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Pathogenic *Vibrio harveyi*, in Contrast to Non-Pathogenic Strains, Intervenes With the p38 MAPK Pathway to Avoid an Abalone Haemocyte Immune Response

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ABSTRACT

Vibrio harveyi is a marine bacterial pathogen responsible for episodic abalone epidemics associated with massive mortalities in France, Japan, and Australia. The aim of this study was the understanding of a possible role of the p38 MAPK in abalone haemocyte responses towards this bacterium. First, the pathogenicity of different *V. harveyi* strains was compared in both immersion and injection trials, and clear differences were detected. The three strains, ORM4, 04/092, and 05/053, all isolated from moribund abalone, induced up to 80% mortalities in immersion or injection challenges (LD_{50} (ORM4) $= 2.5 \times 10^2$ CFU animal^{-1}). The two strains, LMG 4044T and LMG 7890 were non-pathogenic towards abalone in immersion trials, and needed very high numbers for killing by intramuscular injections ($LD_{50} = 8.9 \times 10^4$ and 1.6×10^5 CFU animal^{-1}, respectively). To start unraveling the mechanism explaining these differences, the p38-MAPK, a keyplayer in antimicrobial immune response, was studied. The non-pathogenic strain, LMG 7890 can be eliminated by abalone haemocytes and induces haemocyte phagocytosis and high ROS production. With different concentrations of a p38-specific inhibitor, SB203580, p38 implication was shown. This inhibitor reduced phagocytosis and ROS induction leading to LMG 7890 proliferation. In the case of the pathogenic ORM4 which can not be eliminated by abalone haemocytes, no phagocytosis and ROS production was induced, and a retarded p38 activation was observed. Taken together, our results suggest that p38 MAPK modulation may be one of the ways of virulent *V. harveyi* to attack its host and escape abalone immune response. J. Cell. Biochem. 106: 152–160, 2009.

KEY WORDS: *HALIOTIS TUBERCULATA*; VIRULENCE; MAPK; IMMUNE ESCAPE

Vibrio harveyi is a marine pathogen that affects a large range of animals [Austin and Zhang, 2006]. Since 1998, epizooties of the abalone, *Haliotis tuberculata*, occur in France, both in farm and in wild stocks and were shown to be caused by this vibrio [Nicolas et al., 2002; Travers et al., in press]. This Gram-negative bacterium was also found associated with abalone disease in Australia and Japan [Handlinger et al., 2005; Sawabe et al., 2007]. The disease starts with loss of muscular strength, appearance of white pustules on the foot, and finally a fatal septicaemia, leading to up to 80% mortality within a few weeks [Nicolas et al., 2002; Handlinger et al., 2005].

Abalone immunity is principally based on their circulating cells, the haemocytes [Cheng, 1981; Travers et al., 2008] and the effectors they excrete. Although mollusc haemocytes play also a role in functions as nutrient digestion, metabolite transport, and wound or shell repair [Sparks and Morado, 1988], their main role is in internal defense. They are responsible for chemotaxis, lectin-mediated pathogen recognition, phagocytosis, encapsulation and elimination of invaders via enzymatic destruction and/or by the production of antimicrobial peptides [Cheng, 1981; Mitta et al., 2000; Bachère et al., 2004; Hooper et al., 2007]. For cellular immune responses to be effective, haemocytes must migrate, attach to the invader,

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phagocytose, and finally kill. This is classically done by secretion of the highly toxic reactive oxygen species (ROS) [Soudant et al., 2007].

The first step of the immune response is pathogen recognition, which, in turn, leads to signal transduction and an appropriated cellular response. The MAP Kinase (MAPK) signal transduction pathway is constituted of a family of serine/threonine kinases implicated in many cellular processes, including stress, cell cycle and growth control but also host defense [for reviews Seger and Krebs, 1995; Cowan and Storey, 2003]. Actually, the three most characterized MAPK families are the ERK1/2 (Extracellular Regulated Kinase 1 and 2, or p42/p44), the JNK46/54 (c-jun N-terminal kinases 46 and 54) and the SAPK2/p38 kinases.

These three MAPK families are well conserved during eukaryotic evolution and orthologous genes were recently isolated and characterized in two gastropods, the marine *Littorina littorea* [Iakovleva et al., 2006b] and the freshwater, *Biomphalaria glabrata* [Humphries and Yoshino, 2003, 2006], known as intermediate host in human Schistosomiasis or Bilharziosis. In response to contact with Gram-negative bacteria or in the presence of bacterial lipopolysaccharides, the phosphorylation of p38 MAPK is induced in molluscan haemocytes [Canesi et al., 2002a,c; Iakovleva et al., 2006a], leading to efficient bacterial phagocytosis. The addition of p38 MAPK inhibitors reduces these phagocytosis responses, and thus pathogen degradation [Canesi et al., 2002c; Iakovleva et al., 2006a].

By using different MAPK inhibitors, Zelck et al. have shown that the ERK signaling pathway is involved in phagocytosis, encapsulation and ROS production upon infection of *B. glabrata* haemocytes with the flatworm, *Schistosoma mansoni* [Zelck et al., 2007]. By using the p38 MAPK inhibitor, SB203580 the same authors also demonstrated the involvement of p38 in phagocytosis. It was suggested that the MAPK-induced oxidative burst [Zelck et al., 2007] was mediated by NADPH oxidase recruitment [Humphries and Yoshino, 2003].

Pathogens can act in different manners to undermine the protective abilities of molluscan haemocytes. *Vibrio tapetis* is a pathogen of the manila clam, *Tapes phillipinarum*, which is known to intervene with the haemocyte's cytoskeleton reducing their adhesion properties both for the pathogen and the substratum [Choquet et al., 2003]. Other molluscan pathogens have been shown to reduce haemocyte viability or phagocytosis capacities [Allam and Ford, 2006; Labreuche et al., 2006]. Abalone infected with pathogenic *V. harveyi*, however, show little or no visible host responses based on microscopic examination of living haemocytes or stained tissue sections [Nicolas et al., 2002; Travers et al., in press] and the strategy employed by pathogenic *V. harveyi* strains to avoid abalone immune response is thus far unknown.

Our study aimed at understanding a potential role of the p38 MAPK signal transduction pathways in the haemocyte responses to *V. harveyi* infection. By incubating haemocytes with virulent or non-virulent *V. harveyi* strains, combined or not with a specific p38 MAPK inhibitor, SB203580, we could demonstrate the importance of the p38 MAPK pathway in the elimination of non-virulent *V. harveyi*. However, virulent *V. harveyi* is able to circumvent haemocyte phagocytosis and ROS production processes, leaving the question unanswered how this pathogen escapes its host's defenses.

MATERIALS AND METHODS

BACTERIAL STRAINS

Several *Vibrio harveyi* strains were employed (Table I): three strains isolated from moribund abalone in 1998 (virulent strain ORM4, [Nicolas et al., 2002]) and in 2004 and 2005 (strains 04/092 and 05/053); and two collection strains, LMG 7890 (former *V. carchariae* type strain) and LMG 4044T (*V. harveyi* type strain), both obtained from the "Laboratorium voor Microbiologie" (Universiteit Gent, Belgium). Please note, that *V. carchariae* is a junior synonym of *V. harveyi* [Gauger and Gomez-Chiarri, 2002] and was chosen as being the closest known relative to ORM4 [Nicolas et al., 2002].

CECT4600T is the *Vibrio tapetis* type strain originally isolated from manila clams [Paillard and Maes, 1990; Borrego et al., 1996].

V. harveyi and *V. tapetis* were cultured on Luria Bertani broth with additional salt (LBS, 20 g/L NaCl f.c.) at 28 and 18°C, respectively. Before immersion or injection challenges, or for in vitro contacts, strains were grown overnight and washed twice in 0.2 μm filtered and sterilized seawater (FSSW). Bacterial concentrations were determined by optical density measurements at 490 nm.

ANIMALS

Both wild and farmed abalone, *Haliotis tuberculata*, and wild clams, *Ruditapes philippinarum*, were used in these studies. Adult wild abalone (60–80 mm shell length) were collected from natural populations in the Bay of Roscoff, Brittany (3°58′W, 48°43′N) in September 2005, and in the Bay of Brest, Brittany (4°33′W, 48°21′N) in July 2007. Animals were acclimatized in the laboratory for at least 2 weeks prior to the experiments in 110 L tanks with an open seawater circuit under continuous aeration. Temperature was maintained at about 16°C. During the acclimation period, animals were fed ad libitum on a marine macroalgal diet of *Laminaria digitata* and *Palmaria palmata*. These field abalone were used for all in vitro experiments.

Reproductively mature (= ripe) young abalone (n = 500, 35.0 mm ± 2.2, 2 years old) were transferred from the "France Haliotis" hatchery (Plouguerneau, France) to the animal holding facility of LEMAR in June 2007 and used for all in vivo bacterial challenges.

Adult Manila clams, *R. philippinarum*, (30–40 mm) were collected from a natural clam bed near Bailleron Island in the Gulf of Morbihan

TABLE I. Origin and References for the *V. harveyi* Strains Used in This Study

Name	Origin	Reference
ORM4	Moribund abalone, France 1998	Nicolas et al. [2002]
LMG 7890	Dead brown shark (Carcharinus plumbeus), kidney, Baltimore	Colwell [1982]
LMG 4044T	Dead amphipod (Talorchestiasp.) Woods Hole, MA	Johnson [1965]
Strain 04/092	Moribund abalone, France 2004	This study
Strain 05/053	Moribund abalone, France 2005	This study

(Southern Brittany, France) and maintained in tanks with an open seawater circuit. Clams were only used for providing their haemocytes for the positive control in the CNA-adherence assay.

IN VIVO BACTERIAL CHALLENGES

For immersion challenges, farmed abalone were placed in 5 L tanks with 0.5 μm filtered seawater and maintained at 19°C. Abalone were exposed (or not) during 24 h to 10^5 CFU ml^{-1} of either of the different bacterial strains (ORM4, LMG 7890, LMG 4044T, Strain 05/053 and Strain 04/092). Each group, containing 20 abalone, was tested in triplicate.

For injection challenges, farmed abalone were anesthetized with 5% ethanol, and a triplicate of each group of 10 animals was injected with 50 μl of a 10-fold serial dilution, ranging from 10 to 10^7 bacteria per 50 μl (strains ORM4, LMG 7890, LMG 4044T). Subsequently, abalone were maintained in 0.5 μm filtered seawater at 19°C.

For both types of challenges, seawater was renewed each day and experiments lasted for 7 days. Temperature was monitored on a daily basis, and dead abalone were counted and removed twice a day.

To determine the LD50 values, survival percentages, obtained in the injection challenges, for each quantity of bacteria at days 2.5 were plotted against the bacterial dose, and regression lines were calculated. The 50% survival dose was deduced.

IN VITRO BACTERIAL CHALLENGES

Haemolymph sampling and preparation. Haemolymph from field abalone was withdrawn from the cephalic sinus located at the anterior part of the foot muscle using a 25-Gauge needle and a 5 ml syringe. Individual haemocyte concentrations were quantified using a ''Malassez cell'' and adjusted in serum to 1×10^6 or 5×10^6 cells ml^{-1} for use in each assay below. Cell suspensions were mixed with FSSW (control) or bacteria (haemocyte: bacteria ratio = 1:25) and used in parallel for the bacterial growth, ROS production, phagocytosis, microscopy and Western blot assays as well as for CNA and mortality assays.

Clam haemolymph was only used as positive control for the CNA-adhesion assay and was collected from the posterior adductor muscle using a 25-Gauge needle and a 1 ml syringe.

Haemocyte mortality assay. Aliquots of 100 μl of individual abalone haemocyte suspension (5×10^6 cells ml^{-1}) were placed into individual wells (n = 3 per animal per condition, 6 animals) in a 96-well microplate for 15 min at 18°C before addition of FSSW (control) or bacteria (*V. harveyi* ORM4, ratio = 1:25 and 1:75). After 0, 1, 2, 3, 5, 7, 22 or 28 h, 200 μl of the mitochondrial dehydrogenase substrate, Methyl Thiazolyldiphenyl-Tetrazolium (MTT, 5 mg ml^{-1} in FSSW) was added. Plates were incubated at 18°C for 1 h before adding 100 μl of isopropanol–HCl (0.04 N) to solubilize the precipitated reaction product, formazan. Optical density was measured at 540 nm with a reference wavelength of 620 nm [Lebel et al., 1996]. Results were expressed as the percentage of dead cells relative to that of control haemocytes (FSSW).

Non-adherent cell ratio (CNA) assay. This assay, previously developed on clam haemocytes [Choquet et al., 2003], was used to examine the cytotoxicity of bacteria on abalone haemocytes. The authors demonstrated that clam haemocytes loose adherence specifically in the presence of the clam-pathogenic strain, *Vibrio tapetis*. In the present work, *V. tapetis* (on clam haemocytes) is only used as a positive control. Abalone, or clam (control), haemocytes were aliquoted (300 μl per well) into a 24-well microplate in the presence of FSSW (control) or bacteria (*V. harveyi* ORM4, LMG 7890, LMG 4044T or *V. tapetis* CECT 4600, ratio = 1:25). After 3 h at 18°C, the supernatant was transferred into cytometer tubes containing 300 μl of the fixative formalin (6%). Subsequently, samples were incubated with SYBR Green I fluorescent dye, a DNA intercalator (Molecular Probes, 10^{-3} dilution of the commercial stock solution) for 30 min at room temperature. Samples were analyzed on a FACS-Calibur™ flow cytometer (Becton Dickinson). Results are expressed as the ratio of non-adherent cells incubated with bacteria divided by the number of cells incubated with FSSW. A ratio larger than 1 is indicative of a cytotoxic effect on the haemocytes by the tested bacteria.

Determination of the haemolymph bactericidal response: role of p38 MAPK on the ability of abalone haemocytes to kill bacteria. One milliliter of diluted abalone haemolymph (1×10^6 cells ml^{-1}) was mixed with FSSW or bacteria (*V. harveyi* ORM4 and LMG 7890, ratio 1:25) and 100 μl aliquots were quickly transferred into 1.5 ml tubes. The tubes were incubated at 18°C for 0, 1, 2, 3, 4, 5, and 6 h in two independent experiments. The same protocol was followed for haemolymph treated with 20 μM of the p38 MAPK inhibitor, SB203580 (in DMSO, Sigma), or only DMSO, which were added to the haemocytes, 10 min before bacterial contact. At each timepoint, supernatants were serially diluted in FSSW, plated in duplicate on LBS plates and incubated for 24 h at 28°C prior to counting bacteria. Two independent experiments, each on 3 abalone, were performed.

Phagocytosis assay. The phagocytosis protocol was adapted from Allam et al. [2002]. A sub-sample of abalone haemolymph was diluted (1:1) in FSSW and 400 μl of this solution was added to a 24-well plates (Cellstar, Greiner Bio-one). Haemocytes were allowed to adhere for 15 min at 18°C, and subsequently incubated for 10 min in presence of the p38 inhibitor (SB203580 from 0.04 to 20 μM, f.c.), or only DMSO, before the addition of 10 μl of bacteria (ORM4 or 7890, ratio 1:25) and 100 μl of green fluorescent beads (Fluoresbrite YG Microspheres, 2.00 μm, Polysciences, 1:200 in distilled water).

After 3 h at 18°C, supernatants were removed and trypsin (2.5 mg ml^{-1} in AASH) was added to detach the adherent cells at RT. Plates were shaken for 10 min and anti-aggregant solution AASH (1.5% EDTA, 6.25 g L^{-1} NaCl, in 0.1 M phosphate buffer, pH 7.4) was used to stop the reaction by dilution. By flow cytometry, the beads are identified through their fluorescence. Non-ingested particles were easily distinguished from engulfed ones by their size on a FSC (forward scatter, representing particle size) versus SSC (side scatter, representing internal complexity of particles) and a FSC versus FL1 plot. The phagocytosis index is defined as the percentage of haemocytes phagocyting three or more beads, as previously described [Hégaret et al., 2003].

Reactive oxygen species: production assay. The ability of abalone haemocytes to produce reactive oxygen species, in presence or absence of different bacterial strains (*V. harveyi* LMG 7890 and ORM4), was measured by adapting the protocol previously described [Martello and Tjeerdema, 2001]. Aliquots (100 μl) of abalone haemocytes (1×10^6 cells ml^{-1}), in presence or absence of bacteria (ratio 1:25) and of the inhibitor SB203580 (from 0.02 to 20 μM in DMSO f.c.) or only DMSO, were dispersed into a white 96-well microplate. Just before measurement, a luminol working solution (25 μl of 1 mM luminol in KOH (9.8 g L^{-1})/boric acid (7.8 g L^{-1})) was added. The reactive oxygen species produced by the cells oxidize the luminol in a chemiluminescent reaction producing photons, which were measured each 10 min during 3 h in a Mithras LB 940 luminometer (Berthold technologies). Controls with only bacteria or only cells were performed in parallel. Data were collected during three independent experiments, each on 3 abalone.

Microscopy. Diluted abalone haemolymph (200 μl, 1×10^6 cells ml^{-1}) was incubated in presence (or absence) of SB203580 (or only DMSO), and in presence of *V. harveyi* ORM4 or *V. harveyi* LMG 7890. Cell and bacterial morphology were regularly examined with an inverted Leica DM-IRB microscope equipped with a Retiga 2000R Fast 1394 CC camera (QImaging) to observe bacterial growth and changes in cell morphology.

Western blot analysis. Aliquots (100 μl, 1×10^6 cells ml^{-1}) of abalone haemocyte solution were incubated with or without bacteria (ratio 1:25), in the presence or absence of the inhibitor SB203580 (0.2–20 μM in DMSO f.c.) or only DMSO. At each time point (see Determination of the Haemolymph Bactericidal Response: Role of p38 MAPK on the Ability of Abalone Haemocytes to Kill Bacteria Section), haemocytes were pelleted and resuspended in 100 μl of sample buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8, to obtain whole haemocyte extracts. After boiling, haemocyte extracts were separated on 10% SDS/PAGE gels according to Laemmli [1970] and transferred onto nitrocellulose membranes (Schleicher and Schuell). The membranes were saturated during 1 h in Tris Buffered Saline containing 0.1% Tween-20 (TBST) and 1% bovine serum albumin (Sigma). Blots were incubated overnight with the rabbit anti-phospho-P38-Thr180/Tyr182 antibody (1:1,000) (#9211, Cell Signaling Technology). Secondary anti-rabbit perox-

A

	% of mortalities at day 5 after immersion challenges	LD50 at day 2.5 by injection challenges
ORM4	80 %	2.5×10^2
LMG 7890	0 %	1.6×10^5
LMG 4044T	0 %	8.9×10^4
Strain 04/092	80 %	Nd
Strain 05/053	80 %	Nd

B

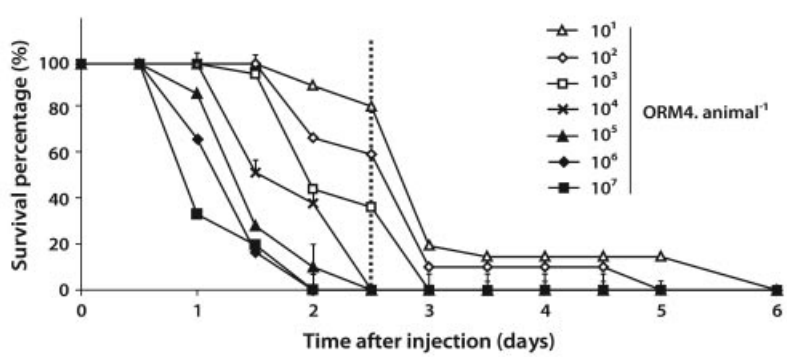

Fig. 1. A: Percentages of farmed abalone mortality cumulated during 5 days of immersion challenges and LD_{50} (CFU $animal^{-1}$) values determined by injection challenges on day 2.5. Results are the means ± SE of three independent experiments containing each triplicates of groups of each 10 (injection) or 20 (immersion) animals. B: Mortalities observed during a week after intramuscular injection of different doses of ORM4 bacteria. The LD50 value was calculated at day 2.5 (dotted line) by a linear regression of the survival values over bacterial doses.

idase-conjugated antibodies (Biorad) were used at a 1:2,000 dilution. After 1 h, blots were washed extensively in TBST and bands were visualized using the ECL chemiluminescence substrate according to the manufacturer's instructions (Amersham). Subsequently, after extensive washing in TBST, the same membrane was incubated overnight with the rabbit anti-actin (20–33) antibody (Sigma) at a 1:3,000 dilution. After 3 washes in TBST, an 1:5,000 diluted anti-rabbit peroxidase-labeled conjugate (Biorad) was added for 1 h. Washing and revelation was done as specified above.

Signals were quantified with the public domain ImageJ program (written by Wayne Rasband, NIH, USA, http://rsb.info.nih.gov/ij/), and the ratio phospho-p38/actin is plotted. Three independent kinetics experiments were analyzed.

STATISTIC ANALYSIS

Statistical analyses (comparison of haemocyte bactericidal response, phagocytosis and ROS production between strains with and without inhibitor) were performed by using the Mann–Whitney *U*-test with significance at $P \leq 0.05$. LD50 were calculated from a regression model of the original data. All statistical analyses were performed at a 95% confidence level using the Statgraphics Plus 5.1 statistics software.

RESULTS

IN VIVO PATHOGENIC EFFECT OF DIFFERENT *V. HARVEYI* STRAINS: IDENTIFICATION OF VIRULENT AND NON-VIRULENT STRAINS

To test the pathogenic potential of different *V. harveyi* strains (Table I) immersion challenge experiments were performed. The three strains (ORM4, 04/092, and 05/053) all isolated from moribund abalone during abalone epidemics, resulted in 80% mortality within 5 days. With the two collection strains, LMG 4044T and LMG 7890, no death was observed during this time period (Fig. 1A). In injection challenges, all strains finally killed abalone, but the LD_{50} values varied considerably. The LD_{50} values for the collection strains, LMG 4044T and LMG 7890, were 8.9×10^4 and 1.6×10^5 CFU animal^{-1}, respectively, whereas that of the ORM4 strain was 2.5×10^2 CFU animal^{-1} (Fig. 1A). For the highest doses of ORM4 (10^7 CFU ml^{-1}), abalone mortality began after only one day upon inoculation; as little as 10 CFU animal^{-1} led to 80% mortality within 3 days (Fig. 1B).

In conclusion, a large difference was detected both in immersion and injection experiments between *V. harveyi* strains, permitting to define pathogenic and non-pathogenic strains.

PATHOGENIC *V. HARVEYI* EFFECTS ON ABALONE HAEMOCYTES

The effect of the pathogenic ORM4 strain on abalone haemocytes was evaluated by testing the viability and adherence capacity of these cells; two traits known from the literature to be affected by *Vibrio* infections. Bacterial charges did not detectably influence haemocyte survival as 97% (SE 5.9%) survived after 28 h (Fig. 2A). In addition, no obvious morphological changes or any release of adherent cells were observed after 3 h of contact with *V. harveyi*, as shown by the non-adherent cell ratios (Fig. 2B). *V. tapetis* on the other hand had a high cytotoxic effect on clam haemocytes (CNA = 9.1, SE 0.9) but not on abalone haemocytes (CNA = 1.1, SE 0.4). None of the *V. harveyi* strains tested induced any loss of adherence on the abalone haemocytes.

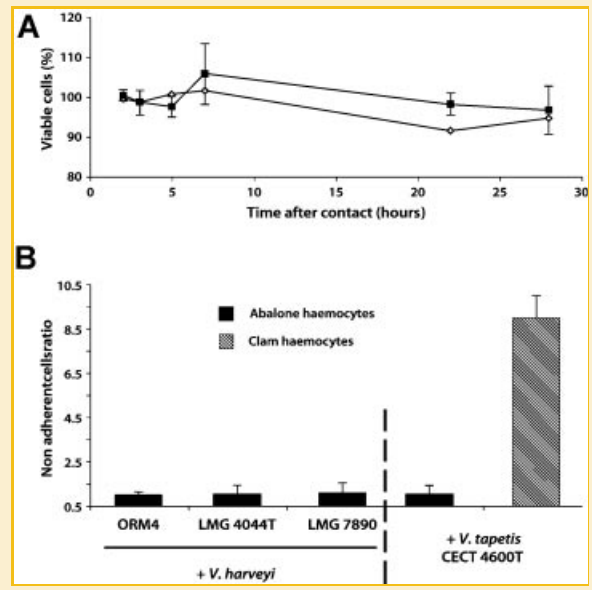

Fig. 2. A: In vitro abalone haemocyte mortalities in the presence of ORM4, measured during a time course with the MTT assay. Filled squares represent the 1:25 haemocyte/bacteria ratio and open diamonds the 1:75 ratio. Values are given as mean ± SE of three independent experiments containing triplicates of two-animal groups. B: In vitro adhesion assay (CNA). Results are given as the ratio of non-adherent cells in presence of bacteria over non-adherent cells in presence of seawater after 3 h of contact. Clam haemocytes were used as positive control. These cells (dashed right column) are known to loose adherence in presence of *Vibrio tapetis*, CECT 4600T. Error bars (SE) are calculated from three independent experiments.

IMPORTANCE OF P38 MAPK IN THE HAEMOCYTE RESPONSE TO PATHOGENIC AND NON-PATHOGENIC *V. HARVEYI*

To study the potential role of the p38 MAPK in signal transduction in abalone haemocytes triggered by bacterial in vitro challenges, p38 MAPK phosphorylation, (corresponding to activation) was evaluated. The non-pathogenic *V. harveyi* strains, LMG 4044T and LMG 7890, both strongly induced phospho-p38 MAPK after 1 h of contact with a "tailing" of expression for up to 5 h (Fig. 3). *V. harveyi* ORM4, on the other hand, induced much slower p38 MAPK phosphorylation kinetics and apparently to a lower level than the non-pathogenic strains. A peak of phospho-p38 appeared from 2 to 4 h of contact (Fig. 3).

To analyze in more detail these observed differences in p38 activation in the antimicrobial haemocyte response, cells were treated (or not) with the p38 MAPK inhibitor, SB253080, and both haemocyte and bacterial survival was estimated each hour during these in vitro contacts. As shown in Figure 4A, p38 MAPK phosphorylation after a 2 h contact with LMG 7890, is largely reduced when haemocytes are incubated with inhibitor, in a dose dependent manner. p38 MAPK phosphorylation is inhibited with the two highest doses of SB203580 tested: 10 μM (partial inhibition) and 20 μM (inhibition).

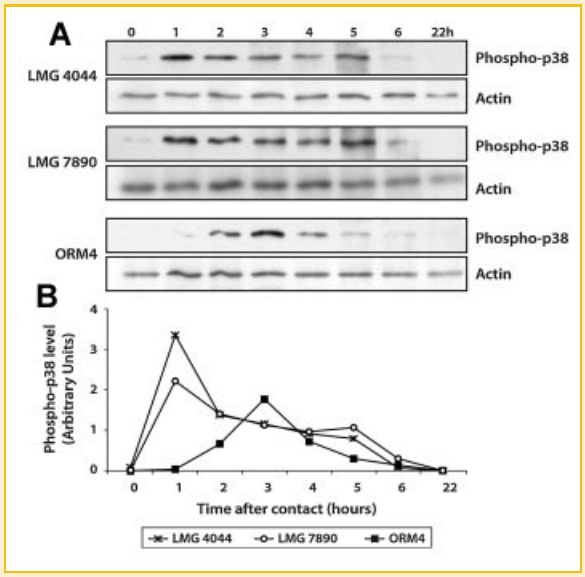

Fig. 3. p38 MAPK activation in field abalone haemocytes. A: Protein extracts of haemocytes incubated during a time course with different strains of *V. harveyi* (ORM4, LMG 7890 and LMG 4044T) were separated on 10% SDS–PAGE gels, blotted and incubated with an anti-phospho-p38 antibody (upper panels). Actin (lower panels), revealed on the same membrane, is given as a loading control. The Western blots shown are a representative example of three independent samplings. B: Quantification of the subpart A-autoradiograms.

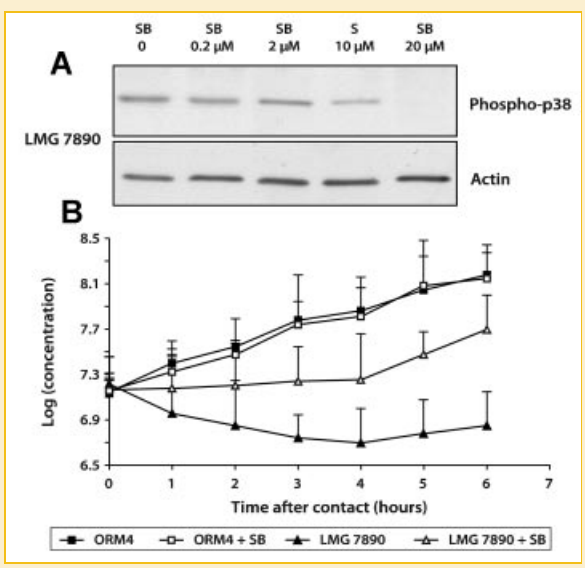

Fig. 4. Effect of the p38 MAPK inhibitor, SB203580. A: p38 phosphorylation. Western blot of protein extracts of abalone haemocytes, incubated for 2 h with LMG 7890, in presence of different doses of SB203580. The Western blot shown is a representative example of four different and independent samples. B: Bacterial growth or mortality. Concentrations of LMG 7890 and ORM4 were determined after contact with abalone haemocytes at 18°C, in presence or absence of 20 μM SB203580. Results are means (+ SE) of plating of triplicates of two independent experiments.

Subsequently, the effect of this inhibitor was tested on the haemocyte bactericidal activity both by plating assays and microscopy observation. The pathogenic strain ORM4 proliferates quickly in total haemolymph; in 6 h the ORM4 concentration increased 1 log unit (0.05, SE) which gives a generation time at 18°C of about 45 min (Figs. 4B and 5). The non-pathogenic strain LMG 7890 on the other hand does not seem to grow well and is apparently rapidly destroyed by the haemocytes (Figs. 4B and 5). When 20 μM of the p38 inhibitor was added, a minimal proliferation of the LMG 7890 strain could be observed (from 1.5×10^7 to 5×10^7 CFU ml^{-1} in 6 h, Figs. 4B and 5), which is significantly different from LMG 7890 without inhibitor ($P < 0.005$). The inhibitor did not have any detectable effect on the pathogenic ORM4 ($P = 0.8$), which grew well both in the presence or absence of the p38 MAPK inhibitor (Fig. 5).

To try to understand the importance of haemocyte phagocytosis and the p38 MAPK role on differential bacterial growth, phagocytosis experiments were performed by flow cytometry.

The basal beads phagocytosis obtained in absence of bacteria was significantly induced by the non-pathogenic *V. harveyi*, LMG 7890, in contrast to pathogenic ORM4 that did not activate nor inhibit basal beads phagocytosis (Fig. 6A, $P < 0.05$). The phagocytosis of fluorescent beads or fluorescent beads mixed with pathogenic or non-pathogenic bacteria, was significantly inhibited by the presence of 20 μM of the p38 MAPK inhibitor (Fig. 6B, $P < 0.05$).

To investigate whether abalone haemocytes produced an oxidative burst when in contact with one of the *V. harveyi* strains,

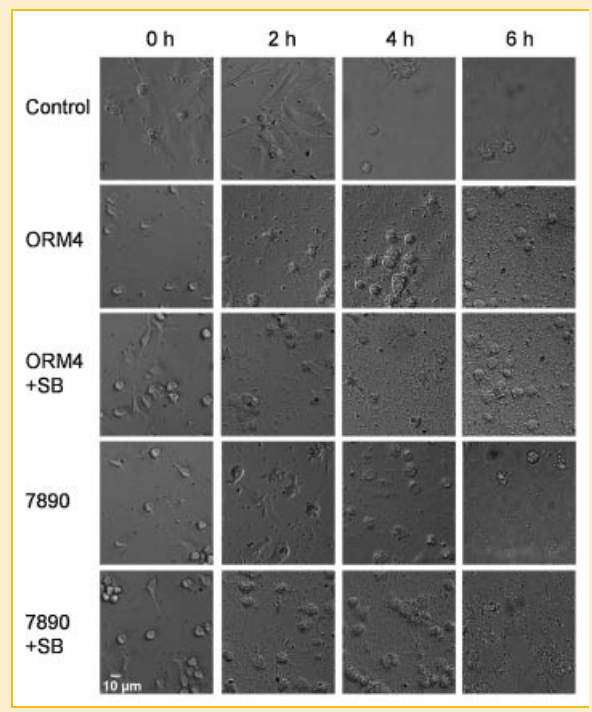

Fig. 5. Cellular morphology and bacterial presence, observed by phase contrast microscopy, at the indicated time points. Abalone cells were incubated at 18°C in presence of the *V. harveyi* strains, LMG 7890 or ORM4, and in presence or absence of 20 μM of the p38 MAPK inhibitor, SB203580 (SB). Barr = 10 μm.

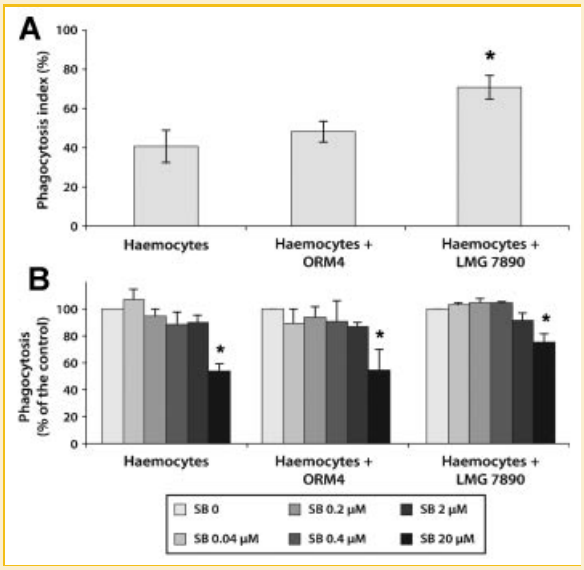

Fig. 6. Fluorescent bead phagocytosis by abalone haemocytes estimated by flow cytometry in presence or absence of the strains, ORM4 and LMG 7890. A: Phagocytosis index expressed as percentage of haemocytes containing three or more beads, in the presence or absence of the bacterial strains. The value measured for LMG 7890 is statistically different from the others and indicated by a star. All results are means of triplicates of two independent experiments (± SE). B: Haemocyte phagocytosis in presence of different doses of the p38 MAPK inhibitor, SB203580 (SB) with or without bacterial strains. Results are given as percentage of phagocytosis index, using the phagocytosis value of cells without inhibitor (control) as a 100%. Statistically significant differences with the controls (SB 0) are indicated by a star ($P < 0.05$).

and to verify a possible role of p38 MAPK activation in this process, ROS production was measured. Significant photon emission was only detected in the presence of the non-pathogenic LMG 7890 strain and can be inhibited with the SB203580 inhibitor (Fig. 7, $P < 0.05$). Inhibition is linear between 5 and 20 µM and almost complete with 20 µM (Fig. 7, Insert).

In comparison, only a very small, non-significant, ROS induction was measured in presence of the ORM4 strain without SB203580 (Fig. 7).

DISCUSSION

To be an efficient pathogen, able to proliferate in haemolymph in presence of mollusc immune cells, *V. harveyi* must prevent or deal with activation of the bactericidal activities of these cells. The present study demonstrates for the first time that pathogenic and non-pathogenic *V. harveyi* cells differ in their "interaction" with the host's defense. The pathogenic ORM4 avoids efficiently the bactericidal response, probably, in part, through a modified or a reduced p38 MAPK activation, both in terms of kinetics and intensity. In contrast to the non-pathogenic strains, the pathogenic ORM4 is able to grow in the presence of haemocytes and prevents normal ROS and phagocytosis inductions.

To evade haemolymph bactericidal activity, *Vibrios* are known to produce compounds that inhibit phagocytosis, as illustrated by the extracellular products of *V. aesturianus*, an oyster pathogen [Labreuche et al., 2006] or the capsules of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, which were suggested to

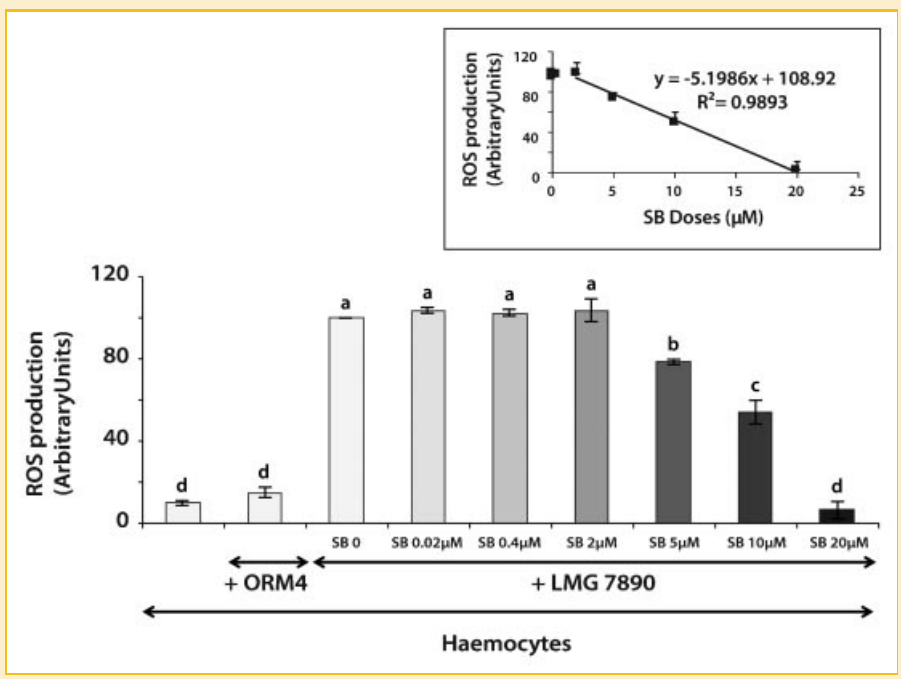

Fig. 7. ROS production estimated by the luminol chemiluminescence assay in abalone haemocytes after bacterial contact, in presence or absence of different doses of the p38 MAPK inhibitor. SB = SB203580 (p38 inhibitor). Results are expressed as relative luminescence units and are means of triplicates of two independent experiments (± SE). Different letters indicate those ROS values which are significantly different from each other ($P < 0.05$). Insert: Plot representation of ROS production (induced by LMG7890) over "inhibitor doses."

prevent bacterial degradation by haemocytes [Genthner et al., 1999; Hsieh et al., 2003; Falklind-Jerkerus et al., 2005; Qadri et al., 2005]. Another rather direct way to avoid phagocytosis is to damage haemocytes [Cheng, 1975; Labreuche et al., 2006], or to prevent the oxidative burst associated with phagocytosis [Pruzzo et al., 2005]. This strategy is employed by *V. anguillarum* [Bramble and Anderson, 1999] or *Vibrio* sp. strain S322 [Lambert and Nicolas, 1998]. Finally, bacteria can overcome the defense response through a deregulation of signaling pathways involved in immune cell activation [Rosenberger and Finlay, 2003; Canesi et al., 2005; Pruzzo et al., 2005]. The lethal factor of *Bacillus anthracis* induces in human an inhibition of p38 MAPK activation in macrophages by cleaving the amino-terminal extensions of the MKK3 and MKK6 MAPK-Kinases which normally activate p38 MAPK [Park et al., 2002]. *Erlichia chaffeensis*, an obligatory intracellular Gram-negative bacterium which infects human monocytes or macrophages, also causes a down-regulation of p38 MAPK activation [Lin and Rikihisa, 2004].

We show here that pathogenic and non-pathogenic *V. harveyi* differ in their capacity to kill abalone both by bath immersion and injection challenges. Also their capacity to grow in the presence of haemocytes, and their ability to activate abalone immune cells are completely different. An efficient immune response can in this case be defined on the basis of the results obtained with the non-pathogenic strains. After a rapid p38 MAPK activation which peaks within 1 h, bacteria are damaged and eliminated through phagocytosis and ROS production. This ''normal'' abalone haemocyte response was shown to be impaired in presence of the p38 MAPK inhibitor, confirming the role of this MAPK pathway proposed in other models for mediating molluscan phagocytosis and ROS production [Canesi et al., 2002a; Humphries and Yoshino, 2006; Iakovleva et al., 2006a]. It is important to note that in this work we always talk about fluorescent bead-phagocytosis (as we are unable, for the moment, to directly measure phagocytosis of our strains) and its induction (or absence of induction) by the LMG 7890 (or ORM4) bacterium. But although bead-phagocytosis certainly only partially reflects genuine vibrio phagocytosis, the induction by LMG7890 and the absence of induction by the pathogen, underline the biological significance of these measures. Moreover, Allam et al. also showed a significant bead-phagocytosis induction by the extracellular products of non-pathogenic *V. splendidus* on clam haemocytes, in contrast to those of the pathogens, *V. tapetis* and *V. anguillarum* (which in this case are able to diminish phagocytosis). Thus intervening with phagocytosis induction is one of the ways vibrios can use to specifically undermine their host's haemocyte responses.

Canesi et al. [2002a] first implicated p38 MAPK in bacterial elimination in mussel haemolymph, and showed that it was inhibited in presence of a specific inhibitor, SB203580 [Canesi et al., 2002b]. Similar implications of p38 MAPK in phagocytosis were subsequently noticed for *Biomphalaria glabrata* and *Lymnaea stagnalis* [Zelck et al., 2007]. However, as in the case of the authors of these reports which used between 20 and 100 μM, we needed to use rather high SB203580 concentrations to get an efficient p38 inhibition. As these concentrations were reported to slightly inhibit other kinases in mammalian cells, it cannot be excluded that one of these is also implicated in the abalone immune response [Davies et al., 2000]. Another reservation should be made concerning eventual plasma cidal effects which may also intervene in the immune response and which were not investigated here.

The pathogenic *V. harveyi* ORM4, however, when compared with the non-pathogenic strains, induced less p38 phosphorylation, and the activation occurred later (between 2 and 4 h post-infection). This bacterium is able to rapidly grow in haemolymph (with a very short generation time) and seems to have the ability to avoid ROS production and phagocytosis induction, and all this, apparently without any detectable cytotoxic effects on the haemocytes.

In conclusion, we show for the first time that the p38 MAPK pathway is implicated in the ''normal'' abalone haemocyte response against a non-pathogenic bacterial intruder. Pathogenic *V. harveyi*, however, can efficiently circumvent the abalone's immune response, by inhibiting or avoiding haemocyte bactericidal activities, in part, through a modification of the p38 MAPK signaling pathway. However, as the virulence factors playing key roles in this strain's pathogenicity strategy are still unknown, their identification and characterization has to be undertaken to elucidate the mechanisms it employs for efficient survival within its host.

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