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NOTE

Purification of *Piscirickettsia salmonis* and associated phage particles

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ABSTRACT: *Piscirickettsia salmonis* was isolated from cell culture using differential centrifugation and purified on a 30% Percol gradient. The purity of the preparation was assessed by transmission electron microscopy and phage-like particles were found to be associated with some of the *P. salmonis* isolates examined. This is believed to be the first report of a phage associated with rickettsia from fish.

KEY WORDS: *Piscirickettsia salmonis* · Rickettsia · Purification · Bacteriophage

Rickettsia-like organisms are an important group of fish pathogens, affecting a variety of fish species from diverse geographical locations in different aquatic environments (Fryer & Lannan 1996). The significance of rickettsiae as a fish pathogen first came to light in 1989, when substantial mortalities were experienced at some coho salmon *Oncorhynchus kisutch* farms in Southern Chile, although the aetiological agent remained unidentified at that time (Cvitanich et al. 1990). Attempts to isolate and identify the causative agent of these outbreaks then followed, and a previously undescribed obligate gram-negative intracellular pathogen was isolated and characterised as *Piscirickettsia salmonis* gen. nov. sp. nov, a rickettsial organism belonging to the order Rickettsiales, family Rickettsiaceae (Fryer et al. 1992).

It is possible to culture the bacterium *in vitro* within fish cell lines (Fryer et al. 1990). However, for many analyses, it is necessary to isolate and purify the organism from the host cell. Because of its intracellular nature, the release of rickettsiae from the cells is often incomplete, and often more than half of the total infectious yield remains associated with intact host cells or cell debris (Moulder 1985). Kuzyk et al. (1996) described a method of purifying *Piscirickettsia salmonis* from tissue culture supernatants using centrifugation in a Percol gradient. This was based on methods previously described by Tamura et al. (1982) and Weiss et al. (1975). A modified version of the method described by Kuzyk et al. (1996) has been used successfully in the present study to obtain several different isolates of *P. salmonis* in very high purity. Upon examination of the purified bacteria by electron microscopy, some of the isolates were found to have associated bacteriophage. This note describes the purification method and is the first description of a phage infecting *P. salmonis*.

Material and methods. ***Bacterial growth and isolation:*** The *Piscirickettsia salmonis* strains presented in Table 1 were maintained in the chinook salmon embryo cell line (CHSE-214; ATCC CRL-1681, Lannan et al. 1984) at 15 to 17°C, using antibiotic-free Eagle's minimal essential medium (MEM) with Earle's salts, supplemented with 2% (v/v) foetal calf serum (FCS) and 2 mM L-glutamine.

Purification of Piscirickettsia salmonis: Six triple flasks (3000 cm^2) (Nunc A/S Roskilde, Denmark) were seeded with CHSE-214 cells at a seeding concentration of 5×10^4 cells ml^{-1}, and incubated for 48 h at 22°C. The culture medium was removed and each flask was then inoculated with 20 ml of supernatant recovered from a *P. salmonis*-infected cell culture. After incubation at 15°C for 2 h, the supernatant was removed and approximately 100 ml of fresh MEM, supplemented as above, was added to each flask. The cells were cultured for between 10 and 15 d until an extensive cytopathic effect (CPE) (approximately 90%) was observed. The cell culture supernatant was collected and centrifuged at 20 000 × *g* for 30 min at 4°C and result-

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ing pellets resuspended in 12 ml Tris-Sucrose buffer (TSB, 33 mM Tris-hydrochloride containing 0.25 M sucrose, pH 7.4). The suspended pellets were homogenised with a Dounce tissue homogeniser (Kinematica, Switzerland) at a moderate speed for 2 min, then gently vortexed for 1 min. Homogenates were centrifuged at 210 × *g* for 10 min at 4°C to remove host cell debris. Three ml aliquots of the homogenate were loaded onto 27 ml of Percoll (Pharmacia Biotech AB, Uppsala, Sweden), prepared at 30% in TSB (v/v) in polycarbonate centrifuge tubes (25 by 89 mm). The tubes were centrifuged at 25 000 × *g* for 60 min at 4°C to allow a self-forming gradient to develop by isopycnic centrifugation. Bands which formed were harvested from the gradient, diluted 10-fold in phosphate buffered saline (PBS: 5.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 116 mM NaCl, 10 mM KCl, pH 7.4) and rickettsiae pelleted by centrifuging at 20 000 × *g* for 30 min at 4°C. The pellet was resuspended with PBS and centrifuged as described above to eliminate residual Percoll from the preparations. Differential pelleting and density gradient centrifugation was performed using a fixed angle rotor (type TFT-70.38) in a Kontron, Centrikon T-1170 ultracentrifuge. Density marker beads (Pharmacia) were used to indicate the density profile of the gradient and the density at which sample bands formed.

Protein concentrations of the rickettsial preparations were determined using a protein determination kit (BioRad Richmond, CA, USA). The rickettsia (1 ml of the preparation) were first lysed using 0.1 to 0.5 mm diameter zirconia/silica beads in a mini bead-beater (Biospec Products. Inc., Bartlesville, OK, USA), beating for 160 s at high speed.

Transmission electron microscopy (TEM): Samples were prepared for TEM by fixing rickettsial pellets with 2.5% glutaraldehyde in 100 mM cacodylate buffer (v/v) at 4°C for 2 h. They were then placed in the cacodylate buffer and rinsed overnight at 4°C. The pellets were post-fixed with 1% (w/v) osmium tetroxide in the same buffer for 1 h, stained with 2% (w/v) uranyl acetate in 30% (v/v) acetone for 1 h in the dark, dehydrated through a series of acetone concentrations, and then embedded in Spurr's embedding medium. Sections, 100 nm in thickness, were prepared and these were stained for 2 min with uranyl acetate saturated in 50% ethanol, before placing the sections in lead citrate for 2 min. They were viewed with a Philips EM 301 electron microscope at 80 kV in transmission mode.

Experimental challenge: Atlantic salmon (20 to 25 g), used for experimental infection, were maintained in a flow-through filtrated and ultraviolet disinfected seawater system at 11°C. Groups of 10 fish were placed in separate tanks (50 l) and were inoculated intraperitoneally with $10^{4.6}$ $TCID_{50}$ ml^{-1} of each of the rickettsial isolates (0.1 ml $fish^{-1}$) shown in Table 1, except the isolate SLGO-95. One fish was sampled from each group 15 d post-inoculation to confirm the presence of the bacteria in the kidney of the fish using an indirect fluorescent antibody technique (IFAT). Bacteria were re-isolated from kidney tissue at this time, by aseptically removing kidney samples and homogenising them in MEM (1:10 w/v). The homogenates were left to stand for 5 min at 22°C to allow large particles to settle out, before placing 0.5 ml of the suspension onto a CHSE-214 cell monolayer, prepared previously in a 25 cm^2 flask. The flask was incubated at 15°C for 2 h, before removing the supernatant and adding 5 ml of fresh MEM supplemented with 2% (v/v) FCS and 2 mM L-glutamine. The cultures were incubated at 15°C for 20 to 30 d and observed daily for the development of a CPE.

IFAT: Air-dried smears, prepared from infected kidneys, were fixed in absolute methanol for 5 min. Samples were incubated with rabbit anti-*Piscirickettsia salmonis* (kindly provided by Prof. J. Fryer, Oregon State University, Corvallis, OR, USA, and diluted 1/1000 in PBS), for 60 min in a humidified chamber at 22°C. Negative controls of PBS were also included. Slides were rinsed thoroughly with PBS and left to stand for 5 min in PBS; then they were rinsed once again before applying goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Sigma-Aldrich, Inc., St Louis, MO, USA) diluted 1/80 in PBS, to the smears for 30 min. Slides were incubated in the dark in a humidified chamber at 22°C during this time, after which they were again washed with PBS as above. Smears were observed for the presence of bacteria under B excitation using an Olympus IMT-2 microscope with a reflected fluorescent attachment, and exciter and barrier filters for fluorescein isothiocyanate.

Results and discussion. A simple and effective method for the purification of *Piscirickettsia salmonis* from cell culture is described, based on the use of Percoll gradients with differential pelleting. The first observation of phage particles associated with *P. salmonis* under TEM is also reported.

Percoll gradients are widely used in the separation of cells, organelles and even viruses (Rickwood 1987). Tamura et al. (1982) used Percoll density gradient centrifugation to purify *Rickettsia tsutsugamushi* and found a 40% Percoll gradient to be optimal for the isolation of the organism, which was located at a density of 1.07 to 1.08 g ml^{-1}. Kuzyk et al. (1996) later used a similar gradient to purify *Piscirickettsia salmonis*, and found 2 bands to form in the gradient; one of a low-density, devoid of rickettsial whole-cell material, and the other a high-density band composed of organisms with the size and morphology of *P. salmonis*. Barnes et

al. (1998), on the other hand, used a diatrizoate meglumine and diatrizoate sodium density gradient centrifugation to purify the organism; however, purified *P. salmonis* preparations contained organisms still associated with CHSE-214 host cells. A simple and effective method, slightly modified from that described by Kuzyk et al. (1996), was used for the purification of *P. salmonis* from cell culture.

After the development of a CPE in infected CHSE-214 cells (Fig. 1) *Piscirickettsia salmonis* was isolated from host cell debris by differential pelleting and density gradient centrifugation. Eight *P. salmonis* isolates, shown in Table 1, were purified using a 30% (v/v) Percoll density gradient. A wide, whitish band with a density of 1.056 to 1.080 g ml^{-1}, as determined from density marker beads, formed within the gradient. The normal CHSE-214 cell proteins had a density of 1.053 to 1.055 g ml^{-1} in the control gradient.

The yield of protein obtained from *Piscirickettsia salmonis* type species LF-89 using this purification protocol was 7.5 mg ml^{-1}. The band at a density of 1.056 to 1.080 was found to be composed of rickettsiae under TEM examination, the size and morphology of which corresponded to that expected for *P. salmonis* (Fig. 2), pleomorphic and ca 0.5 to 1.5 µm in diameter (Fryer & Lannan 1994). The preparations were mainly composed of whole-cell *P. salmonis*, with only very small amounts of fragmented material.

All isolates in Table 1 were examined by TEM, and virus-like bodies were found to be associated with 2 of the *Piscirickettsia salmonis*, LF-89 and VQ 013 (Figs. 3 & 4). These phage-like particles were polyhedral in shape and had a short rigid tailpiece attached to the vertex of the polyhedron. The diameter of the phage particles was approximately 110 to 130 nm. The TEM examinations demonstrated that phage particles were within the cell of *P. salmonis* and also attached to the cell walls of the bacterial cells by the tip of their tail and appeared to eventually lyse the host cell (Fig. 5). There was no evidence that the CHSE-214 cell line used to propagate the *P. salmonis* isolates was infected with any phage-like particles.

Morel (1974) cited by Buchanan (1978) was the first to observe phages associated with the obligate parasitic bacterium *Porochlamydia buthi*, and described them as short-tailed polyhedral particles. Although virus-like bodies have been previously associated with rickettsia or in rickettsia (Buchanan 1978, Wright et al. 1978,

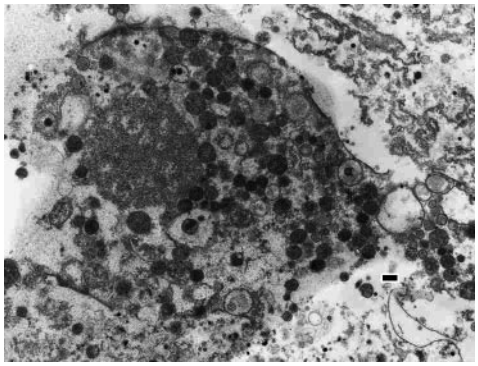

Fig. 1. Transmission electron micrograph of a rickettsia-infected CHSE-214 cell. Numerous rickettsia-like organisms (strain LF-89) of varying sizes are present within and also being released from the cell cytoplasm, 7 d post-inoculation. Scale bar = 0.5 µm

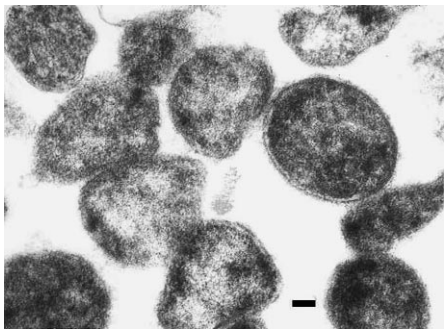

Fig. 2. Rickettsia-like organisms (strain LF-89) purified by Percoll density gradient centrifugation. Scale bar = 0.1 µm

Table 1. *Piscirickettsia salmonis* isolates examined in this study

Strain	Source	Country
LF-89 ATCC VR 1361	Coho salmon *Oncorhynchus kisutch*	Chile[a]
AVG 5/268	Atlantic salmon *Salmo salar*	Ireland[b]
VQ 013	Rainbow trout *Oncorhynchus mykiss*	Chile[c]
SRS-UACH	Coho salmon *Oncorhynchus kisutch*	Chile[c]
SRS-4	Coho salmon *Oncorhynchus kisutch*	Chile[c]
R-29	Atlantic salmon *Salmo salar*	Chile[c]
SLGO-95	Coho salmon *Oncorhynchus kisutch*	Chile[d]
R980769	Sea bass *Dicentrarchus labrax*	Greece[e]

The *P. salmonis* isolates were kindly provided by:
[a]Dr John Fryer (Department of Microbiology, Oregon State University, Oregon, USA) (Fryer et al. 1992)
[b]Dr Roy Palmer (Aquatic Veterinary Group, University College of Galway, Ireland)
[c]Dr Carlos Farias (Univ. Austral de Chile, Valdivia, Chile)
[d]Dr Pedro Smith (University of Chile, Santiago, Chile)
[e]Infected fish tissue was supplied by Dr Athanassios Prappas (National Fish Disease Laboratory, Athens, Greece)

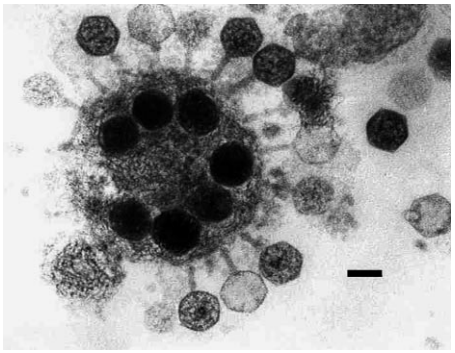

Fig. 3. Numerous bacteriophage particles attached to individual rickettsia-like bodies (strain LF-89). Scale bar = 0.1 µm

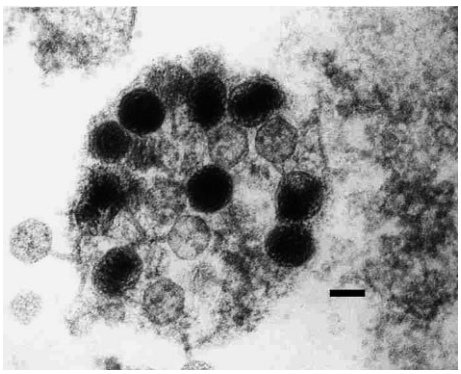

Fig. 4. Rickettsial organism (strain VQ 013) being lysed by virus-like bodies. Scale bar = 0.1 µm

Shaw & Moloo 1993), there have been no reports of viral particles associated with rickettsia isolated from fish.

No mortalities occurred in the groups injected with the isolates associated with the phage particles or in the other groups apart from 2 dead fish infected with isolate SRS-4 during the fourth week of the experimental period. The isolates were recovered from all remaining experimentally infected fish, which were sampled at the end of Week 4. After re-isolation, isolates LF-89 and VQ 013 were re-examined for the presence of the phage under TEM; however, no phage-like particles could be observed in either isolate.

The use of bacteriophages as a biological control for diseases of cultured fish has aroused much interest in recent years, especially since no drug residues and drug toxicity are associated with this type of therapy (Wu et al. 1981, Nakai et al. 1999). Control is based, firstly, on the fact that growth of the bacteriophage is able to out-compete bacterial growth and, secondly, infection by the bacteriophage is very specific for its host. However, potential difficulties associated with phage therapy need to be considered. For example, it is necessary to understand the heterogeneity and ecology of both the phage and the bacterium, and to be able to select highly virulent phages against target bacteria. It is also important to establish if bacterial strains have become resistant to the phage, and to understand which factors of the immune response of the vertebrate host are able to inactivate the phages

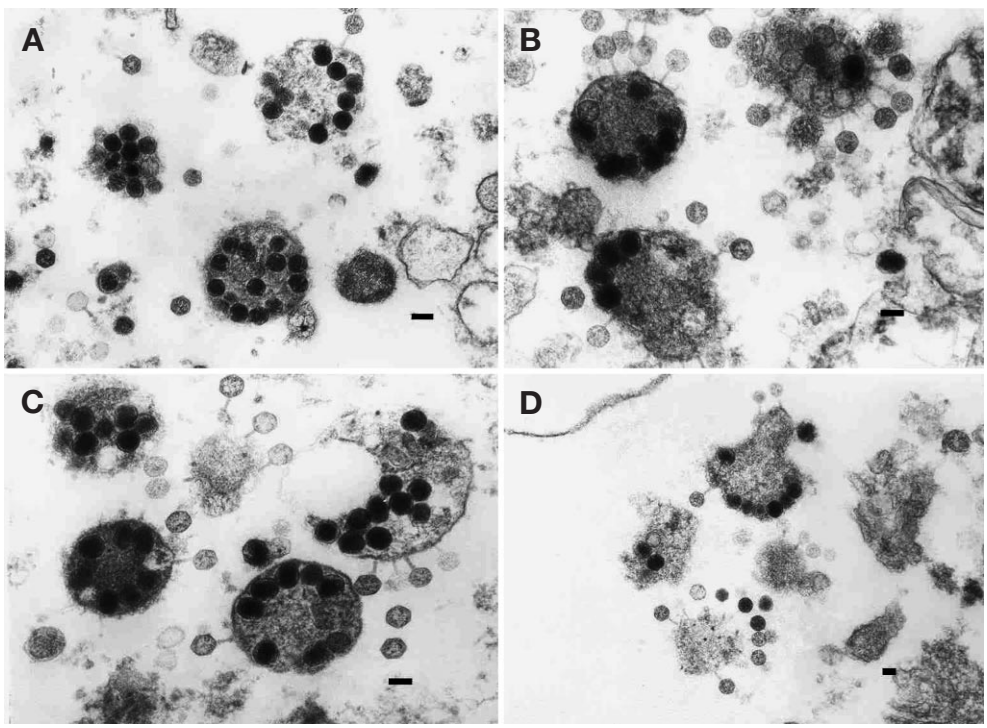

Fig. 5. Transmission electron micrograph of rickettsia-like organisms associated with bacteriophage. (A) Individual organisms filled with viral particles (strain LF-89). Scale bar = 0.15 µm. (B,C) Rickettsia-like bodies attached to numerous phage particles (strain VQ 013). Scale bars = 0.1 µm. (D) Lysed rickettsia-like organism (strain VQ 013). Scale bar = 0.1 µm

and lyse bacteria (Barrow & Soothill 1997). These points are important when developing an effective phage therapy. It would appear from this study that the phage particles are able to grow successfully within the rickettsia, when the bacterium is grown *in vitro* within cell culture, while they disappeared when the rickettsia were cultured *in vivo* during experimental challenges. It shows that the phages might have been introduced during the cultivation of the bacterium. Differences in the culture environment of the rickettsia grown *in vivo* and *in vitro* may, in part, explain why the phages appear unable to grow within the rickettsia whilst in the host animal.

Phage therapy may provide an alternative to the problems associated with antibiotic therapy; however, it is suggested that the requirements of the phage *in vivo* need to be elucidated. Further work to characterise the bacteriophages associated with *Piscirickettsia salmonis* is currently underway.

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