the genus *Vibrio*. Following the summer mortality outbreaks reported in France between 1998 and 2000, *Vibrio harveyi* strains were isolated from moribund abalones, allowing in vivo and in vitro studies on the interactions between abalone *H. tuberculata* and *V. harveyi*. This work reports the development of primary cell cultures from abalone gill tissue, a target tissue for bacterial colonisation, and their use for in vitro study of host cell—*V. harveyi* interactions. Gill cells originated from four-day-old explant primary cultures were successfully sub-cultured in multi-well plates and maintained in vitro for up to 24 days. Cytological parameters, cell morphology and viability were monitored over time using flow cytometry analysis and semi-quantitative assay (XTT). Then, gill cell cultures were used to investigate in vitro the interactions with *V. harveyi*. The effects of two bacterial strains were evaluated on gill cells: a pathogenic bacterial strain ORM4 which is responsible for abalone mortalities and LMG7890 which is a non-pathogenic strain. Cellular responses of gill cells exposed to increasing concentrations of bacteria were evaluated by measuring mitochondrial activity (XTT assay) and phenoloxidase activity, an enzyme which is strongly involved in immune response. The ability of gill cells to phagocyte GFP-tagged *V. harveyi* was evaluated by flow cytometry and gill cells-*V. harveyi* interactions were characterized using fluorescence microscopy and transmission electron microscopy. During phagocytosis process we evidenced that *V. harveyi* bacteria induced significant changes in gill cells metabolism and immune response. Together, the results showed that primary cell cultures from abalone gills are suitable for in vitro study of host-pathogen interactions, providing complementary assays to in vivo experiments.

Keywords: *Haliotis tuberculata* ; *Vibrio harveyi* ; Gill cell culture ; Pathogenicity ; Phenoloxidase ; Phagocytosis

1. Introduction

Diseases affecting cultured molluscs are caused by protozoans, fungi, bacteria or viruses, leading to severe damages at any stage of production from larvae to adults (Paillard et al. *2004*). Bacterial diseases like Brown Ring disease (BRD) and Juvenile oyster disease (JOD) caused high mortalities in hatcheries and were commonly described in larval and adult bivalves (Lauckner *1983*; Paillard et al. *2004*; Paillard and Maes *1989*; Boettcher et al. *1999*, *2000*). Summer mortalities caused by Vibrio bacterial strains have recently been described in various cultured mollusc as the Pacific oyster Crassostrea gigas (Le Roux et al. *2002*; Saulnier et al. *2010*) and the European abalone Haliotis tuberculata (Nicolas et al. *2002*). European abalone stocks are currently threatened by several pathogens causing severe mass mortalities in wild or farmed populations, among them Xenohaliotis californiensis, Haplosporidium sp. and Vibrio harveyi (Nicolas et al. *2002*; Azevedo et al. *2006*; Balseiro et al. *2006*; Huchette and Clavier *2004*).

Vibrio harveyi (Syn. V. carchariae) is a marine bacterium common in the ocean (Gauger and Gomez-Chiarri *2002*), and known to be pathogenic in a large range of vertebrates and invertebrates including molluscs (Austin and Zhang *2006*). Abalone diseases due to the pathogen V. harveyi have been already described for Haliotis diversicolor (Nishimori et al. *1998*), for Haliotis laegivata (Handlinger et al. *2005*; Vakalia and Benkendorff *2005*; Dang et al. *2011a*) and for H. tuberculata (Nicolas et al. *2002*), in tropical or temperate seawater, causing septicaemia. Since 1998, summer mass mortality outbreaks of the European abalone H. tuberculata reported along the north coast of Britanny (France) have been associated with Vibrio bacteria (Nicolas et al. *2002*). Bacterial strains were isolated from both farmed and wild abalones, allowing in vivo and in vitro studies on the interactions between abalone H. tuberculata and V. harveyi (Nicolas et al. *2002*; Travers et al. *2008b*, *2009*). Vibrio harveyi is an opportunistic bacterium becoming pathogenic if immunity is compromised due to summer heat stress, poor water quality or reproduction period (Cheng et al. *2004a*, *b*; Vakalia and Benkendorff *2005*; Hooper et al. *2007*; Travers et al. *2008b*; Mottin et al. *2010*; Dang et al. *2011a*, *b*; Latire et al. *2012*). Considering the development of aquaculture in Europe (Huchette and Clavier *2004*), the evolution of

reliable diagnostic tools is of major importance for the control and prevention of bacterial diseases (Paillard et al. 2004). Cell cultures provide alternative and controlled experimental models for basic research as well as with various applications in pathology, ecotoxicology and natural compounds production (Rinkevich 1999, 2011, Auzoux-Bordenave and Domart-Coulon 2010). Although there is currently no cell lineage in marine invertebrates, primary cell cultures provide suitable tools for complementary *in vivo* studies. Invertebrate immune defence is mainly mediated by circulating hemocytes (Smith 1991), including phagocytosis, encapsulation, production of antimicrobial compounds as well as the phenoloxidase cascade that is an important reaction of melanisation involved in immunity (Cheng 1981, Bachère et al. 1995, Hooper et al. 2007, Travers et al. 2008c).

Antibacterial activity of abalone hemolymph has been demonstrated against *Vibrio* using *in vitro* assays (Vakalia and Benkendorff 2005, Travers et al. 2009, Dang et al. 2011a, 2011b) such as phagocytosis, ROS activity or phenoloxidase activity, and secretion of antibacterial peptides or lipophilic antibacterial compounds. The immune response of abalone hemocytes has been previously shown to change according to the pathogenicity of bacterial strains (Travers et al. 2009). Thus pathogen strains would be able to proliferate in contact with hemolymph avoiding phagocytosis and preventing immune response. Moreover, only few host responses were investigated based on cell morphology or using cultured hemocytes (Nicolas et al. 2002, Travers et al. 2008c). While primary culture of hemocytes have been previoulsy used for studying host-pathogen interactions at a cellular level (Boulo et al. 1991, Ford and Ashton-Alcox 1993) there are only few assays using other target tissues such as mantle, digestive gland or gills (Dang et al. 2011b, Faucet et al., 2003).

The present work aimed at studying abalone *Haliotis tuberculata*-*Vibrio harveyi* interactions in gill primary cultures. Explant cultures were developed from the gills, a target tissue for bacterial infection, and subcultured to perform *in vitro* pathogenicity assays. The characterization of cell morphology, viability and the physiological status of the cells were monitored over culture time using flow cytometry and semi-automated assay (XTT). Gill cell cultures were used to investigate *in vitro* the mode of action of two bacterial strains of *V. harveyi*. The responses of gill cells to *V. harveyi* bacteria, including phagocytosis, were evaluated by using a combination of semi-quantitative enzymatic *in vitro* assays (XTT, phenoloxidase), flow cytometry analyses, as well as fluorescence and transmission electron microscopy analysis.

Material and methods

Animals

European adult abalone (*Haliotis tuberculata*), 40 to 60 mm shell length, were collected from the France-Haliotis farm (Plouguerneau, Brittany). The animals were maintained at the laboratory in an 80 L tank supplied with aerated natural seawater. Abalones were fed once a week with red algae *Palmaria palmata*. Two days before experiments, abalones were starved and seawater was UV treated to prevent contamination.

Bacterial strains

Two *Vibrio harveyi* strains were used for *in vitro* pathogenicity assays: a virulent strain ORM4 isolated from moribund abalone in 1998 (Nicolas et al. 2002) and a non pathogenic strain LMG7890 (former *V. carchariae* type strain) isolated from dead brown shark *Carcharinus plumbeus* (Colwell 1982). For microscopic observations and bacterial phagocytosis assays, GFP-tagged *V. harveyi* strains were used (Travers et al. 2008a).

The two bacterial strains were cultured on Luria Bertani Agar (Sigma, St. Louis, MO, USA) supplemented in salinity (to 20 $g.L^{-1}$) and incubated at 28°C for 24h. For *in vitro* contact, bacterial strains were washed in sterilized seawater. Bacterial concentrations were determined by optical density measurements at 490 nm. For all tests controls consisted of cells without bacteria, and cells with heat inactivated bacteria.

Gill cell primary culture

Primary cultures were performed according to the explant method previously developed for mollusc mantle culture (Auzoux-Bordenave et al. 2007) and recently adapted to the gills by Gaume et al. (2012). The gills were aseptically dissected and placed for 2 days in an antiseptic solution (= natural filtered sea water containing 200 U/mL penicillin–200 lg/mL streptomycin (Sigma), 250 lg/mL gentamycin (Sigma) and 2 lg/mL amphotericin B (Sigma)). Tissues were then minced into 2-3 mm^3 explants which were placed to adhere onto the bottom of plastic 6-well plates. After 30 min of adhesion, explants were covered with 1 mL modified Leibovitz (Sigma) L-15 medium adjusted to 1100 $mosm.L^{-1}$ by addition of mineral salts and supplemented with 100 $U.mL^{-1}$ penicillin-streptomycin (Sigma), 200 $\mu g.mL^{-1}$ gentamycin (Sigma) and 1 $\mu g.mL^{-1}$ amphotericin B (Sigma) to limit external contamination. The pH was adjusted to 7.4. Explant primary cell cultures were incubated at 18°C in a humidified incubator and observed daily with an inverted phase contrast microscope (Telaval 3, Jena).

After 4-5 days of primary culture, a large quantity of gill cells was generated from the explants and the cells were collected. Cell suspension was strained on a 70µm mesh filter (Cell strainer, VWR Internat.), resuspended in modified L-15 medium and assessed for cell density using a hemocytometer. Cells were subcultured in multi-well microplates to perform *in vitro* assays. For optimal cellular response, cell density was adjusted to 500,000 cells/well in 24-well microplates (Dutscher) and 150,000 cells/well in 96-well microplates (Dutscher).

Cell population distribution

Analyses were performed using FACS Calibur flow cytometer (Becton Dickinson) equipped with a 488nm laser. 1000 events were counted for each sample. Results are expressed as cell cytogram indicating the size (FSC value), the complexity (SSC value), and the level of fluorescence. Cells were incubated for 30 min with SYBR Green fluorescent

dye (Molecular Probes) at 10^{-3} dilution of the commercial stock solution. Gill cells were gated to estimate the population distribution in terms of forward scattering (FSC, size) and side scattering (SSC, granularity and volume).

Phagocytosis assay

Phagocytosis assays were performed by flow cytometry according to the protocol for hemocytes adapted from Allam et al. (2002). After four days explant culture, gill cells were collected and diluted in L-15 culture medium. Phagocytosis index was defined by the percentage of cells ingesting three or more fluorescent beads, and was measured by flow cytometry. 10µl of bead solution (Fluorospher, Invitrogen) was added to 200 µl of cell suspension (700 beads per cells), and were incubated for 1 hour at room temperature in the dark. Flow cytometry analysis was performed as described previously. Results are expressed as cell cytogram indicating the size (FSC value), the complexity (SSC value), and the level of fluorescence.

To measure the ability of gill cells to phagocyte the two *Vibrio harveyi* strains, the same protocol was used. GFP-tagged bacterial strains were added to the culture and incubated at room temperature in the dark for 1 hour, 2 hours and 20 hours. Fluorescence was measured by flow cytometry. Data obtained were the means ±SE of three values expressed as percentage of fluorescence, and statistical analysis was performed using a two-sample *t*-test, with significance at $P<0.005$.

XTT Reduction Assay

Cellular activity was measured by the XTT assay (Roche Laboratories, Meylan, France) based on the reduction of a tetrazolium salt (XTT-sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) into yellow formazan salt by active mitochondria (Mosmann 1983). The XTT assay, previously adapted to mollusc cells, provides a global evaluation of the number of viable cells through the measurement oftheir mitochondrial activity (Domart-Coulon et al. 2000). Assays were performed in 96-well microplates. For this, 50 lL of a mixture of XTT/PMS (N-methyl dibenzopyrazine methyl sulfate) was added to 100 µL of cells/well. Plates were incubated for 20h at 18°C, and the intensity of the coloration was measured by spectrophotometry at 490 nm with a 655 nm reference wave-length (BioTek plate reader). For *in vitro* assays, bacterial concentration was adjusted in seawater, and 50 µL of this mixture were added to the cell culture for 1, 5 and 20 hours. After the required incubation time, XTT assay was performed as described before.

Data obtained were the means ±SE of six values expressed as optical density (OD). The effects of bacteria on cell viability were expressed as percentage of the control (cells without contaminant). Statistical analysis was performed using a two-sample *t*-test, with significance at $P<0.005$.

Phenoloxidase Activity

Since the phenoloxidase enzyme is strongly involved in immune response, the assessment of its activity is frequently used to evaluate the cell response to external contaminants (Bachère et al. 1995, Cerenius et al. 2008, Hellio et al 2007). Phenoloxidase activity assays were performed in 96-well microplates. 50 µL of TrisHCl buffer (0.2M, pH 8.0) and 10 µl L-DOPA (L-3,4-dihydrophenyl-alanine, 20 mM, Sigma) were added to supernatant of 200000 cells. The microplate was mixed gently for 10s prior measurement. Detection of phenoloxidase activity was carried out by the measurement of L-3-4-dihydroxyphenylalanine (L-DOPA) transformation in dopachromes. The colorimetric reaction was measured in triplicate at room temperature every 10 min for 100 min at 492 nm, and expressed as changes in optical density per mg of protein. Controls consisted of TrisHCl and L-DOPA without cells or bacteria, and were performed in parrallel assays. Protein amount in each test, cellular extract and controls, were determined by the Bradford method (Bradford 1976), with serum albumin as a standard. Data obtained were the means ±SE of six values expressed as OD. The impact of bacteria on the phenoloxidase activity was expressed as percentage of control (cells without contaminant). Statistical analysis was performed using a two-sample *t*-test, with a significant at $P<0.005$.

Fluorescecet microscopy

Gill cells, four days old subcultured, were incubated in the presence of the two GFP-tagged bacterial strains for one or two hours and immediately observed with a direct fluorescence microscope (Olympus).

Transmission electron microscopy

Four days old subcultured gill cells were incubated for 2 h with V. harveyi bacterial strains. For ultrasructural analysis, gill cells were fixed with 2% glutaraldehyde in 0.1 M Sörensen phosphate buffer containing 0.6 M sucrose at pH 7.4. Cell samples were post-fixed in 2% OsO4 in So¨rensen phosphate buffer (0.18M = 5.33g/L NaH2PO4.2H2O (Sigma) and 20,7g/L Na2HPO4 (Sigma) in distilled water (pH 7.4)) for 1 h at room temperature, then rinsed three times in So¨rensen buffer, dehydrated in ethanol and embedded in Spürr's resin (Electron Microscopy Sciences, Aygnesvives, France). Ultra-thin sections (about 50 nm)were cut on an Ultracut Microtome (Ultramictomes Reichert-Jung Ultratome E) with a diatome Ultra 45° diamond, and mounted on formavar-coated Cu-grids (Electron Microscopy Sciences), stained with uranyl acetate 2% in 50% ethanol and observed at 75kV withHitachiH-7100 transmission electron microscope (Tokyo, Japan) equipped with a digital CCD Hamamatsu Camera.

Results

Cell culture from abalone gills

After one day of primary culture, 80% of the explants adhered to the flask bottom and gill cells started to migrate out of

192 the explant (Fig 1a). During the first three days of culture, a large quantity of gill cells was generated from the explants
193 and the cells were spread out onto the bottom of the flask. Gill cell population consisted of a majority of rounded
194 epithelial cells (Fig 1a), glandular cells (Fig 1b) and hemocytes (Fig 1c). The ciliary activity of epithelial cells within
195 the gill filaments demonstrated the vitality of explant primary cultures, according to previous observations from Gaume
196 et al (2012). Typical hemocyte morphological cell types were recognized *in vitro* according to previous studies on
197 abalone hemocytes (Auzoux-Bordenave et al. 2007, Travers et al. 2008c): the majority appeared small round and
198 brightly coloured, amoeboid-like, and fiboblastic-like (Fig 1c), most of them adhering strongly to the plastik flask
199 bottom.
200 Ultrastructural observations of cultured cells by TEM allowed the characterization of different cell morphotypes: gill
201 cells consisted of ciliated epithelial cell with cilia, microvilli and numerous mitochondria (Fig 1d) while glandular cells
202 contained mucopolysaccharide vesicles (Fig 1e). Hyalinocytes exhibited a large round nucleus containing electron
203 dense chromatin nucleoli (Fig 1f) and granulocytes contained electron dense granules and exhibited pseudopodia (Fig
204 1g).
205 After 4 to 5 days of primary culture, cells originated from the explants were subcultured in multi-well plates for *in vitro*
206 assays. Subcultured cells exhibited the same morphotypes all along culture time, fibroblastic cells being less numerous
207 compared to the explants culture.
208 Flow cytometry analysis allowed the comparison of freshly isolated gill (Fig. 2a) cells and 3-day-old subcultured gill
209 cells (Fig. 2b). Results showed that freshly dissociated gill cells were mainly composed of small cells with a relative
210 low intracellular complexity. In contrast, subcultures were mainly composed of larger cells harbouring relatively high
211 intracellular complexity, showing the selection of cells following the transfer in subculture.
212 Gill cells were successfully subcultured in multi-well plates and cell viability was evaluated by the XTT assay. A linear
213 relationship between mitochondrial activity and cell number was previously evidenced at any time of cell culture (data
214 not shown). Figure 2c shows the XTT response of subcultured gill cells as a function of cell density, over a 24 days
215 period. The evolution of XTT response (absorbance) showed a slight decrease during the first three days, and an
216 increase in global cell metabolism with cell density overtime. The two concentrations tested, 100,000 and 200,000 cells
217 per well, allowed to adjust the optimal cell density for further *in vitro* assays to 500,000 cells per well in 24 well-plates
218 and to 150,000 cells per well in 96 well-plates respectively.
219
220 *Phagocytosis*
221 After 3-4 days of explant cell cultures, cells were collected, and were brought into contact with fluorescent beads or
222 with pathogenic and non pathogenic *V. harveyi* at different concentrations to evaluate their phagocytosis capacity. Cell
223 population distribution was estimated according to the size (FSC, forward scattering) against complexity (SSC, side

scattering) of gill cells stained with SYBR Green after 1 h of contact with fluorescent beads (Fig. 3a). Gill cells which ingested three or more beads displayed a bigger size and a higher complexity than cells which did not ingest beads. Smaller cells did not absorb fluorescent beads. Gill cells were able to phagocyte more than 80% of fluorescent beads as estimated by the phagocytosis index after one hour of incubation (Fig. 3a), and up to 70% of pathogenic and non pathogenic bacterial strains after two hours of contact (Fig 3b, c). For concentrations of 25 or 75 bacteria per cell, the percentage of phagocytosis was significantly increased between one and two hours, with a higher phagocytosis for LMG7890 than for ORM4. When bacteria were placed into saturation relative to gill cell density (100 bacteria per cell) the same phagocytosis ability was observed for the two bacterial strains.

Bacterial phagocytosis by gill cells was investigated by TEM and fluorescent microscopy using GFP-tagged bacteria. Control cells did not display any bacteria, either on the outside or the inside of cells. After two hours of contact with both bacterial strains, many bacteria were present around the cells while some of them appeared internalized within the host cells (Fig 4). Some cells appeared to be damaged. Two cell morphotypes were able to phagocytose *Vibrio*: the hemocytes (Fig. 4a) and the epithelial cells (Fig. 4b). Figure 4b shows an encapsulated bacterium in the cytoplasm, residual body after digestion, and condensed mitochondria in an hyperphosphorylation oxydative state. Details of endocytosed bacteria were observed in epithelial cells, with lysosome I digesting bacteria and lysosome II dispensing their enzymatic content (Fig 4c). Fluorescence microscopy observations with GFP-tagged bacteria allowed to localize *V. harveyi* bacteria in close proximity to the host epithelial cells and potentially inside the host cell cytoplasm after two hours of contact (Fig 4d). Similar observations were made for the pathogenic ORM4 and the non-pathogenic LMG7890 bacterial strains.

Effect of V. harveyi on gill cell viability

The effects of *Vibrio harveyi* bacterial strain on gill cell metabolism were evaluated by the XTT assay (Fig 5). Gill cells from 4-day-old subcultures in 96-well plates were exposed to increased concentrations of bacteria (heat inactivated bacteria and 10, 50 and 100 live bacteria per cell), and their XTT responses were measured up to 20 hours, and compared to the metabolism activity of gill cell control. Figure 5 showed that metabolic activity of gill cells decreased in the presence of the two bacterial strains from the first hour, whereas it remained constant for 20 hours for control cells and cells supplemented with dead bacteria. The metabolic activity of gill cell was shown to significantly decrease as a function of bacterial concentration. At a concentration of 100 bacteria per cell XTT response of gill cell decreased to 90% after 20 hours of contact with the pathogen *V. harveyi* ORM4 and 80% with the non pathogen LMG7890. A decrease of 50% of viability was observed at a density of 50 bacteria per cell after 5 hours of contact with ORM4, while this level was reached only after 20 hours with LMG7890, even with a density of 100 bacteria per cell.

Effect of V. harveyi on phenoloxidase activity

The immune response of gill cells exposed to bacteria was measured by the phenoloxidase activity, an enzyme strongly involved in the immune response. Phenoloxidase activity of gill cells remained stable for 18 hours without bacterial strains or in presence of dead bacteria, but strongly decreased in a dose dependant manner in presence of the pathogenic and the non pathogenic *V. harveyi*, as shown in Figure 6. For low concentrations (density of 25 bacteria per cell) phenoloxidase activity was slightly reduced during the first five hours, but a significant decrease of phenoloxidase activity was shown after 18 hours of contact. Phenoloxidase activity decreased after 5 hours of contact, whith a reduction of 40 to 60% of phenoloxidase activity with concentration of 100 and 200 bacteria per cell respectively, for both strains compared to the control. Similar results were obtained for the pathogenic and the non pathogenic *V. harveyi*.

Discussion

In this work, we developed primary cell cultures from *Haliotis tuberculata* gills, which are a target tissue for bacterial infection in marine mollusks. As previously observed in primary cultures of mollusc tissues, the explant method provided a suitable *in vitro* model that contained all the cell types present in the tissue of origin (Kleinschuster et al. 1996, Auzoux-Bordenave and Domart-Coulon 2010). Gill cells were successfully sub-cultured for up to 24 days with a persistent metabolic activity as demonstrated by the XTT assay. The decrease of mitochondrial activity over the first four days following subculture initiation may be attributed to the adaptation of cells to *in vitro* conditions. Abalone gill cell primary culture was first performed by Suwattana et al. (2010) on *Haliotis asinina*. These authors reported viable cell cultures for up to 28 days but observed many contaminations due to the initial presence of bacteria in gills before culture. Contaminations by bacteria or fungi remain a real problem in primary cell culture, especially for tissues that are directly exposed to seawater such as the gills and the mantle (Rinkevich 1999, Van der Merwe et al. 2010). In the present work, the addition of antimicrobial components to the culture medium allowed to prevent bacterial and fungal proliferation *in vitro* without damaging gill cell viability.

Gill cell cultures exhibited the main morphotypes previously described in mollusc gills and hemocytes *in vivo*, ie. epithelial cells, including glandular cells, and the two categories of hemocytes, i.e. hyalinocytes and granulocytes (Auzoux-Bordenave et al. 2007; Travers et al. 2008c, de Oliveira et al. 2008, Manganaro et al. 2012).

Because circulating hemocytes are the first target cells involved in the immune response of marine molluscs, previous studies mainly focused on the interactions between abalone hemocytes and *Vibrio* bacteria. *In vitro* immunity assays based on hemocytes culture allowed to evidence the antibacterial activity of abalone hemocytes exposed to *Vibrio* species (Vakalia and Benkendorff 2005, Dang et al. 2011b, Travers et al. 2009). Significant variations of antibacterial activity of hemocytes were observed depending on environmental conditions such as temperature, quality of seawater,

diet, or spawning period (Travers et al. 2008b, Mottin et al. 2010, Dang et al. 2011a, b). Furthermore, immune depression and antibacterial activity were reported in abalones affected by viruses or bacteria in hemolymph but also in the digestive gland (Travers et al. 2009, Dang et al. 2011a).

Since the gills are in direct contact with seawater, they represent a target tissue for the entrance and adhesion of bacteria. Moreover the mucus covering the gill filaments may attract or serve as nutrient for various microbes (Rosenberg and Falkovitz 2004, Sharon and Rosenberg 2008). Although the entrance routes of *Vibrio harveyi* bacteria in abalone have not yet been identified, recent studies evidenced that the gills might be directly infected by bacteria (Travers et al. 2008a). In decapod crustacea, the gills have been shown as the main site of bacterial accumulation, suggesting a role of this tissue in the interactions with pathogens (Martin et al. 1993, Alday-Sanz et al. 2002, Burgents et al. 2005).

Using cell cultures from gill tissues, the present work aimed at studying the interactions between *Vibrio harveyi* bacteria and gill cells from the abalone (*Haliotis tuberculata*). Flow cytometry analysis combined with microscopic observations evidenced that cultured gill cells were able to phagocyte both pathogenic and non pathogenic *V. harveyi* strains. Vibrios are known to produce compounds that inhibit host hemocyte responses and especially phagocytosis (Labreuche et al. 2006, Allam and Ford 2006). Furthermore, Vibrio bacteria develop rapidly in the presence of hemocytes and have been shown to interact directly with the hemocytes, preventing immunity and phagocytosis (Travers et al. 2009). Phagocytosis is one of the first lines of defence in molluscs and is mainly performed by hemocytes. Fluorescent microscopy and TEM analysis allowed the identification of internalized bacteria both within hemocytes and epithelial cells. The presence in infected cells of condensed mitochondria in hyperphosphorylation oxydative state demonstrated a high activity induced by bacterial phagocytosis. Furthermore, the identification of lysosomes within the host cells, provided further evidence of bacterial endocytosis *in vitro*. Lysosomes, which are involved in the degradation of the bacterial cell wall, play a crucial role during phagocytosis by secreting hydrolytic enzymes. The use of lysosomes as biomakers to assess the cytotoxicity of external contaminants has been previously reported on abalone hemocytes (Latire et al. 2012).

The gill cell capacity of phagocytosis, measured by flow cytometry was upper than 80%. Only after two hours of contact with the two bacterial strains, gill cells were able to phagocyte 70% of bacteria. Travers et al. (2009) reported an increase in the capacity of hemocytes to phagocyte fluorescent beads in the presence of the non-pathogenic LMG7890 bacterial strain. In the same way, we observed that the phagocytic ability of gill cells was higher in the presence of the non-pathogenic strain compared to the pathogenic one. Despite the ability of abalone cells to phagocyte *V. harveyi*, our study evidenced that both viability and immune response of gill cells were directly affected by the two bacterial strains. Although the LMG7890 strain is not pathogenic for abalones *in vivo*, Travers et al. (2009) reported that a direct injection could induce mortalities, but to a lower extent compared to ORM4 strain. In this study, the presence of

pathogenic and non-pathogenic bacteria significantly decreased the cell viability in a concentration dependent manner, as measured by metabolic activity assay (XTT). The sharp decrease of cell viability observed in the presence of ORM4 bacteria confirmed the virulence of this strain against abalone gill cells. These results differed from those obtained in abalone hemocytes, showing that ORM4 bacterial strain had no significant effect on hemocytes viability, even after 28 hours of contact (Travers et al. 2009).

In addition to the effects on global cell metabolism, our results demonstrated that the two bacterial *V. harveyi* strains induced a significant decrease of the phenoloxidase activity *in vitro*. Phenoloxidase has been previously detected in the hemolymph of several invertebrates and its activity has been shown to be directly involved in the immune response (Coles and Pipe 1994, Lacoue-Labarthe et al. 2009). Recent studies reported the use of phenoloxidase activity to measure the immune response of abalone hemocytes exposed to various environmental stressors such as temperature, salinity, trace metals and pathogens (Cheng et al. 2004a, b, Travers et al. 2008b, Mottin et al. 2010, Dang et al. 2011b). Since hemocytes are very abundant in gill tissues, the phenoloxidase activity measured *in vitro* may be partly due to the hemocytes. Nevertheless, our results are consistent with previous studies using phenoloxidase activity as a marker to measure the immune response of mollusc to external contaminants (Bachère et al. 1995, Hellio et al. 2007).

In this study, we evidenced that primary cultures of abalone gills provide suitable models for *in vit*ro pathogenicity assays. This work demonstrates for the first time that *V. harveyi* bacteria induced significant alterations on gill cells viability and immune responses, although important phagocytic activity was detected in these target cells. Further studies using other immune indicators such as ROS activity, combined with lysosome quantification in target cells will help specify the mode of action of *Vibrio harveyi* on abalone cells.

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List of figures

Figure 1. Abalone gill cell primary cultures observed under inverted phase contrast microscope (a, b, c) and transmission electron microscopy (d, e, f, g). a: One-day-old gill explant primary gill cells culture from *Haliotis tuberculata* showing the outgrowth of cells from an explant in a 6-well plate, b: Three day-old explant primary culture

showing cell spreading around the flask bottom. Cell population consisted of rounded epithelial cells (ec), and glandular cells (gc) c: Three day-old explant culture showing hemocytes represented by small hyalinocytes (b) and fibroblastic like cells (f). Transmission electron micrograph of four day-old cell culture, d: epithelial cell, e: mucous cell, f: hyalinocyte, g: granulocyte. ci: cilia, nu: nucleus, mi: microvilli, mu: mucous vesicle, p: pseudopodia of the phagocytosis vesicle in formation, lv: lipofuschin vesicles.

Figure 2. Flow cytometry analysis and mitochondrial activity of gill cells of *Haliotis tuberculata*. Characteristics of cell popoulations of freshly mechanically dissociated gill (a) and gill cells after 3 days in subculture (b), size (FSC) against internal complexity (SSC) density plot representation, after 30min of incubation with SYBR Green. Evolution of the XTT response of gill cells in subculture (c) at two densities: 100,000 and 200,000 cells/well in 96-well microplates. Data are the means ± SE of ten values expressed as OD 490nm/655nm.

Figure 3. Phagocytosis by *Haliotis tuberculata* gill cells estimated by flow cytometry. Phagocytosis index was expressed as percentage of gill cells containing three or more beads after one hour of contact (a). Percentage of phagocytosis of gill cells in presence of the pathogenic ORM4 (b) and the non-pathogenic LMG7890 (c) bacterial strain after one and two hours of contact with three densities of bacteria: 25, 75 and 100 bacteria/cell. Data are the means ± SE of triplicate experiment. Significant differences ($p<0.05$) from absorbances measured in control cells are indicated by an asterisk.

Figure 4. Phagocytosis by *Haliotis tubeculata* gill cells after two hours of contact observed by TEM and fluorescent microscopy. a: internalization of ORM4 bacteria by hemocytes, b: cytoplasm of an epithelial cell showing internalized bacteria, lysosomal body and condensed mitochondria, c: detail of lysosome digesting bacteria, d: fluorescent micrograph showing ORM4-GFP tagged bacteria in close association with gill cell cytoplasm. b: bacteria around cells, ec: epithelial cell, lb: lysosomal body, pl: primary lysosome, sl : secondary lysosome , nu: nucleus, m: mitochondria, ml: lysosomal multilamellar membrane, p: pseudopodia of the phagocytosis vesicle, arrows: internal bacteria.

Figure 5. Effect of the pathogenic strain ORM4 (a) and the non-pathogenic LMG7890 strain (b) of *V. harveyi* on the metabolic activity of gill cells. XTT response from 4-day-old subcultures after 1, 5 and 20 hours of contact with dead bacteria (D), and for ratio of 10, 50 and 100 bacteria/cell. Data are the means ± SE of six values of two independent experiments. Significant differences ($p<0.05$) from absorbances measured in control cells are indicated by an asterisk.

Figure 6. Effect of the pathogenic strain ORM4 (a) and the non-pathogenic LMG7890 strain (b) *V. harveyi* on immune

response of gill cells, measured by the phenoloxidase activity (PO) after 1, 5 and 18 hours of contact with bacteria killed by boiling (D), and for ratio of 25, 75, 100 and 200 bacteria per cell. Data are the means ± SE of three values of independent triplicate experiment. Significant differences ($p<0.05$) from control cells are indicated by an asterisk.

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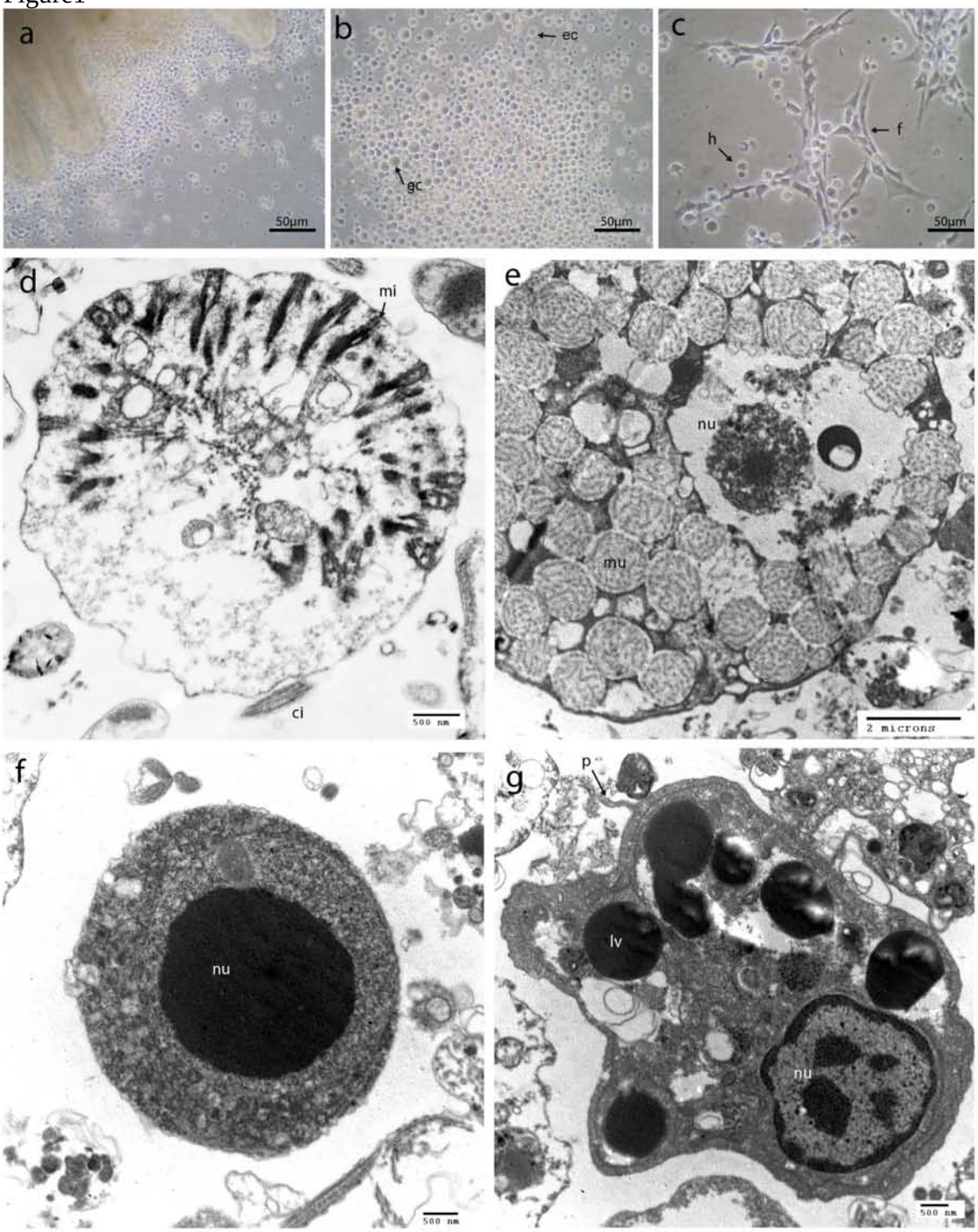

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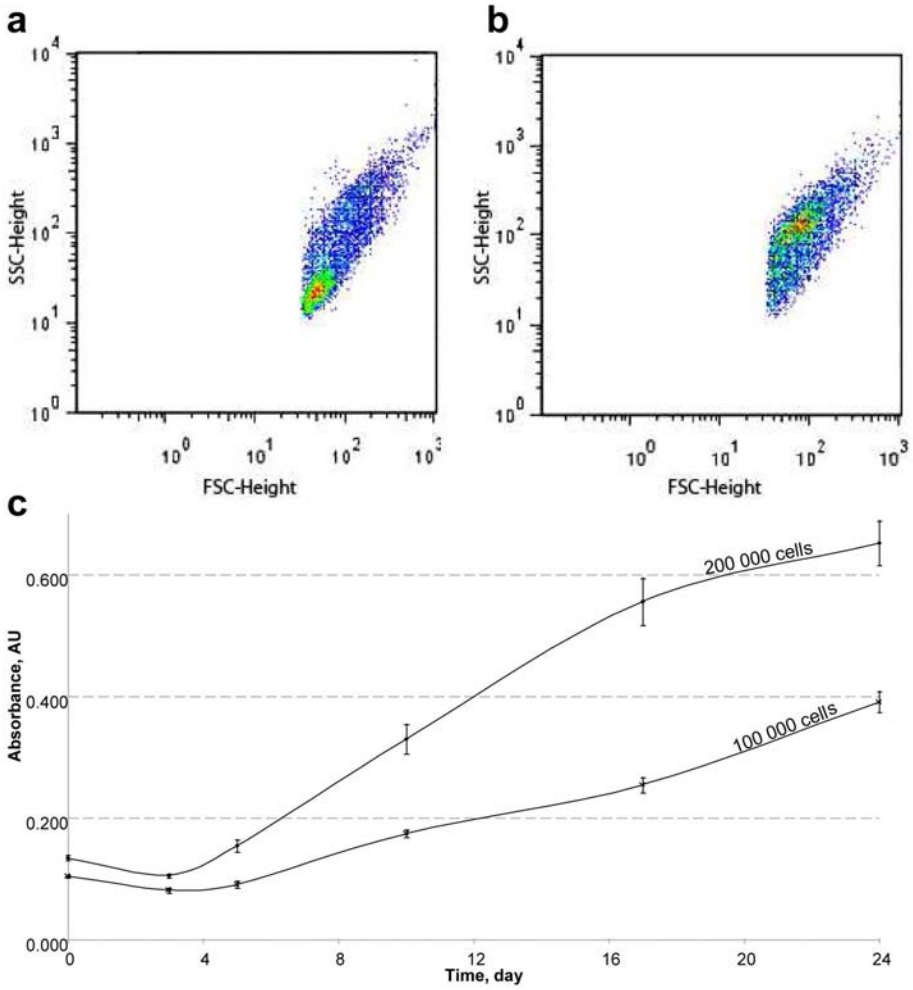

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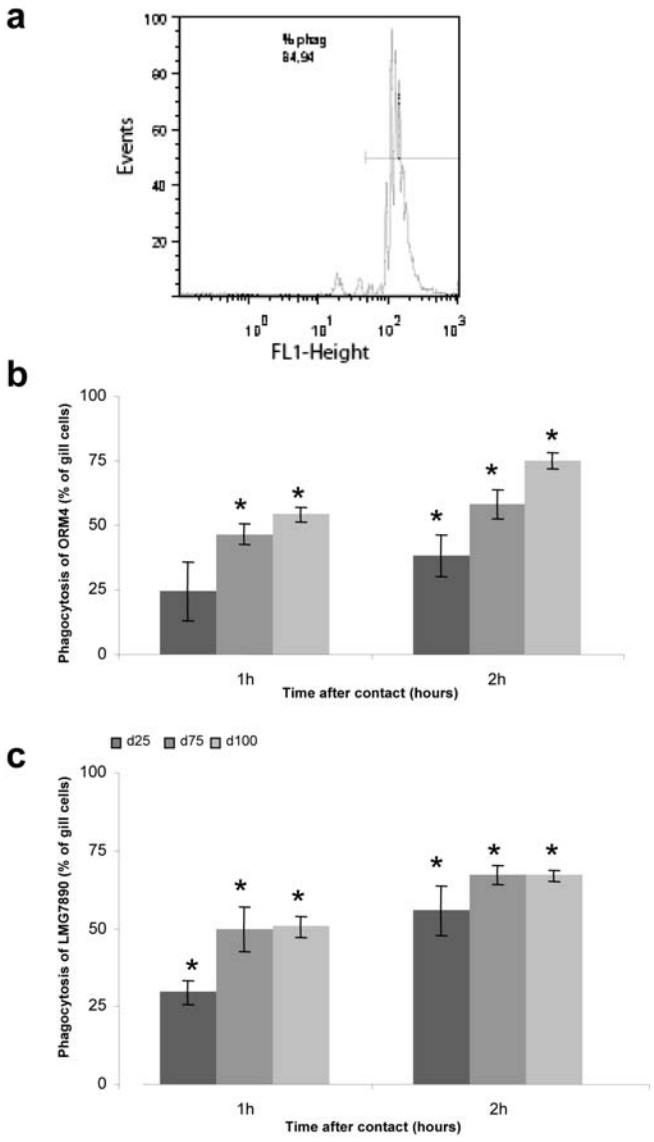

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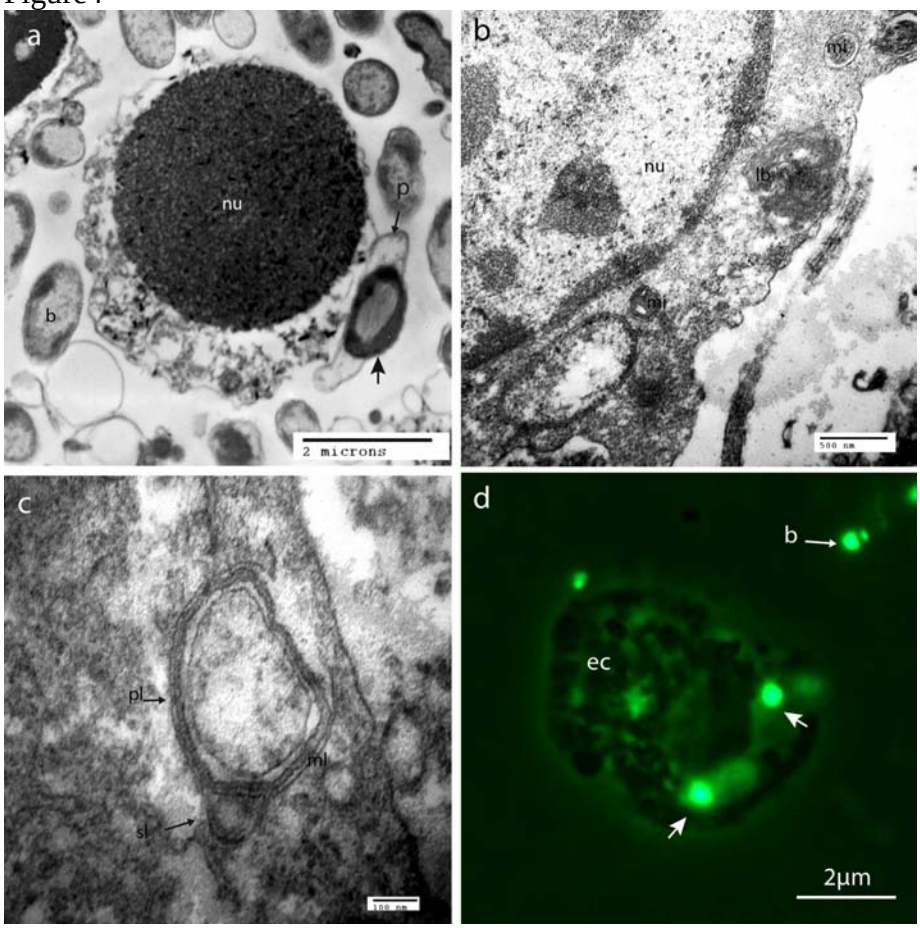
Figure4



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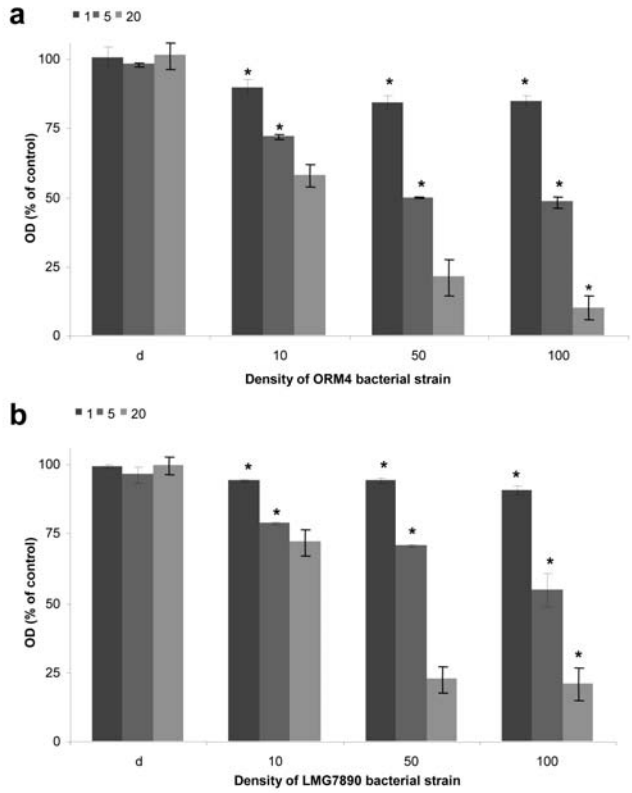

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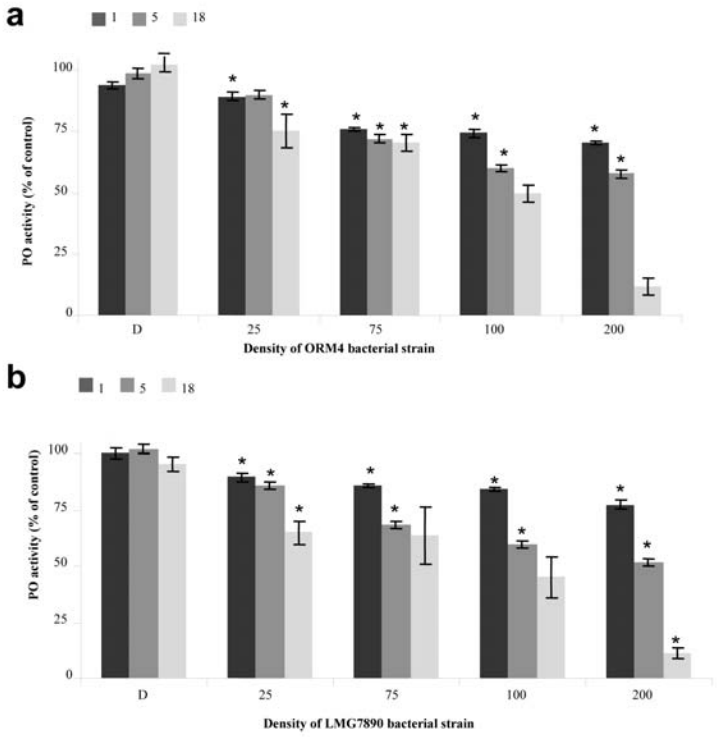

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