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Evidence of exotoxin secretion of *Piscirickettsia salmonis*, the causative agent of piscirickettsiosis

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Abstract

Piscirickettsia salmonis is the aetiological agent of piscirickettsiosis, a disease which affects a variety of teleost species and that is particularly severe in salmonid fish. Bacterial-free supernatants, obtained from cultures of three isolates of *Piscirickettsia salmonis,* were inoculated in Atlantic salmon, *Salmo salar* L., and in three continuous cell lines in an effort to determine the presence of secretion of extracellular products (ECPs) by this microorganism. Although steatosis was found in some liver samples, no mortalities or clinical signs occurred in the inoculated fish. Clear cytotoxicity was observed after inoculation in the cell lines CHSE-214 and ASK, derived from salmonid tissues, but not in MDBK, which is of mammalian origin. The degree of cytotoxicity of the ECPs was different among the *P. salmonis* isolates tested. The isolate that evidenced the highest cytotoxicity in its ECPs exhibited only an intermediate virulence level after challenging fish with bacterial suspensions of the three *P. salmonis* isolates. Almost complete inhibition of the cytotoxic activity of ECPs was seen after proteinase K treatment, indicating their peptidic nature, and a total preclusion of the cytotoxicity was shown after their incubation at 50 °C for 30 min. Results show that *P. salmonis* can produce ECPs and at least some of them are thermolabile exotoxins that probably play a role in the pathogenesis of piscirickettsiosis.

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Introduction

At present, there are several fastidious Gram-negative bacteria that replicate intracellularly in their hosts, in either obligate or facultative mode (Mauel, Ware & Smith 2008), and are associated as causative agents of important diseases in aquatic animals including molluscs, crustaceans, and teleost fish. This emergent group of pathogens has been named loosely as rickettsia-like organisms (RLOs) and/or *Piscirickettsia*-like organisms (Mauel & Miller 2002). Within this bacterial group, *Piscirickettsia salmonis* was the first microorganism to be confirmed as a highly virulent pathogen, being the aetiological agent of piscirickettsiosis, a devastating disease in salmonid fish (Fryer & Hedrick 2003). It has also been reported that tissues of rainbow trout, *Oncorhynchus mykiss* (Walbaum),with red-mark syndrome in Scotland and with strawberry disease in the United States, two pathological conditions that are probably connected, are positive to RLOs 16S rRNA sequence by polymerase chain reaction and to *P. salmonis* by immunohistochemistry (Metselaar *et al.* 2010), which suggests that this bacterium could cause other fish diseases besides piscirickettsiosis.

A significant number of studies have been carried out on *P. salmonis*. This has allowed some important advances such as obtaining a more accurate taxonomic classification and understanding, at least partially, the immune response against it and the routes of entrance in its hosts (Fryer *et al.* 1992; Smith *et al.* 1999; Marshall *et al.* 2007). Nevertheless, the virulence factors of this

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pathogen are poorly known. This work was therefore initiated in order to investigate if *P. salmonis* produces extracellular products (ECPs) by studying their possible biological effects either *in vitro* in fish cell lines and/or *in vivo* in salmonid fish.

Materials and methods

Fish

In total, 560 Atlantic salmon, *Salmo salar* L., presmolts, held in fibreglass tanks supplied with a flow-through system of fresh water, were used. Some fish ($n = 400$) were employed for testing the virulence of the *P. salmonis* isolates and the remainder to test the effect of bacteria-free supernatants.

Cells

Monolayers of the chinook salmon, *O. tshawytscha* (Walbaum), embryo CHSE-214 (Lannan, Winton & Fryer 1984), Atlantic salmon kidney ASK (Devold *et al.* 2000) and Madin-Darby bovine, *Bos taurus* L., kidney MDBK (Madin & Darby 1958) cell lines were used. All of them were grown in the absence of antibiotics and antimycotics. Salmonid cells (CHSE-214 and ASK) were incubated at 17 °C and mammal cells (MDBK), unless explained, at 37 °C. The CHSE-214 and MDBK cells were cultured using Eagle′s minimal essential medium with Earle′s salts (Auto-mod; Sigma) supplemented with L-glutamine (EMEM) and with 10% of foetal calf serum (EMEM-10). The ASK cells were grown using L-15 Leibovitz medium with L-glutamine supplemented with β-mercaptoethanol and with 10% of foetal calf serum (all from Gibco).

Bacteria

Three isolates of *P. salmonis,* named *Ps-1, Ps-2* and *Ps-3,* obtained from rainbow trout, Atlantic salmon and coho salmon, *O. kisutch* (Walbaum), respectively, were used in this study. Bacteria were isolated from fish suffering piscirickettsiosis outbreaks from three different sea sites in the south of Chile. After the primary isolation, bacteria were preserved at −196 °C in liquid nitrogen until used. They were cultured in all the experiments in monolayers of the CHSE-214 cell line and incubated at 17 °C. Bacterial titration was carried out using the end-point dilution method (Reed & Müench 1938) in 96-well microplates.

Virulence test of *P. salmonis* isolates

Ten experimental groups, with 40 fish each, were used**.** Fish were distributed equally in two 2.5-m^3 tanks, each having 200 tagged animals in total, with 20 fish per experimental group. Fish were inoculated with three different doses (10^3, 10^4 and 10^5 $TCID_{50}$ $fish^{-1}$) of each isolate. Bacterial suspensions were intraperitoneally i.p. injected (0.1 mL per individual) in fish of the two tanks (i.e. 40 fish per dose of each isolate). Forty sham-inoculated fish (20 individuals per tank), injected i.p. with 0.1 mL of a sterile suspension of CHSE-214 cells, were used as control of the assay. Mortalities were recorded up to 46 post-inoculation (p.i.) days. Dead fish were examined by necropsy. In addition, kidney smears, fixed in absolute methanol, were Gram stained and also analysed by an indirect immunofluorescence test (IFAT) (Lannan, Ewing & Fryer 1991) to confirm the presence of *P. salmonis* in these tissues.

Toxicity test in fish of *P. salmonis*-free supernatants (*Ps*-fs)

Four experimental groups with 40 fish each were used. Fish of three groups were injected i.p. with 0.1 mL of a *Ps*-fs obtained from isolates *Ps-1*, *Ps-2* and *Ps-3*, respectively. The fish belonging to the remaining group were sham inoculated. Fish of each group were allotted equally in two 100-L tanks (eight tanks in total). *Piscirickettsia salmonis*-free supernatants were obtained from supernatants of CHSE-214 cells infected with *P. salmonis* and harvested when cytopathic effect reached approximately 100%. Those supernatants were centrifuged twice at 5 °C. The first centrifugation was at 1000 ***g*** for 15 min. The pellet was discarded and then the supernatant centrifuged at 10 000 ***g*** for 1 h. The pellet was again discarded and finally the supernatant was filtered through a membrane of mixed cellulose ester (DISMIC-25AS; Toyo Roshi Kaisha, Ltd) with 0.2-μm size pores. The experiment lasted 47 days p.i. At the end of the assay, three fish per group were euthanized by anaesthetic overdose (benzocaine at 200 mg L^{-1}) to obtain tissue samples (gills, heart, kidney, liver and spleen) for histological examination.

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Cytotoxicity test of *Ps*-fs in CHSE-214, ASK and MDBK cell cultures

Fresh monolayers of cells grown in 25-cm^2 flasks were inoculated in duplicate with 1 mL of individual *Ps*-fs obtained from the isolates *Ps-1*, *Ps-2* and *Ps-3*. Inoculated cells were observed daily until day 25 p.i. under an inverted optical microscope and their morphology was compared with sham-inoculated cell monolayers. CHSE-214 and ASK cells were always incubated at 17 °C, while MDBK cells were incubated in two different temperature regimes: at 37 °C during all the assay or at 17 °C for 7 days after the inoculation time and then at 37 °C until the end of the experiment at day 25 p.i.

Titration of *Ps*-fs cytotoxicity and effect of temperature and proteinase K on its cytotoxic ability in CHSE-214 cell cultures

Only the *Ps*-fs obtained from the *Ps-3* isolate was tested. Unless otherwise explained, the incubations were at 17 °C and the general method described above was used in these assays. For titration, 10 fold dilutions of *Ps*-fs were used. For the temperature test, the *Ps*-fs were incubated at 50 and 60 °C for 30 min and then inoculated in cell monolayers. Proteinase K assay was carried out treating a *Ps*-fs with 3.5 μM of this enzyme (United States Biological). After a 48-h incubation period, the enzymatic reaction was stopped adding AEBS (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; Sigma) at 0.5 mM. Solutions were inoculated in cell cultures 40 min after AEBS addition. The following solutions were used as controls of this assay: (*a*) *Ps*-fs obtained from *Ps-3* isolate; (*b*) EMEM-10 with proteinase K (3.5 μM); (*c*) EMEM-10 with proteinase K (3.5 μM) and AEBS (0.5 mM); (*d*) EMEM-10 with AEBS (0.5 mM) and (*e*) EMEM-10. After an incubation period of 48 h, solutions *a*, *b* and *e* were directly inoculated in cells. In solutions *c* and *d*, the AEBS was added after incubation for 48 h and 40 min later they were inoculated in the cell monolayers.

Results

Virulence of *P. salmonis* isolates

Disease was reproduced with the three isolates inoculated. Sick fish showed clinical signs of piscirickettsiosis, and dead fish exhibited gross pathology consistent with this disease. Examination of kidney smears of dead fish by IFAT showed the presence of *P. salmonis* in these tissues. No clinical signs or mortality occurred in the mocked-inoculated fish. Total cumulative mortalities of salmon inoculated with doses of 10^3, 10^4 and 10^5 $TCID_{50}$ fish^{-1} were, respectively, 82.5%, 100% and 100% for isolate *Ps-1*; 50%, 67.5% and 87.5% for isolate *Ps-2* and finally, 65%, 77.5% and 100% for isolate *Ps-3*. Within each of these doses, there were significant differences in survivability among fish groups (Log rank test $P \leq 0.05$). The order of the isolates, depending on the survivability they caused in the inoculated fish, was *Ps-2* > *Ps-3* > *Ps-1* (Fig. 1).

Ps-fs toxicity in fish

No diseased fish or mortalities were observed throughout the assay. Histopathology showed lesions in the liver of the fish inoculated with the *Ps-3* isolate characterised by severe steatosis and focal necrosis. Livers of fish inoculated with isolates *Ps-1* and *Ps-2* had moderate steatosis, but similar findings were observed in sham-inoculated fish.

Cytotoxicity test of *Ps*-fs in cell cultures

Monolayers of CHSE-214 cells inoculated with *Ps*-fs of each of the three isolates showed morphological abnormalities compared with the sham-inoculated cells. The most dramatic and fastest changes occurred in the cells inoculated with a *Ps*-fs prepared from the isolate *Ps-3*, which exhibited

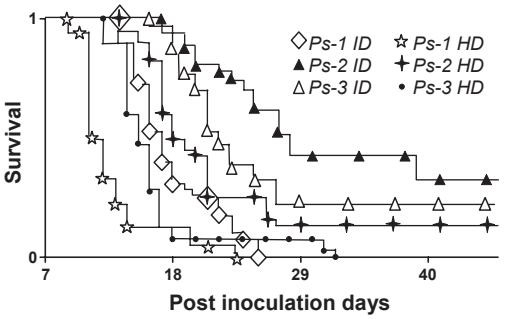

Figure 1 Survival curves of Atlantic salmon inoculated with high (*HD*) and intermediate (*ID*) doses of three *Piscirickettsia salmonis* isolates (*Ps-1*, *Ps-2* and *Ps-3*). *HD* and *ID* were 10^5 and 10^4 $TCID_{50}$ fish-1, respectively.

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from day 4 p.i. foci of rounded refringent cells with morphology resembling cells infected with whole *P. salmonis*. In addition, boundaries between cells were conspicuous and some cells showed pleomorphism, hypertrophy and cytoplasm vacuolation (Fig. 2). Cytotoxicity progressed with time reaching about 80% of the monolayer at day 15 p.i. Cells exposed with *Ps*-fs of isolates *Ps-1* and *Ps-2* also showed cytotoxicity, but the morphological changes appeared later (at day 10 p.i.), with only about 50% of the monolayer being affected at day 25 p.i. A similar pattern, but with a milder cytotoxic effect, was observed in ASK cells. No evidence of cytotoxicity was observed in MDBK cells either when they were incubated at 37 °C or when they were held for 7 days after inoculation at 17 °C and then at 37 °C.

Titration

Ps-fs of the isolate *Ps-3* in CHSE-214 cells showed cytotoxic effect up to dilution 10^{-2}.

Temperature effect

No cytotoxic effect was caused by *Ps*-fs after they were subjected to either 50 or 60 °C for 30 min.

Proteinase K

The cytotoxic effect of the *Ps*-fs treated with proteinase K was completely inhibited until day 18 p.i., but from day 19 to 25 p.i. the intercellular boundaries were more distinctly, although subtly, observed. As expected, cells inoculated with solution *a*, exhibited a clear cytotoxic effect from day 4 p.i. Addition of AEBS did not cause cytotoxicity (solution *d*) and suppressed the proteinase K effect on cells (solution *c*).

Discussion

Bacterial pathogens contain genes that code for virulence factors which in turn allow these organisms to infect and frequently cause disease in their hosts (Mims, Nash & Stephen 2000; Alberts *et al.* 2008). To succeed as infectious agents, pathogenic bacteria must colonize their host, reach an appropriate niche, avoid host defences, replicate, exit from the invaded eukaryote and be transmitted to a new susceptible host (Mims *et al.* 2000; Gyles & Prescott 2010). In spite of these basic steps of all bacterial infections, the specific mechanisms and virulence factors that pathogens use to cause disease in their hosts are as diverse as these microorganisms themselves (Alberts *et al.* 2008).

There are no published works related to ECPs of *P. salmonis* and their pathogenic function, but there is some information about other possible virulence mechanisms of this bacterium. Surface projections that would allow the attachment of *P. salmonis* to the chorion of the salmonid ova have been observed by scanning electron microscopy (Larenas *et al.* 2003). Generation of antibiotic resistance of *P. salmonis* has been reported (Smith *et al.* 1996), although the molecular mechanisms that would explain this phenomenon are unknown. The ability of *P. salmonis* to survive and replicate in the cytoplasm of macrophages has been documented (McCarthy *et al.* 2008). A similar pathogenic strategy is also used for other intracellular pathogens (Alberts *et al.* 2008). Besides macrophages, *P. salmonis* also invades a variety of cells that are normally non-phagocytic (Branson & Nieto Díaz-Muñoz 1991; Cvitanich, Gárate & Smith 1991). Therefore, it is likely that this bacterium induces its own endocytosis by such cells. On the other hand, structural features of the predominant *P. salmonis* lipid A, representing the hexaacyl

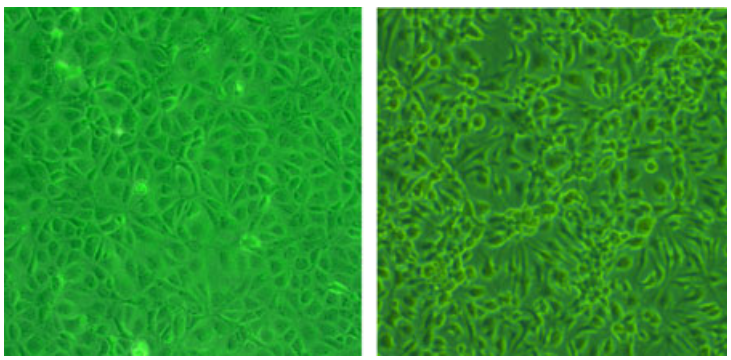

Figure 2 Monolayers of CHSE-214 cells. Left: uninoculated. Right: cytotoxic effect 9 days after inoculation with extracellular products of *Piscirickettsia salmonis* (isolate *Ps-3*). Phase contrast 100×.

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form, show a high degree of similarity with these classical forms of enterobacterial lipid A (Vadovič, Fodorová & Toman 2007). This finding suggests that the lipopolysaccharides (LPS) of this bacterium may have a strong endotoxic activity, which could explain the cause of the disseminated intravascular coagulation described in cultured coho salmon infected with *P. salmonis* (Cvitanich *et al.* 1991). The transcriptomic response of Atlantic salmon experimentally inoculated with *P. salmonis* showed a down-regulation of genes involved in the adaptive immune response, G-protein signalling pathway and apoptotic process, which may be reflective of virulence mechanisms used by this pathogen to survive, replicate and escape host defences (Tacchi *et al.* 2011).

Our results in show that *P. salmonis* secretes ECPs and at least one of their components has cytotoxic effects *in vitro* and probably mediates some tissue damage *in vivo* in salmonid fish infected with this microorganism. The almost complete inhibition of the *in vitro* effect of the *P. salmonis* ECPs by proteinase K treatment indicates their peptidic nature and therefore they can be categorized as exotoxins. The slight residual cytotoxicity of ECPs may have been caused by an incomplete hydrolysis of proteins because of an insufficient incubation time and/or dose used of the proteinase K or, although unlikely, by the presence of some proteins resistant to that enzyme (Butler *et al.* 1991; Dabaghian *et al.* 2008). Another hypothetical explanation could be the action of LPS released to the culture medium (Brunson & Nicolson 1978).

The effect of these exotoxins seems to be selective depending on the target cells because any of the ECPs of the three *P. salmonis* isolates tested produced damage in eukaryotic cells (MDBK) and all of them caused higher cytotoxicity in CHSE-214 compared with ASK cells. On the other hand, it appears that the quality and/or the quantity of the secreted ECPs may vary depending on the *P. salmonis* strain. This is supported by the fact that the degree of the cytotoxic effect of the ECPs in the fish cell lines was clearly different according to the isolate used to obtain these supernatants.

There was no clear association between the virulence degree of the isolates, measured through fish inoculation with bacterial suspensions, and the cytotoxic effect of their ECPs on cell monolayers. The isolate *Ps-3* showed only an intermediate virulence, compared with the other two isolates, but its ECPs had the highest cytotoxic effect on cell cultures. In turn, ECPs of the most virulent isolate (i.e. Ps-1) exhibited a lower cytotoxicity compared with those of the isolate *Ps-3*. These findings, along with the fact that the ECPs injection in fish did not reproduce the disease, suggest that there are other virulence factors besides the exotoxins evidenced here that are important in the pathogenicity of *P. salmonis* in salmonid fish.

The *in vitro* toxigenic potency of *P. salmonis* ECPs appears to be significant because they still cause cytotoxicity in cultured cells after a 10^{-2} dilution. Conversely, it seems that the *in vivo* potency of these exotoxins would be low because, as already mentioned, fish injected with undiluted ECPs did not show clinical signs of piscirickettsiosis. These last results, however, should be interpreted cautiously because the inoculation procedure used in this work, in terms of dose, administration via and/or other factors, may have not been adequate to evidence the pathogenic effect of these ECPs. Probably, *P. salmonis* secretes ECPs permanently while infecting the host, therefore a single dose, which was the method used here to inoculate the fish, may not replicate the effect produced in the natural course of the disease. Histopathology of fish, both sham inoculated and inoculated with ECPs of the three isolates, showed some level of liver steatosis, but the severity of such lesions were clearly higher in fish inoculated with ECPs of the isolate *Ps-3*. This finding is consistent with the higher cytotoxicity of ECPs *in vitro* shown by the *Ps-3* isolate, suggesting an association between the *in vitro* and *in vivo* effect of exotoxins of *P. salmonis*. Nevertheless, because the histopathology was carried out only on a limited number of animals and, in addition, this was not a time-course study these results are not conclusive.

Although there are no publications referring to *P. salmonis* ECPs, many authors have reported their presence and significance as virulence factors in a number of other Gram-negative salmonid pathogens, including bacteria of the genera *Aeromonas*, *Flavobacterium*, *Moritella*, *Photobacterium*, *Tenacibaculum*, *Vibrio* (*Listonella*) and *Yersinia* (Austin & Austin 2007; Tobback *et al.* 2007; van Gelderen, Carson & Nowak 2009; Bjornsdottir, Gudmundsdottir & Gudmundsdottir 2011; Frans *et al.* 2011). Bacteria belonging to the genera *Francisella* and *Coxiella* are phylogenetically related to *P. salmonis,* and thus, it is worth

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discussing, at least briefly, if their virulence mechanisms include the ability to secrete exotoxins. With respect to *Francisella tularensis*, the most well known species of this genus, there is no consensus opinion on the synthesis of exotoxins, and genes encoding toxins have not been found in this species. Nevertheless, in *F. novicida*-like isolates, which are close relatives of *F. tularensis*, genes coding for putative RTX exotoxins have been recently found (Siddaramappa *et al.* 2011). In the case of *Coxiella burnetii,* no exotoxins have been described, and LPS is, indeed, the only defined virulence factor (Gilk, Voth & Heinzen 2009). However, it should be noted that *C. burnetii* isolates have genes encoding a type IV secretion system predicted to deliver to the host cytosol effector proteins that mediate the formation of the parasitophorous vacuole, in which it replicates, and other cellular events (Voth *et al.* 2011).

In summary, the main conclusions of this work are that *P. salmonis* secretes ECPs, which contain heat-labile exotoxins, that produce a selective cytotoxicity *in vitro* depending on the type of targeted cell; the quality and/or quantity of the secreted exotoxins is heterogeneous among *P. salmonis* strains; and, finally, these exotoxins probably play a role in the pathogenesis of piscirickettsiosis. It is important to know the virulence factors of pathogenic microorganisms for several practical reasons including the development of vaccines, therapeutic and diagnostic methods and to discover epidemiological markers. As is also widely known, toxoids have been successfully employed to prevent some important bacterial diseases in humans and domestic animals, and therefore, this is an obvious potential use of *P. salmonis* exotoxins. Although this work has provided some information about the virulence factors of *P. salmonis,* it is evident that further knowledge is required to fully understand the mechanisms of pathogenicity of this bacterium. Identification and characterization of the *P. salmonis* exotoxins is one, among many, of the tasks that remain to be carried out, and the use of genomic and proteomic tools would be very helpful in this respect.

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