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Productive Infection of *Piscirickettsia salmonis* in Macrophages and Monocyte-Like Cells From Rainbow Trout, a Possible Survival Strategy

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ABSTRACT

Piscirickettsia salmonis is the etiologic agent of the salmonid rickettsial septicemia (SRS), an endemic disease which causes significant losses in salmon production. This intracellular bacterium is normally cultured in salmonid epithelial cell lines inducing characteristic cytopathic effects (CPEs). In this study we demonstrate that *P. salmonis* is able to infect, survive, replicate, and propagate in the macrophages/monocytes cell line RTS11 derived from rainbow trout spleen, without inducing the characteristic CPEs and the host cells showing the same expression levels as non-infected control cell. On the other hand, bacteria were capable of expressing specific proteins within infected cells. Infected macrophages cease proliferation and a fraction of them detached from the plate, transform to non-adhesive, monocyte-like cells with proliferative activity. Productive infection of *P. salmonis* into salmonid macrophage/monocyte cells in culture provides an excellent model for the study of host–pathogen interactions, almost unknown in the case of *P. salmonis*. Our results suggest that the infection of cells from the salmonid innate immune system without inducing an important cell death response should lead to the persistence of the bacteria and consequently their dissemination to other tissues, favoring the evasion of the first line of defense against pathogens. J. Cell. Biochem. 108: 631–637, 2009.

KEY WORDS: *P. SALMONIS*; RTS11 CELL LINE; MACROPHAGES; MONOCYTES

Piscirickettsia salmonis is the etiological agent of the salmonid rickettsial septicemia (SRS), or piscirickettsiosis [Fryer et al., 1990, 1992; Fryer and Hedrick, 2003], a systemic infection of cultured salmonids which causes significant losses in aquaculture [Mauel and Miller, 2002; Fryer and Hedrick, 2003]. The disease was first reported in coho salmon [Bravo and Campos, 1989; Gaggero et al., 1995], but infectivity was also demonstrated in all salmonid cultured species such as the Atlantic salmon (*Salmo salar*), Chinook salmon (*Oncorhynchus tshawytscha*), and rainbow trout (*Oncorhynchus mykiss*) from the south of Chile to the northern hemisphere. These species do not develop the disease during the freshwater phase of growth but it occurs 6–12 weeks after introduction into seawater [Cvitanich et al., 1991].

P. salmonis was described as a non-motile, not encapsulated, pleomorphic but generally cocoid bacteria, with a diameter from 0.2 to 1.5 μm [Bravo and Campos, 1989; Rojas et al., 2007]. It is an obligate intracellular bacterium which replicates within membrane-bound cytoplasmic vacuoles in tissues from infected fishes and in cell cultures derived from fish, that was reported unable of growing in cell-free media [Cvitanich et al., 1991; Fryer and Hedrick, 2003]. However, two recent reports have suggested that the bacterium may be able to grow on artificial cell-free media [Mauel et al., 2008; Mikalsen et al., 2008]. Nevertheless, little is known about its infective strategy mainly because of experimental limitations imposed by the intracellular nature of the bacterium.

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The pathogen may be routinely cultivated in a number of different cell lines from salmonids and others related fish species. The cell line CHSE-214 (ATCC CRL 1681), derived from embryonic kidney of Chinook salmon is normally used to propagate this organism. Growth is optimal at 15–18°C in antibiotic-free Eagle's Minimum Essential Medium (MEM) supplemented with fetal bovine serum (FBS). After 7–10 days of infection infected cells display a cytopathic effect (CPE) in the form of clusters of rounded cells [Lannan et al., 1984; Fryer et al., 1990, 1992].

A growing number of marine fish cell lines are now available; most of them originating from pooled embryos and normal tissues. Salmonids and cyprinids provide the greatest number of cell lines, reflecting their economic importance [Lannan et al., 1984; Fryer and Lannan, 1994] and representing important models to study fish pathogens.

The salmonid cell lines CHH-1 from *Oncorhynchus keta*, CSE-119 from *Oncorhynchus kisutch*, or RTG-2 from *O. mykiss*, and the non-salmonid cell lines EPC from *Cyprinus carpio* and FHM from *Pimephales promelas* are permissive to *P. salmonis*, while other fish cell lines have not been assayed for their ability of being infected by this bacterium. All in vitro cultures described present epithelioid morphology except CSE-119 cell line, which is fibroblastic [Lannan et al., 1984; Fryer and Lannan, 1994; Almendras et al., 1997]. It is thus of importance to assay the infective capacity of *P. salmonis* in other cell types in order to understand infective strategies of the bacterium and increase our knowledge of piscirickettsiosis.

The first immune system-derived cell line established from rainbow trout spleen was named RTS11 [Ganassin and Bols, 1998]. Under the original culture conditions, in the presence of a high serum concentration (20–30%), this cell line presents two cell types with different morphological and functional characteristics. Most cells are small, round, and non-adherent, with a kidney shaped nucleus and a limited, slightly granular cytoplasm, suggesting a monocyte-like morphology. These cells coexist with a smaller population of large and adherent cells with a well spread granular cytoplasm and an oval to round nucleus surrounded by prominent vacuoles, representing the typical morphology of macrophages. Additionally, these authors have described that the macrophage-like cells are phagocytic for latex beads and yeast cells, they present esterase and acid phosphatase activities and take up DiI-acetylated low-density lipoprotein. On the other hand, the round cells presenting monocyte-like morphology are non-phagocytic, esterase negative, and inactive for DiI-Ac-LdL uptake.

RTS11 attachment ability of macrophage-like cells is dependent on serum concentration; their adhesion increases dramatically when serum is removed from the culture [Ganassin and Bols, 1998]. Considering this serum dependence, in this study we have increased the macrophage population in the RTS11 cell line by decreasing serum concentration in the medium, in order to induce adherence and macrophage differentiation rather than proliferation.

In this work, we demonstrate that *P. salmonis* is able of infect, survive, replicate, and propagate in this salmonid monocytes/macrophages cell line. Our results open up the possibility of future research for studying the biological interaction of *P. salmonis* and other salmonid pathogens, with cells that play a key role in the innate and adaptive immune response of fish to the pathogen. Additionally, it will be possible to understand survival strategies of the bacterium which includes resistance to killing by macrophages.

MATERIALS AND METHODS

P. SALMONIS

The prototype strain LF-89 (ATCC VR 1361) was propagated in CHSE-214 cells in MEM (Gibco BRL) supplemented with 15 mM Hepes, 10 mM sodium bicarbonate, and 10% FBS (Gibco BRL) [Rojas et al., 2007].

RTS11 CELLS

Cells were grown at 20°C in Leibovitz's L-15 medium (Sigma) supplemented with 15% FBS (Gibco BRL), replicated each 15 days by dividing the cells into two 25 cm^2 flasks (Falcon) along with their spent medium, and adding an equivalent volume of fresh growth medium.

In order to increase adherent cells for experimental assays, cells were seeded onto poly-L-lysine coated coverslips in 6-well plates with L-15 medium supplemented with 7.5% FBS and then cultivated for 7 days to 50–70% confluence.

INFECTION ASSAYS

Monolayers containing adherent macrophages-like cells (3,500 to 3,700 cells/coverslips) were washed with phosphate-buffered saline (PBS) and infected with *P. salmonis* at a multiplicity of infection (MOI) of 50. One milliliters of the supernatant from CHSE-214 infected cells was centrifuged at 700*g* to eliminate debris and *P. salmonis* was recovered from the supernatant by centrifugation at 13,000*g* for 45 min. Then, bacteria were suspended in L-15 medium, added to each tissue culture plate well, and allowed to adsorb to the cells for 1 h at 20°C. Adherent macrophages were then rinsed three times with PBS and incubated with fresh medium until 2, 5, or 10 days post-infection. Conditioned medium containing round non-adherent cells detached by effect of infection were centrifuged at 300*g* for 20 min. Cells present in the sediment were suspended in a minimum volume of PBS and recovered onto microscope slides using a cytocentrifuge Hettich Mikro 22.

IMMUNOFLUORESCENCE STAINING

Macrophage cells adhered onto coverslips and non-adherent monocyte-like cells obtained by cytospin were fixed and permeabilized at 2, 5, and 10 days post-infection with methanol–acetic acid 3:1(v/v) for 10 min at 20°C. Cells were then washed three times in PBS and incubated in the dark for 1 h at 20°C with a 1:75 (v/v) dilution in BSA 1%, saponine 0.1% in PBS of the oligoclonal antibody anti-*P. salmonis* (SRS Inmunotest Bios Chile) conjugated to FITC. Afterwards, cells were washed three times with PBS, mounted for fluorescence with Vectashield mounting medium (Vector Lab., Inc.) and viewed with a Nikon Eclipse 400 fluorescence microscope. Color photography was performed with a Nikon Coolpix 4500 digital camera.

DUAL-FLUORESCENT LABELING OF F-ACTIN AND *P. SALMONIS*

Cells were fixed and permeabilized as described above. For actin staining, cells were incubated for 40 min with 1:40 (v/v) of

phalloidin–rhodamine solution (Molecular probes) in the dark. Cells were then incubated with anti-*P. salmonis* in conditions already defined. After washing with PBS, coverlips and cytospin preparations were mounted and examined using a Zeiss LSM Confocal Scanning Laser microscope.

PHAGOCYTOSIS ASSAY

Adherent cells and round non-adherent cells detached by infection effect were assessed for their phagocytic ability. Cells were tested by incubation for 2 h in a humidity chamber with a suspension of crystal violet-stained yeast (*Saccharomyces cereviseae*) previously counted in a hemocytometer. Cells were then washed with PBS–0.05% Tween-20, fixed with methanol–acetic acid, and observed by phase-contrast microcopy.

CELL COUNT AND VIABILITY

Cell were seeded in 6-well plates, cultured for 7 days, and then infected with *P. salmonis*. At 0, 2, 4, 6, 8, 10, 12, and 15 days post-infection, non-adhesive round monocyte-like cells detached by the effect of infection and macrophages detached with 0.1% trypsin were collected by centrifugation at 300*g* for 20 min. Both cell types were suspended in PBS and the total number of cells was counted in a hemocytometer. Possible damage of infected cells was analyzed using a trypan blue dye exclusion assay. Cell suspensions were mixed 1:1 (v/v) with 0.4% trypan blue solution (Sigma) and incubated for 3 min at room temperature. Cell number and viability at each time post-infection were determined in triplicate using three independent wells.

TITRATION

Infectious titers were evaluated at days 2, 5, and 10 post-infection by the method of Reed and Muench [1938]. $TCID_{50}$ was defined as the dilution of pathogen required to infect 50% of a given number of inoculated cells in culture. $TCID_{50}$ assay end-point values were determined by serially diluting supernatants of RTS11 infected cells in a 96-well plate with 8 wells for each dilution.

RNA ISOLATION

Total RNA from infected cells and uninfected controls was extracted with Trizol reagent (Gibco BRL) following the manufacturer's protocol. The RNA pellets were dissolved in diethylpyrocarbonate (DEPC) treated water, quantified spectrophotometrically at 260 nm and stored at −80°C. Prior to RT-PCR, RNA samples were treated with 1 U of DNase (Promega)/μg of RNA. Single-stranded cDNA templates were prepared from 1 μg of total RNA.

EXPRESSION OF BACTERIAL CHAPS AND CELLULAR EF1α mRNAs

For mRNA detection of the bacterial protein Chaps, cDNA was synthesized using ThermoScript RT-PCR (Invitrogen) following manufacturer's recommendation. Specific cDNA was then amplified by PCR using primers and conditions described previously [Marshall et al., 2007].

To analyze mRNA expression of the cellular protein EF1α, specific cDNA was synthesized using oligo-dT and M-MLV reverse transcriptase (Invitrogen). PCR was carried out in a total reaction volume of 10 μl with EF1α sense primer 5′-gtctacaaaatcggcggtat-3′ and EF1α antisense primer 5′-cttgacggacacgttcttga-3′. PCR conditions were denaturation at 94°C for 15 min, followed by 35 cycles consisting of 94°C for 30 s, 56°C for 30 s, and 72°C for 10 s and a final extension step at 72°C for 10 min.

Amplicons were analyzed by 2% agarose gel electrophoresis containing 1× Tris–borate buffer and stained with ethidium bromide for 30 min.

RESULTS

DETERMINATION OF INFECTION OF THE MACROPHAGES/MONOCYTES CELL LINE RTS11 BY *P. SALMONIS*

Non-phagocytic monocyte-like cells detached by infection and adherent macrophage-like cells showing phagocytic activity were examined 5 days after infection for the presence of the bacteria by fluorescence microscopy. Figure 1A shows phagocytic activity of macrophage cells (thin arrows) and the absence of this phagocytic property in monocyte-like cells (thick arrows) of the RTS11 cell line. In Figure 1B representative images of both cell types infected with *P. salmonis* are shown. Green fluorescence confirmed the presence of bacteria in both cell types (Fig. 1B). From randomly selected fields containing more than 500 cells we estimated that 40% of cells were infected. Additionally, stained F-actin filament associated to *P. salmonis* was observed in monocyte like cells as well in macrophages (Fig. 2). These data clearly indicate that RTS11 cells were infected with *P. salmonis*.

A significant increase in the number cells infected with *P. salmonis* was detected with increasing days post-infection. In contrast, none appear in non-infected control cells (Fig. 3). These results indicate that the pathogen survives and multiplies in the infected cells.

TITRATION OF *P. SALMONIS* IN INFECTED RTS11 CELLS

The $TCID_{50}$ method was applied to determine the infectivity maintenance of *P. salmonis* after growth in RTS11 cell culture. *P. salmonis* increased the $TCID_{50}$ level even after as many as 10 days post-infection (Table I). Improved infectivity is consistent with increased presence of the bacteria detected by immunocytochemistry, both results indicating rickettsial viability and multiplication inside RTS11 cells.

EFFECT OF *P. SALMONIS* INFECTION ON CELL GROWTH AND VIABILITY IN RTS11 MACROPHAGES AND MONOCYTE-LIKE CELLS

Figure 4 shows cell proliferation of macrophages and detached monocyte-like cells from infected and control non-infected cultures. Two days after infection, a decrease in the proliferative activity of control non-infected and in infected macrophages is observed. However, while control cells recover, infected macrophages show a clear decrease in their proliferative activity (Fig. 4A). Contrarily, infected monocyte-like cells show a decrease in proliferative activity early after infection, as compared to control non-infected cells, but recovers by day 6 post-infection maintaining their proliferative activity up to 12 days post-infection (Fig. 4B). These results were confirmed by BrdU incorporation (data not shown).

To determine whether *P. salmonis* infection resulted in macrophage killing, trypan blue dye exclusion was measured as

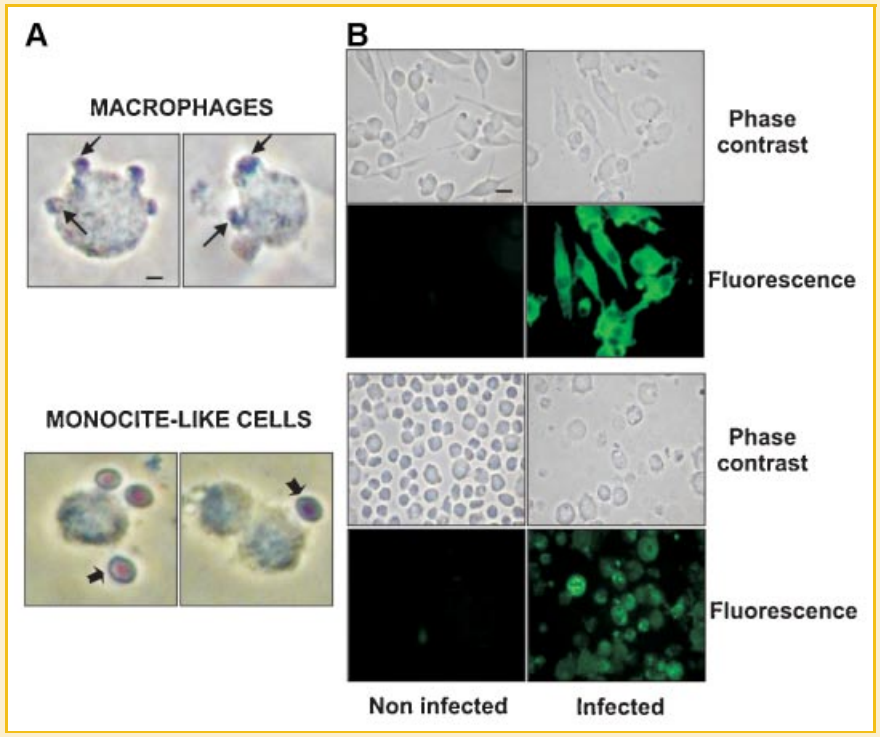

Fig. 1. Fluorescence microscopy images of macrophages and monocyte-like cells infected with *P. salmonis*. A: Cells were incubated for 2 h with a suspension of crystal violet stained *Saccharomyces cereviseae*. (→ yeasts being phagocytated by macrophages, ➡ non-engulfed yeasts in a monocyte-like cells culture). B: Five days after infection with *P. salmonis*, phagocytic (macrophages) and non-phagocytic (monocyte-like) cells were fixed in methanol–acetic acid 3:1 (v/v) for 10 min at room temperature and then incubated for 1 h with an anti-*P. salmonis*-FITC antibody diluted 1:75 (v/v) in BSA 1%, saponine 0.1% in PBS. Scale bar: A, 5 μm. B, 20 μm.

an indicator of induced cytotoxicity. Overall, low-level of cytotoxicity was observed ranging from 10% to 15% for macrophages and 5% to 10% for monocytes (Fig. 5).

EXPRESSION OF A BACTERIAL GENE CHAPS IN INFECTED CELLS

Figure 6 shows that the bacteria expressed Chaps mRNA within infected cells at the different times of infection evaluated, a signal that was not detected in control non-infected cells. Therefore, *P. salmonis* infected, survived and multiplied within infected cells maintaining the capacity to express a specific gene. As an internal control, mRNA expression of the cell protein EF1α was evaluated in non-infected and infected cells. No alterations in the relative expression levels of this mRNA at different times post-infection were observed. These results were confirmed by real-time RT-PCR (not shown).

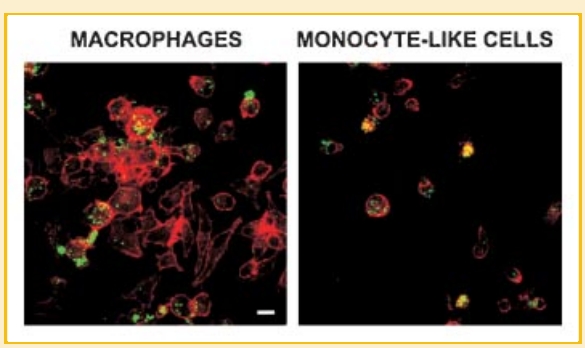

Fig. 2. Confocal microscope images of spleen trout macrophages and monocyte-like cells infected with *P. salmonis*. Infected cells were fixed and permeabilized with methanol–acetic acid 3:1 (v/v) for 10 min at room temperature. Then they were incubated for 1 h with 1:75 (v/v) dilution of anti-*P. salmonis*-FITC antibody for bacterial detection, and for 40 min with 1:40 (v/v) of falloidin–rodamin to detect cell actin filaments. Scale bar, 20 μm.

DISCUSSION

Our results show that *P. salmonis* infects and replicates inside trout macrophages and monocyte-like cells without inducing an important cell death event during the 15 days of infection evaluated. Interestingly, RTS11 infected cells did not present the characteristic CPE described as criterion of susceptibility to *P. salmonis* in other cell lines [Lannan et al., 1984; Almendras et al., 1997]. Clusters of rounded, vacuolized, or detached cells were absent even at advanced times post-infection in cells showing a high number of bacteria.

Permissive fish cell lines such as CHSE-214 derived from chinook salmon (*O. tshawytscha*) or EPC from common carp (*Cyprinus carpio* L.) showed a rapid CPE upon infection with *P. salmonis*. On the other hand, CPE was detected only at 45 days post-infection with this bacteria in cells derived from the brown bullhead (BB) (*Ictalurus nebulosus*) [Almendras et al., 1997] while it is observed before 14 days post-infection in Sf21cells from the insect *Spodoptera*

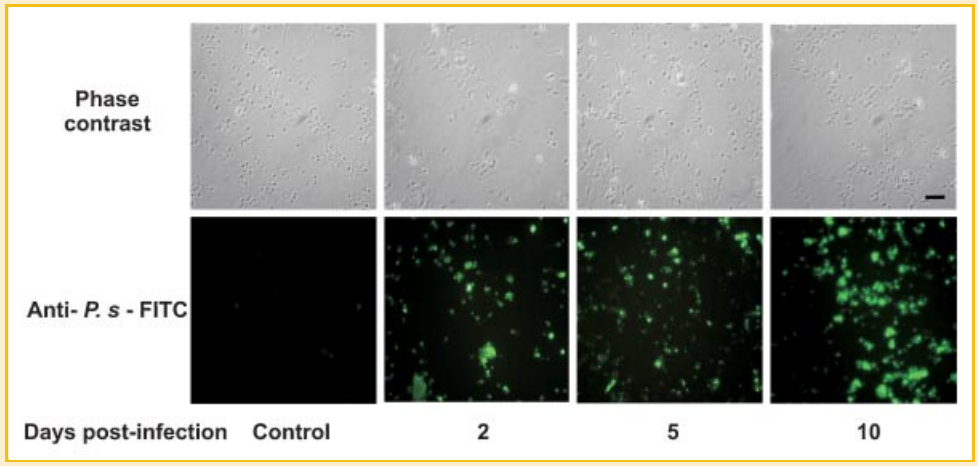

Fig. 3. Intracellular multiplication of *P. salmonis* in macrophages RTS11. At different points post-infection, cells were fixed and permeabilized with methanol–acetic acid 3:1 (v/v) for 10 min at room temperature, and incubated for 1 h with 1:75 (v/v) dilution of anti-*P. salmonis*-FITC antibody. Scale bar, 150 μm.

frugiperda [Birkbeck et al., 2004]. The delayed onset of CPE observed in some non-fish or non-salmonid cell lines suggest that they derive from hosts that are not ideal for *P. salmonis* and this is not the case for the rainbow trout cell line RTS11.

Infected macrophages stop proliferation as measured by number of cells (Fig. 4) and by BrdU incorporation in DNA as well (data not shown). A fraction of infected macrophages detached from the plate and were recovered in the population of cells corresponding to non-adhesive, monocyte-like cells. Interestingly, while in infected macrophages stop proliferation is a long-lasting effect, monocyte-like cells detached from them recover their proliferative activity after an initial decrease. Considering that non-infected macrophages also present a reduction both in number of cells and in BrdU incorporation, at least part of this effect could be a characteristic of this cell type. Recently the presence of *P. salmonis* was demonstrated by transmission electron microscopy within cytoplasmic vacuoles in macrophage-enriched fractions obtained from rainbow trout head kidney at different times after infection [McCarthy et al., 2008]. It was proposed but not proven that the presence of *P. salmonis* in kidney cells was the result of the survival and replication capacity of these bacteria in these cells. Indeed, our results point to the survival and multiplication of *P. salmonis* in macrophages and monocyte-like cells, without an evident damage of host cells. Then bacterial capacity to survive and multiply inside phagocytes may be a mechanism to become "unavailable" for the salmonid host innate immune system, in order to evade the first line of defense against pathogens. Indeed, the innate cellular response is the most important protective mechanisms in fish [Ellis, 2001].

TABLE I. Titer of *P. salmonis* in RTS11 Macrophages

Days of infection	$TCID_{50}$
2	ND
5	$10^{2.88}$ (±0.55)
10	$10^{5.44}$ (±1.49)

P. salmonis infectivity was determined at different times of infection by $TCID_{50}$ assay in 96-well plates with 8 wells per dilution. Cells were infected with serial dilutions of suspensions from RTS11 infected cells. Values are the average of three independent experiments ± standard deviations. ND, non-determined.

Mammalian rickettsial survival strategy has been the subject of several studies to determine how these obligate intracellular pathogens survive within phagocytic cells. *Rickettsia akari* the

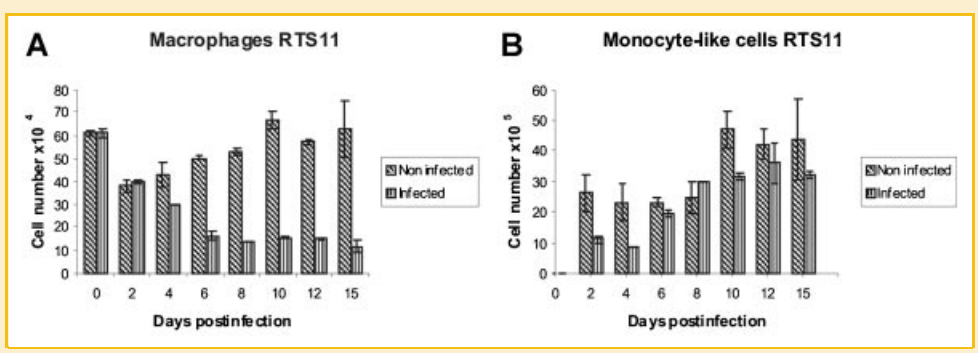

Fig. 4. Effect of *P. salmonis* infection on RTS11 cell proliferation. Monolayers of macrophages were infected with *P. salmonis* at a MOI 50, and recovered with 0.1% trypsin at different days of infection. Macrophages (A) and monocytes-like cells (B) detached by effect of infection were centrifuged at 300*g* for 20 min and suspended in PBS. Total cell number at each time post-infection was determined for three independent wells. Values are the average of triplicates ± standard deviations.

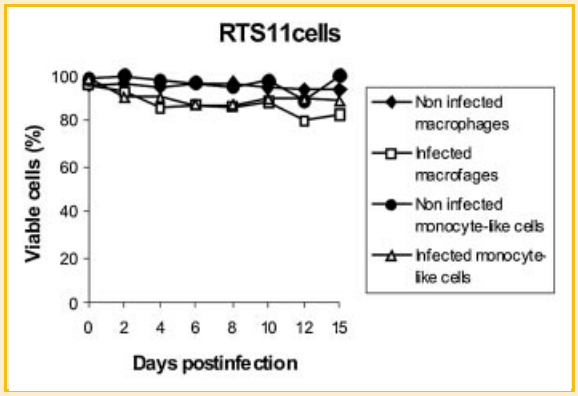

Fig. 5. Effect of *P. salmonis* infection on RTS11 cells viability. Monolayers of macrophages were infected with *P. salmonis* at a MOI 50, and at different days of infection recovered with 0.1% trypsin. Macrophages and monocyte-like cells detached by effect of infection were centrifuged at 300*g* for 20 min and suspended in PBS. Cell suspensions were incubated with the same volume of trypan blue solution and then used for viable cell counts. Viability at each time post-infection was determined for three independent wells. Values are the average ± standard deviations of triplicates.

causative agent of rickettsialpox and *R. typhi*, which produces murine typhus are capable of infecting and surviving within peritoneal macrophages as well as in the macrophage-like cell line P388D1. Nevertheless these pathogens multiply and produce lysis of their target cells, a mechanism which allow rickettsiae to be released and to infect the neighboring cells [Radulovic et al., 2002]. While *P. salmonis* was originally named on the basis of similarities to certain rickettsial species, the 16S rDNA sequence places it within the gamma subdivision of the Proteobacteria, rather than within the alpha subdivision with the genus *Rickettsia* [Fryer et al., 1992; Fryer and Hedrick, 2003].

In summary, survival and proliferation of *P. salmonis* within trout macrophages and monocytes may contribute to the establishment of piscirickettsiosis. The lack of damage of these infected cells

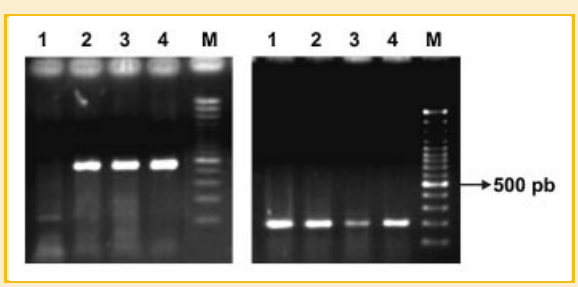

Fig. 6. Transcriptional expression of genes Chaps from *P. salmonis* (A) and EF1α from salmonid cells (B) in non-infected and infected cells. At 2, 5, and 10 days post-infection total RNA from infected and control cells was extracted with Trizol reagent and treated with 1 U/μg of DNase. cDNA was synthesized using ThermoScript or M-MLV reverse transcriptase from bacterial or cellular RNA, respectively. Primers and PCR conditions were as described in Materials and Methods Section. Lanes 1: Non-infected cells; 2–4: Cells 2, 5, and 10 days post-infection, respectively; M: 100 bp ladder.

should lead to the permanence of the bacteria in salmonids and, after dissemination to other tissues, should result in pathogenesis. A combination of intracellular growth of the bacteria and regulation of host cell death, while avoiding pathogen destruction, may be considered as the basis for *P. salmonis* pathogenesis. Taking into account that the molecular mechanism of *P. salmonis*-induced macrophages death is not known, future studies should consider whether apoptosis and other cell death mechanisms are involved in this fish disease.

The demonstration of the infection and propagation of this important pathogen in salmonid macrophage cultures represents an excellent model for future research of host–pathogen interaction of *P. salmonis* or other salmonid pathogens, and may provide valuable information in relation to the survival strategy used by this pathogen within cells that play a key role in the innate and adaptive immune responses.

We propose that *P. salmonis* interaction with phagocytic cells located throughout different tissues of the fish and which are involved in early detection of invading microorganisms, is pivotal in the establishment of piscirickettsiosis infection and the resulting pathogenesis.

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