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Effect of serum factors on the survival of *Renibacterium salmoninarum* within rainbow trout macrophages

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ABSTRACT: While the phagocytosis of *Renibacterium salmoninarum* by rainbow trout macrophages *in vitro* occurred in the absence of serum factors, opsonized bacteria once phagocytosed survived for a longer period than bacteria not exposed to serum. Immune sera favoured survival to a greater extent than non-immune sera. Although heat-inactivated immune sera reduced the extent of bacterial killing, bacterial persistence was enhanced to such an extent when both complement and antibodies were present, that opsonized bacteria grew faster within macrophages than extracellularly. The effect of serum was observed following 16 h, but not 3 h, of prior exposure of the bacteria to serum, suggesting that opsonization per se was not the basis for improved survival, but that the serum induced an adaptative change in the bacterium.

KEY WORDS: *Renibacterium salmoninarum* · Rainbow trout · Macrophages · Serum Survival · Killing

INTRODUCTION

Bacterial kidney disease (BKD) caused by the Gram-positive bacterium *Renibacterium salmoninarum* is a major problem in the culture of salmonids all over the world (Fryer & Sanders 1981). Circumstantial evidence for the intracellular nature of *R. salmoninarum* infection has long been provided by the histopathology accompanying the disease (Young & Chapman 1978, Bruno & Munro 1986). We have previously demonstrated (Bandín et al. 1993) that *R. salmoninarum* can survive in contact with rainbow trout macrophages for up to 4 d, but thereafter killing of the bacterium was observed. At present no information is available concerning the mechanisms that allow the persistence of *R. salmoninarum* within macrophages or the intracellular antimicrobial mechanisms which determine the decline of the pathogen as measured in the *in vitro* assays.

Phagocytosis followed by intracellular killing represents an important effector mechanism for eradication of infectious agents. Opsonization is the process of preparation for phagocytosis of microorganisms which are otherwise not recognized or which have developed strategies for preventing their engulfment and is primarily mediated by antibodies and complement (Kaufmann & Reddehase 1989).

Studies focused on the opsonic effect of fish antibodies and/or complement are not very frequent. However, opsonizing activity of anti-*Aeromonas salmonicida* and anti-*Yersinia ruckeri* antibodies has been demonstrated in rainbow trout *Oncorhynchus mykiss* after the inactivation of complement (Griffin 1983, Sakai 1984, Michel et al. 1991). The involvement of rainbow trout complement in the phagocytosis of *A. salmonicida* and *R. salmoninarum* has also been reported (Sakai 1984, Rose & Levine 1992).

In most of the cases studied opsonization is to the benefit of the host because potentially infectious agents succumb to intracellular killing. However, some

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pathogens can use the opsonizing process to their own advantage, facilitating their entry into host cells (Moulder 1985, Kaufmann & Reddehase 1989).

In the present work the effect of normal and immune serum on the phagocytosis of *Renibacterium salmoninarum* as well as on the induction or avoidance of killing of the phagocytosed bacteria was studied.

MATERIALS AND METHODS

Bacterial strains. Two virulent autoagglutinating strains of *Renibacterium salmoninarum* were used in the present study: MT 420 (isolated from Atlantic salmon *Salmo salar*, Scotland) and RPC1 (coho salmon *Oncorhynchus kisutch*, Spain). The reference strain ATCC 33209 (American Type Culture Collection, Rockville, MD, USA) was also utilized. The working cultures were maintained on Mueller-Hinton Agar (Difco Laboratories, MI, USA) supplemented with 0.1% cysteine hydrochloride (MHA-C) at 15°C for no more than 7 d and stored frozen at –70°C in Mueller-Hinton Broth plus cysteine with 15% v/v glycerol until used.

Fish and immunization procedures. Rainbow trout *Oncorhynchus mykiss* (200 to 500 g body weight) were held in tanks supplied with fresh running water at 12 to 15°C. The fish were anaesthetized with tricaine methanesulfonate (MS 222) during immunization and bleeding procedures.

Formalin-killed *Renibacterium salmoninarum* ATCC 33209 (2×10^9 cells ml^{-1}) was emulsified in an equal volume of Freund's complete adjuvant. Fish were inoculated intraperitoneally with 0.5 ml of this emulsion. Twenty days after inoculation a booster injection, identical in composition to the primary injection, was performed and serum was collected after a further 29 d.

Antiserum and complement. Blood of immunized and non-immunized fish was obtained by bleeding from the caudal vein. The blood was allowed to clot at 4°C overnight; the serum was removed, divided into aliquots and stored at –80°C.

Isolation of trout macrophages. Macrophages were obtained from the pronephros of rainbow trout following the procedure described by Graham et al. (1988). Briefly, the head kidney was removed and disrupted through a 100 µm nylon mesh with Leibovitz' medium (L-15, Gibco BRL, Life Technologies Ltd, Paisley, Scotland) containing 2% inactivated (56°C, 30 min) foetal calf serum (iFCS), 1% penicillin-streptomycin (P/S, Gibco) and 10 U heparin ml^{-1} (Sigma Chemical Co., St Louis, MO, USA). This cell suspension was layered onto a 34 to 51% Percoll gradient. Following centrifugation at $400 \times g$ for 25 min, the band of cells separated at the interface was collected, centrifuged for 5 min at the same speed and resuspended. The viable cell concentration was determined by trypan blue exclusion, and 100 µl of 2×10^7 cells ml^{-1} in L-15 medium supplemented with 0.1% iFCS and 1% P/S was added to 96-well microtitre plates (A/S Nunc, Roskilde, Denmark). After 3 h incubation at 18°C, non-adherent cells were removed by washing with L-15 medium and the remaining monolayers were maintained in L-15 medium, with 5% iFCS and 1% P/S, for 1 to 3 d before use. The number of adherent macrophages was determined by counting the nuclei released following lysis in 0.1 M citric acid, 1% Tween 20 and 0.05% crystal violet as described by Chung & Secombes (1988).

Agglutination tests. A quantitative agglutination test was performed following the procedure of Roberson (1990) by using microtitre plates with serial 2-fold dilutions of 25 µl aliquots of the antiserum. Cell supensions of each strain (10^9 cells ml^{-1}) were used as antigen. For this assay saline solution (0.02% NaCl) was used to avoid autoagglutination observed with phosphate-buffered saline (PBS). The titre was recorded as the reciprocal of the highest dilution of the antiserum which gave positive agglutination after incubation overnight at 15°C. Bacterial suspensions in saline were used as a control.

Electrophoresis and western blotting analysis. Cell surface extracts of *Renibacterium salmoninarum* strains were obtained as previously described (Bandín et al. 1992). Briefly, bacteria grown on MHA-C were scraped off the plates with saline solution and centrifuged at $7000 \times g$ for 10 min (4°C). The cellular pellets were resuspended in 3 ml of 10 mM Tris [hydroxymethyl] amino-methane (Tris), pH 8 plus 0.3% NaCl and disrupted by sonic treatment (Branson sonifier 250). After centrifugation at $10000 \times g$ for 1 min, the supernatant fluids were transferred to another tube and centrifuged again for 60 min at $20000 \times g$ (4°C). The resultant pellets were dissolved in distilled water and stored frozen at –30°C until use. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970).

Proteins fractionated by electrophoresis were electroblotted from the gel onto nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA, USA). After transfer, nitrocellulose membranes were blocked for 1 h with 3% gelatin in Tris-buffered saline (TBS) before immunostaining.

Gelatin blocked membranes were washed in TBS plus 0.05% Tween-20 (TTBS) and then incubated for 1 h in trout serum (1:40). Membranes were washed again and then incubated for 1 h in rabbit anti-rainbow trout immunoglobulin (1:1000). After further washing membranes were incubated with goat-anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad) diluted 1:3000. Bands were visualized by incubating mem-

branes in 0.1 M carbonate buffer (pH 9.8) containing tetrazolium blue (0.3 mg ml^{-1}) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (0.15 mg ml^{-1}). Finally, blots were rinsed in distilled water for approximately 3 min and air-dried.

Incubation of *Renibacterium salmoninarum* with fish sera. *R. salmoninarum* in 0.02% NaCl were incubated for 3 or 16 h at 15 °C with 1:5 saline-diluted immune and non-immune sera obtained from rainbow trout. In some experiments complement coexisting with antibodies in the immune sera was heat-inactivated (30 min at 44°C) following the recommendations of Sakai (1984). After the incubation period bacteria were removed by centrifugation (12000 × *g*, 5 min), washed 3 times with saline and adjusted to 5×10^8 bacteria ml^{-1}.

Determination of the internalization of *Renibacterium salmoninarum* by fluorescence microscopy. To differentiate attached bacteria from internalized bacteria the technique described by Devrets & Campbell (1991) was used. This procedure relies on the labelling of bacteria with fluorescein isothiocyanate (FITC isomer I, Sigma) and the use of ethidium bromide (EB) as a quenching agent. EB is a dye, excluded by live eukaryotic cells, which fluoresces red-orange and causes FITC-labelled targets to shift from green to red fluorescence (Fattorosi et al. 1989). *R. salmoninarum* cells were harvested and adjusted to an optical density (OD_{600}) of 0.6 in 0.1 M $NaHCO_3$. Live bacteria were labelled by the addition of FITC to a final concentration of 0.1 mg ml^{-1} and incubated at 25°C for 60 min. Bacteria were pelleted at 12000 × *g* for 5 min and washed free of unbound FITC with saline. According to a plating assay this labelling did not affect the viability of *R. salmoninarum*. It is also known that this treatment does not disturb the bacterial surface (Mundi et al. 1991). Macrophage monolayers were prepared in 24-well plates by adding 1 ml of 2×10^7 cells per well, incubating for 3 h and washing to remove non-adherent cells. The monolayers in 1 ml of L-15 plus 5% iFCS were inoculated with 100 µl of bacteria at a ratio of 1:10. After centrifugation of the bacteria onto the monolayers for 5 min at 150 × *g*, infected cultures were incubated at 16°C for 1, 3, 5 and 7 d. At the end of each incubation period, infected macrophages were scraped off the wells and both cells and medium transferred to a tube. Then, 100 µl aliquots were removed and mixed with EB (50 µg ml^{-1} final concentration) and a 10 µl drop was immediately placed on a glass slide, overlaid with a coverslip, and evaluated by fluorescence microscopy under oil immersion. In all the experiments viability of macrophages was determined by trypan blue exclusion. In preliminary experiments with suspensions of *R. salmoninarum* labelled with FITC, the addition of EB was shown to produce a colour shift from green to red.

Bactericidal assays. The *in vitro* assay used was a modification of that reported by Graham et al. (1988) and has been previously described (Bandín et al. 1993). Briefly, wells containing viable macrophages in 100 µl L-15 medium plus 5% iFCS were inoculated with 20 µl of serial dilutions of normal or serum-exposed *Renibacterium salmoninarum* in saline. After centrifugation as described above, the infected cultures were incubated at 16°C for 1, 3, 5 and 7 d. At the end of each incubation period the supernatants were removed from the wells and the intracellular bacteria released by the addition of 50 µl of cold sterile distilled water. Then 100 µl of L-15 with 5% iFCS was added to each well to support the growth of the surviving bacteria for 6, 4, 2 and 0 d, respectively. In control wells, macrophages were lysed by the addition of 50 µl distilled water. After 10 min the water was removed and 100 µl L-15 with 5% iFCS was added followed by 20 µl of serial dilutions of *R. salmoninarum* in saline. These wells were incubated for 7 d. After this incubation time, viable bacteria present in the wells were quantified by the addition of 10 µl 3[4,5-di-methylthiazoyl-2-yl] 2,5-diphenyltetrazolium bromide (MTT, 5 mg ml^{-1}) which is reduced in proportion to the amount of viable bacteria present. The optical density was read at 620 nm 15 min later on a multiscan spectrophotometer (Flow). Experiments were carried out in quadruplicate and repeated 3 times (i.e. cells from 3 fish) for each bacterial strain tested. These data were examined statistically, by a 2-tailed Student's *t*-test. Results are expressed as means of data from 3 fish ± standard deviations.

RESULTS

Antibody response of rainbow trout

The agglutinating titres in the immunized fish were low, ranging from 4 to 32. However, when SDS-PAGE-separated *Renibacterium salmoninarum* cellular antigens were immunostained with the antiserum obtained in rainbow trout, antibodies were detected against the major and other cell-surface proteins of the bacterium (Fig. 1).

Double fluorescence microscopy

The utilization of a double fluorescence microscopy technique allowed identification of attached and internalized bacteria. Live bacteria were labelled with FITC and checked to confirm that the labelling did not affect their viability (data not shown). The mixture of labelled bacteria and macrophages was counterstained with EB to change the fluorescence of extracel-

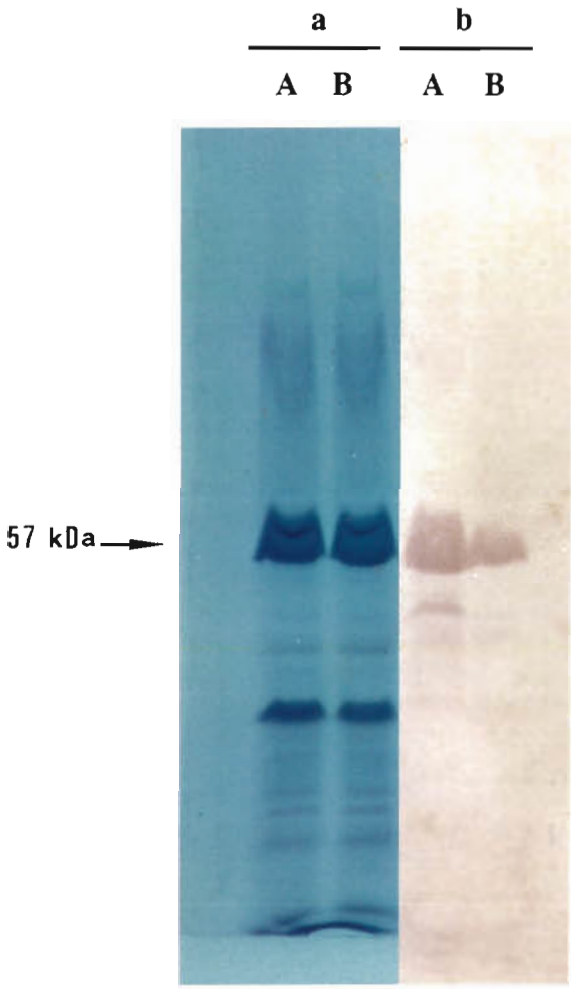

Fig. 1. (a) *Renibacterium salmoninarum*. SDS-PAGE of membrane proteins from 2 strains. Lane A: ATCC 33209; Lane B: RPC1. (b) Western blot of membrane proteins preparation from *R. salmoninarum* isolates tested with trout serum anti-*R. salmoninarum* ATCC 33209. Lane A: ATCC 33209; Lane B: RPC1

lular bacteria. As a result of the addition of EB, which is excluded by live cells, attached but not internalized bacteria fluoresced red, whereas intracellular *Renibacterium salmoninarum* fluoresced green. In all the phagocytosis assays after addition of EB more than 85% of cell-associated bacteria fluoresced green (bacteria from 100 macrophages), indicating that they were internalized and not merely adherent (Fig. 2).

Survival of opsonized *Renibacterium salmoninarum* within trout macrophages

The ability of *Renibacterium salmoninarum* strains opsonized with immune and non-immune rainbow trout serum to survive within macrophages was determined. Identical results were obtained with the 2 strains tested. Figs. 3 & 4 show the results obtained with strain RPC1.

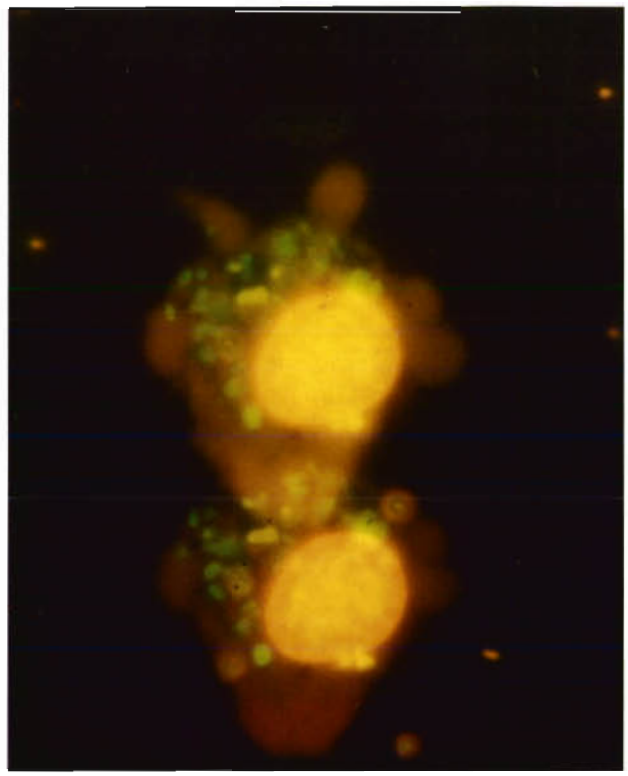

Fig. 2. Fluorescence micrograph of macrophages and phagocytosed FITC-labelled *R. salmoninarum* RPCI with ethidium bromide added. Extracellular bacteria fluoresce red-orange while intracellular bacteria remain green

When the bactericidal assay was performed with bacterial isolates previously incubated with non-immune sera for 16 h, it was observed that *Renibacterium salmoninarum* was able to survive up to 5 d (Fig. 3B), which represents an increase of 1 d in the bacterial persistence with respect to the killing assays performed with untreated bacteria (Fig. 3A). However, a significant decrease ($p < 0.05$) in the number of viable bacteria was observed at longer incubation times (7 d) (Fig. 4B). Figs. 3 & 4 show the results obtained with RPC1 strain.

When immune serum was employed, *Renibacterium salmoninarum* strains were able to survive for the 7 d duration of the assay (Fig. 4C). In addition, it should be pointed out that opsonization with immune serum not only stopped killing, as shown in Fig. 4C, but also slightly increased the growth of intracellular bacteria with respect to the control (bacteria incubated with lysed macrophages). Although the addition of heat-inactivated antiserum (antibody alone) favoured bacterial persistence (data not shown), the survival was notably enhanced when both antibody

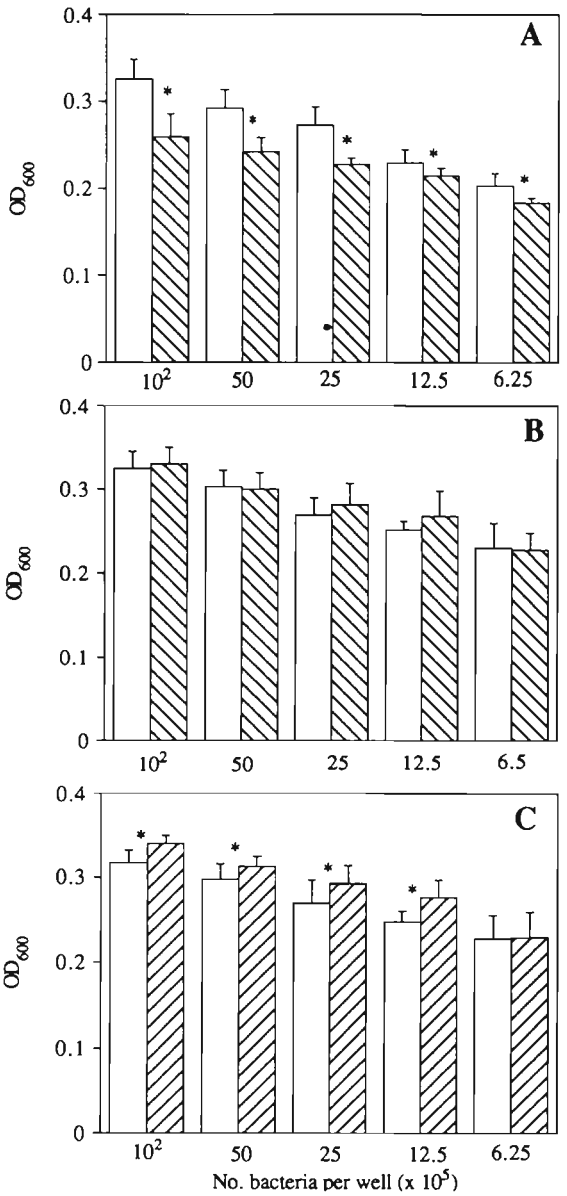

Fig. 3. Infection of rainbow trout macrophages with *Renibacterium salmoninarum* RPC1 for 5 d. Results are the mean of MTT reduction ± SD for 3 experiments. (▨) 5 d with live macrophages / 2 d in L-15 plus lysed macrophages; (□) control, 0 d with live macrophages / 7 d in L-15 plus lysed macrophages. *Significant differences ($p < 0.05$) between each data point and the control. (A) untreated bacteria; (B) non-immune serum coated bacteria; (C) immune serum coated bacteria

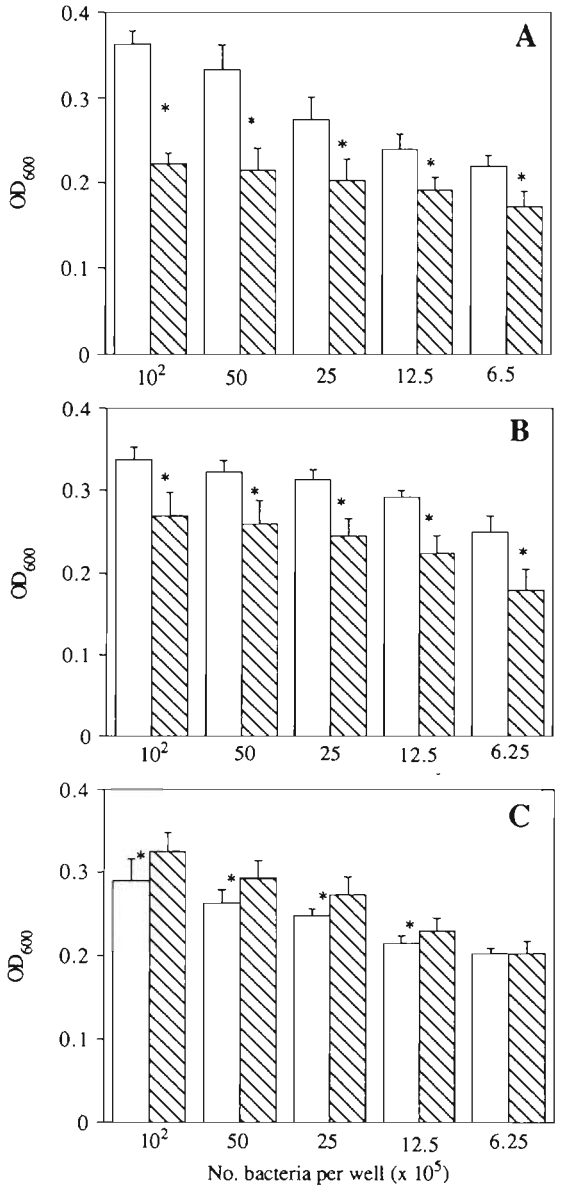

Fig. 4. Infection of rainbow trout macrophages with *Renibacterium salmoninarum* RPC1 for 5 d. Results are the mean of MTT reduction ± SD for 3 experiments. (▨) 7 d with live macrophages / 0 d in L-15 plus lysed macrophages; (□) control, 0 d with live macrophages / 7 d in L-15 plus lysed macrophages. *Significant differences ($p < 0.05$) between each data point and the control. (A) untreated bacteria; (B) non-immune serum coated bacteria; (C) immune serum coated bacteria

and complement were utilized. No protective effect was observed when bacteria were incubated with the serum for 3 h.

DISCUSSION

The internalization assay performed with FITC-labelled bacteria and EB demonstrated that *Renibacterium salmoninarum* is efficiently ingested by trout phagocytes in the absence of serum factors, which is in contrast with results reported by Rose & Levine (1992). Phagocytosis in the absence of serum suggests a direct cell-bacterium interaction and the hydrophobic surface of *R. salmoninarum* (Daly & Stevenson 1987, Bruno 1988, Bandín et. al. 1989) may be important in promoting binding of the pathogen to the fish macro-

phages. In addition, the presence of a high number of internalized bacterial cells (85%) at different time points (1, 3, 5 and 7 d) strongly supports the view that *R. salmoninarum* is an intracellular pathogen.

Although our results indicate that serum is not necessary in the phagocytosis of *Renibacterium salmoninarum*, we did observe differences in the viability of untreated and opsonized bacteria when incubated with trout macrophages. Thus, while untreated bacteria survived for 4 d, bacteria opsonized with non-immune serum were able to survive for up to 5 d, and the viability was increased up to 7 d if immune serum was employed. Indeed the data indicated that intracellular growth of the bacteria opsonized with immune serum was enhanced compared with the control (opsonized bacteria incubated with lysed macrophages). These findings seem to indicate that both complement and antibodies would be implicated in the protection of *R. salmoninarum* against the bactericidal activity of trout macrophages.

In mammals the capacity of antibodies of the IgG class to promote phagocytosis is well established. However, mammalian phagocytes do not bear receptors for IgM on their surface, so IgM itself cannot act as an opsonin (Hiemstra 1992). Although no fish group so far investigated has any Ig class other than IgM, opsonization of bacteria with fish antibodies has been reported to favour phagocytosis (Griffin 1983, Sakai 1984, Michel et al. 1991)

With the aim of determining the effect of antibodies on the interaction of *Renibacterium salmoninarum* with the macrophages, complement activity was depleted by heat-inactivating immune serum. The incubation of bacteria with antibodies alone slightly extended bacterial persistence (data not shown). However, it was observed that survival was clearly enhanced when both complement and antibodies were present.

In mammals it has been demonstrated that IgM is a very effective activator of the classical pathway of the complement system, so IgM-coated particles can be opsonized with complement components. We hypothesize that although IgM-coated *Renibacterium salmoninarum* seems to receive a slight protection against the bactericidal activity of macrophages, bacteria could also be opsonized with complement components extending the survival period.

It is interesting to point out that the protective effect of sera was obtained when bacteria were incubated with it for 16 h but no extension of the survival time was observed with 3 h of exposure to sera. Bacteria have a remarkable ability to alter their metabolism in response to changing environmental conditions such as temperature, pH, osmolarity and mutrient availability. The bacterial outer membrane is the prime structure through which these adaptations are mediated (Brown & Williams 1985). It may be possible that incubation of *Renibacterium salmoninarum* in trout serum for 16 h but not 3 h resulted in a physiological adaptation of the bacterium to conditions similar to those *in vivo* which enabled prolonged survival within macrophages.

The complexity of developing a protective anti-BKD vaccine has been assessed by different authors. It has been reported that salmonids seem to be able to produce antibodies against experimental *Renibacterium salmoninarum* bacterins, but it has not been clearly shown that the presence of such antibodies confers protection (Paterson et al. 1981, 1985, Munro & Bruno 1988). In fact, the results obtained in the present work suggest that the presence of antibodies would favour the survival of *R. salmoninarum* within salmonid macrophages and future studies should be focused on the stimulation of a cell-mediated response.

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