INFECTION AND IMMUNITY, Dec. 1996, p. 5205–5210 Vol. 64, No. 12
0019-9567/96/$04.00+0

Antigenic Characterization of the Salmonid Pathogen *Piscirickettsia salmonis*

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Received 1 July 1996/Returned for modification 15 August 1996/Accepted 20 September 1996

***Piscirickettsia salmonis*, the etiological agent of salmonid rickettsial septicemia, was purified from infected immortal chinook salmon (*Oncorhynchus tshawytscha*) embryo cells by a combination of differential and Percoll density gradient centrifugation. Immune sera from rabbits immunized with purified whole cells of *P. salmonis* reacted with four protein antigens and two carbohydrate antigens with relative molecular sizes of 65, 60, 54, 51, 16, and ~11 kDa, respectively. The carbohydrate antigens appear to be mainly core region lipo-oligosaccharide with lesser amounts of lipopolysaccharide. Serum from convalescent rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Oncorhynchus kisutch*) reacted with several minor immunoreactive protein antigens between 10 and 70 kDa in size and a carbohydrate antigen with a relative molecular size of ~11 kDa. The salmonid immune system did not appear to elicit a strong humoral response against this intracellular pathogen. Indirect immunofluorescence microscopy, immunogold transmission electron microscopy, and biotin labeling of intact *P. salmonis* cells suggest that the immunoreactive antigens identified with rabbit antisera are surface exposed and differ significantly from those identified with salmonid antisera.**

The order *Rickettsiales* historically encompassed any intracellular bacterium, and taxonomy was based on only a few phenotypic characteristics (9). More recently, 16S rRNA sequence similarity studies have helped to better define the taxonomy of the order *Rickettsiales* (9). Rickettsiae cause a variety of medically significant diseases in humans including typhus fever, Rocky Mountain spotted fever, and boutonneuse fever (24, 35). Rickettsiae are also agriculturally significant and are the etiological agents of a variety of veterinary diseases (26).

Piscirickettsia salmonis is the first rickettsia to be isolated from an aquatic poikilotherm (13). *P. salmonis* is the etiological agent of salmonid rickettsial septicemia (SRS) and is an economically significant pathogen of salmonids that is responsible for extensive mortalities in the South American aquaculture industry. *P. salmonis*, a gram-negative obligate intracellular bacterium, was first observed in 1989 in a diseased, moribund coho salmon from a saltwater net pen site on the coast of Chile (3). It is now known that *P. salmonis* is geographically more widespread than was initially suspected and has recently been observed in Ireland (27), Scotland, and Norway and on the Pacific coast of Canada (4). *P. salmonis* was thought to be confined to the saltwater stage of the salmonid life cycle, but Gaggero et al. (15) have recently isolated this pathogen from juvenile coho salmon and rainbow trout during their freshwater stage; thus *P. salmonis* appears to be emerging.

P. salmonis has been observed to infect a wide range of salmonid species and causes a systemic infection that targets the kidney, liver, spleen, heart, brain, intestine, ovary, and gills of salmonids (7). Pleomorphic, predominantly coccoid bacteria that range in diameter from 0.5 to 1.5 μm are found within cytoplasmic vacuoles of cells from infected tissues (3). While initially difficult to culture, *P. salmonis* LF-89 was successfully isolated from the kidney of a diseased adult coho salmon on an immortal chinook salmon embryo cell line (13). Fryer et al. (14) conducted a 16S rRNA sequence similarity study which placed *P. salmonis* in its own genus and species within the order *Rickettsiales* and family *Rickettsiaceae. P. salmonis* is most closely related to *Coxiella burnetii* and *Wolbachia persica*, with 87.5 and 86.3% sequence similarity, respectively (14). *P. salmonis* appears to belong within the tribe *Ehrlichieae* because of its cytoplasmic incompatibility (14).

Efficacy of antibiotic treatment of SRS is poor because of the intracellular nature of *P. salmonis*, thereby making management of the disease difficult (23). To effectively prevent and control SRS, vaccine development is the only logical alternative. However, vaccines prepared from whole-cell bacterins of mammalian rickettsiae have shown disappointing protection in trials (35). Elucidation of the molecular pathogenesis of *P. salmonis* is therefore necessary as a prelude to vaccine development.

Cultivation of rickettsiae is inherently expensive and difficult because of their obligate intracellular nature (16). Therefore, it is impractical to obtain purified antigens from rickettsiae for molecular characterization studies and vaccine preparation (31). Until the advent of recombinant DNA technology, knowledge regarding proteins of rickettsiae was based upon monoclonal antibody studies (31). Recombinant DNA technologies have allowed a variety of rickettsial proteins to be cloned and characterized (10, 18, 31, 34). Protection studies conducted with cloned rickettsial antigens have shown protection against rickettsial infection, thereby establishing a research strategy that can be applied to developing vaccines against other rickettsiae (10, 16, 35). This study describes the purification of *P. salmonis* from host cell material and the identification of immunogenic *P. salmonis* surface antigens.

MATERIALS AND METHODS

Bacterial strains, growth, and isolation. *P. salmonis* strains were routinely passaged on a chinook salmon embryo cell line, CHSE-214 (ATCC CRL-1681),

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at 17°C in Eagle's minimal essential medium (MEM) with Earles salts supplemented with 10% newborn calf serum. Type strain *P. salmonis* LF-89 was obtained from the American Type Culture Collection (ATCC VR-1361) and is herein referred to as *P. salmonis.*

P. salmonis BC-95 was isolated from kidney tissue of a moribund Atlantic salmon displaying SRS-like symptoms (7) obtained from a commercial net pen site in the Pacific Northwest following the protocol of Lannan and Fryer (22). Briefly, 1 g of kidney tissue was aseptically removed from the mortality and homogenized in 3 ml of MEM. A 100-μl volume of a 10^{-3} dilution of the homogenate was used to inoculate a 25-cm^2 monolayer of CHSE-214 cells. The monolayer was incubated at 17°C and monitored for cytopathic effects.

P. salmonis (Atlantic) and *P. salmonis* (coho) were isolated in cell culture on CHSE-214 monolayers from kidney tissue of dead Atlantic and coho salmon fry. Salmon fry were experimentally infected with *P. salmonis* by 0.1-cm^3 intraperitoneal injections of cell culture supernatant from a CHSE-214 culture infected with *P. salmonis*. Identities of the isolates were confirmed as *P. salmonis* by using a latex bead agglutination diagnostic kit (Microtek International, Saanichton, British Columbia, Canada) and by Western blot (immunoblot) analysis with rabbit anti-*P. salmonis* serum.

Purification of *P. salmonis*. A protocol for purifying *P. salmonis* was developed by combining and modifying the protocols of Tamura et al. (32) and Weiss et al. (36). A 6,320-cm^2 Nunc cell factory (Nunc, Naperville, Ill.) was seeded with cell line CHSE-214 and infected with ~450 ml of cell culture supernatant from fully lysed CHSE-214 monolayers infected with *P. salmonis*. Infection was allowed to continue for 14 to 17 days until cytopathic effects obliterated the entire monolayer. Upon destruction of the monolayers cell culture supernatants were collected and pelleted at 10,000 × *g* for 30 min at 4°C. Pellets were resuspended in MEM and homogenized in a 15-ml Dounce tissue homogenizer (VWR, Toronto, Ontario, Canada). The homogenized suspension was centrifuged at 200 × *g* for 10 min at 4°C to pellet large host cell debris. The supernatant was filtered twice through AP20 glass microfiber prefilters (Millipore, Bedford, Mass.) and pelleted at 17,600 × *g* for 15 min at 4°C. Pellets were resuspended in TS buffer (33 mM Tris-HCl, 0.25 M sucrose; pH 7.4). Samples were loaded onto Percoll (Pharmacia Biotech AB, Uppsala, Sweden) gradients with a final concentration of 40% and centrifuged in a fixed angle rotor (type JA-14) at 20,000 × *g* for 60 min at 4°C in a Beckman J2-21 centrifuge. Bands were collected by aspiration, diluted with phosphate-buffered saline, pH 7.4 (PBS) (29), and pelleted at 20,000 × *g* for 10 min at 4°C. Pellets were washed twice with PBS. Contents of the bands were negative stained with 0.5% phosphotungstic acid (PTA) and analyzed by transmission electron microscopy (TEM) on a Philips EM 300 at an accelerating voltage of 75 kV.

Antibody generation. Percoll purified whole cells of *P. salmonis* were fixed overnight at 4°C in PBS containing 5% formalin. A New Zealand White rabbit was administered 230 μg of *P. salmonis* protein antigen emulsified in Freund's complete adjuvant by subcutaneous and intramuscular injections. Prebleed serum was collected prior to the primary immunization. The rabbit was boosted with an additional 230 μg of *P. salmonis* protein antigen emulsified in Freund's incomplete adjuvant at 3- and 5-week intervals after the primary immunization. Serum was collected and titers were determined by enzyme-linked immunosorbent assay (ELISA) (11) using formaldehyde-inactivated whole-cell *P. salmonis* as the antigen and goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Caltag Laboratories, San Francisco, Calif.) as the second antibody. The ELISA was developed with *p*-nitrophenyl phosphate as previously described (5). The titer of the rabbit anti-*P. salmonis* serum was 1/200,000.

Rainbow trout anti-*P. salmonis* serum was obtained from convalescent trout 28 days after sublethal challenge. Rainbow trout were administered an intraperitoneal injection of cell culture supernatant from *P. salmonis*-infected CHSE-214 monolayers containing ~10^6 50% tissue culture infective doses of *P. salmonis* per ml.

Immunofluorescence microscopy. Air-dried samples were fixed in −20°C acetone/ethanol (60:40) for 20 min. Samples were incubated for 30 min with rabbit anti-*P. salmonis* serum (diluted 1/200 in PBS) and then washed 5 min with PBS. Slides were then incubated for 45 min with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (diluted 1/40 in PBS) (Caltag Laboratories) and then washed three times with PBS. Preparations were observed using a photomicroscope equipped with an UV light source and excitation and barrier filters for fluorescein isothiocyanate.

Electrophoresis. Protein analysis was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the protocol of Laemmli (20) as modified by Ames (2). Whole-cell lysates of cell line and *P. salmonis* samples were routinely analyzed by using 12% polyacrylamide gels with 5% stacking gels.

Silver staining. SDS-PAGE samples were digested with 1 μl of stock proteinase K (PK) solution (1 mg/ml) per 10 μl of SDS-PAGE sample for 1 h at 60°C. PK digestion was stopped by boiling for 10 min. A 12 to 17% continuous gradient polyacrylamide gel was used for silver staining and immunoblot analysis of PK-digested samples following the method of Fling and Gregerson (12). Silver staining followed the method of Tsai and Frasch (33).

Western blot analysis. Western blotting was carried out as previously described by Collinson et al. (5). *P. salmonis* antigens were detected by using either rabbit anti-*P. salmonis* serum (diluted 1/2,000) followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (diluted 1/4,000) or rainbow trout anti-*P. salmonis* serum (diluted 1/500) followed by mouse anti-Atlantic salmon IgM (diluted 1/1,000) and goat anti-mouse IgG conjugated to alkaline phosphatase (diluted 1/4,000) (Caltag Laboratories). Salmonids only have a single antibody isotype that is an IgM-like tetramer that lacks a J chain (25). Immunoblots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as previously described (5).

Biotinylation of surface proteins. Percoll-purified *P. salmonis* cells and freshly harvested CHSE-214 cells were surface labeled with the impermeant modification reagent sulfo-NHS-biotin (Pierce, Rockford, Ill.) and analyzed following the method of Dooley and Trust (8). To minimize potential CHSE-214 host cell protein contamination of the *P. salmonis* preparation, *P. salmonis* cells were washed, centrifuged, and resuspended with PBS a minimum of four times before biotinylation was conducted.

Immunogold electron microscopy. Samples prepared for TEM were fixed with 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide in 0.2 M sodium cacodylate buffer. Fixed samples were dehydrated in a series of graded ethanol steps and propylene oxide and embedded in epoxy resin Epon 812. Thin sections of samples were mounted on formvar-coated nickel grids. Osmium was destained from sections by using 0.2% sodium metaperiodate. Sections were washed three times with water then blocked with PBS containing 3% bovine serum albumin. Sections were incubated with rabbit anti-*P. salmonis* serum (diluted 1/200), washed three times with PBS, and then incubated with goat anti-rabbit IgG conjugated to 5-nm gold particles (diluted 1/50) (Caltag Laboratories) and then washed three times with PBS. Samples were fixed briefly with 4% glutaraldehyde in PBS and stained with 1% uranyl acetate in 50% ethanol. Thin sections were viewed with a Philips EM 300 at an accelerating voltage of 75 kV.

RESULTS

Purification of *P. salmonis*. *P. salmonis* was separated from CHSE-214 debris by differential sedimentation and density gradient centrifugation. High-purity *P. salmonis* was required to generate polyclonal rabbit serum with low cross-reactivity to host cell material. The sample loaded onto the Percoll gradient was analyzed before and after Percoll density gradient centrifugation by PTA negative staining TEM. A 40% Percoll density gradient gave the greatest separation of *P. salmonis* from CHSE-214 debris. PTA negative staining of the homogenized cell culture sample before density gradient centrifugation showed that it contained a large amount of cellular debris (results not shown) of either *P. salmonis* or CHSE-214 origin making the preparation unsuitable for generation of high-specificity antiserum. Following density centrifugation, a minor well-resolved low-density band near the top of the gradient and a major diffuse high-density band in the lower half of the gradient were collected separately. PTA negative staining demonstrated that the low-density band was devoid of whole-cell material that resembled *P. salmonis*. The high-density band was mainly composed of material consistent with the size and morphology of *P. salmonis* and some less abundant vesicular material. The high-density band containing whole-cell *P. salmonis* was used to generate polyclonal rabbit antiserum. Immunofluorescence light microscopy by using the anti-*P. salmonis* rabbit serum was conducted on the high-density band (Fig. 1). The predominant immunoreactive material in the preparation was coccoid cells ranging from 0.7 to 1.2 μm in diameter, consistent with the size and morphology of *P. salmonis* (Fig. 1). The anti-*P. salmonis* rabbit serum also reacted with *P. salmonis* isolated from kidneys of moribund salmonids suffering from SRS, as determined by immunofluorescence microscopy and immunoblot analysis.

Surface-exposed antigens of *P. salmonis*. In order to characterize the antigenic profile of *P. salmonis*, Western blot analysis of *P. salmonis* was carried out by using anti-*P. salmonis* rabbit serum (Fig. 2A). PK digestion was used to determine if any observed antigens may have been carbohydrate. Six *P. salmonis* immunoreactive antigens were observed at relative molecular sizes of 65, 60, 54, 51, 16, and ~11 kDa (Fig. 2A). PK digestion destroyed all immunoreactive antigens except the 16- and 11-kDa antigens (Fig. 2B). Anti-*P. salmonis* serum did not significantly cross-react with any CHSE-214 proteins even when the

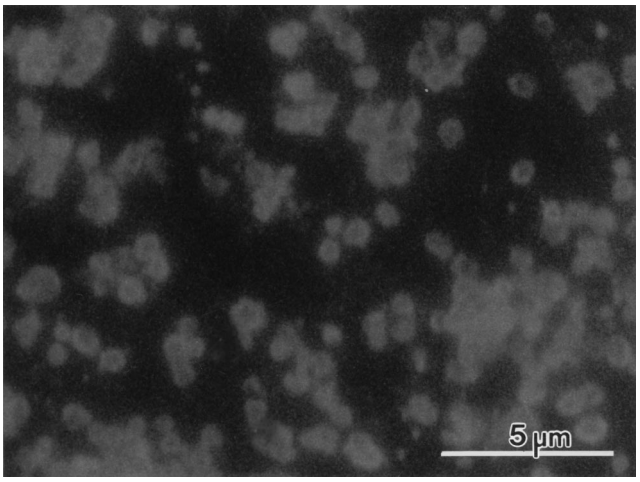

FIG. 1. Indirect immunofluorescence microscopy of *P. salmonis*. Material from the high-density band collected from the Percoll density centrifugation of *P. salmonis* material is shown. Antigen was detected with rabbit anti-*P. salmonis* serum. Goat anti-rabbit IgG conjugated to fluorescein isothiocyanate was used as the second antibody. Magnification, ×4,500 (bar = 5 μm).

immunoblot was allowed to develop overnight (results not shown). Anti-*P. salmonis* serum also was found not to react with CHSE-214 host cells or salmonid kidney tissue by analysis of infected cells with immunofluorescence microscopy (results not shown).

Percoll-purified *P. salmonis* and freshly harvested CHSE-214 cells were surface biotinylated with the impermeant alkylating reagent sulfo-NHS-biotin to help determine if any immunoreactive proteins comigrated with major *P. salmonis* surface proteins. Biotinylated proteins were detected by streptavidin-conjugated alkaline phosphatase (Fig. 3). One major *P. salmonis* protein was clearly evident around 28 kDa, and four major proteins were evident between 45 and 80 kDa (Fig. 3). Several other minor proteins were also evident. These five major proteins could easily be differentiated from CHSE-214 proteins.

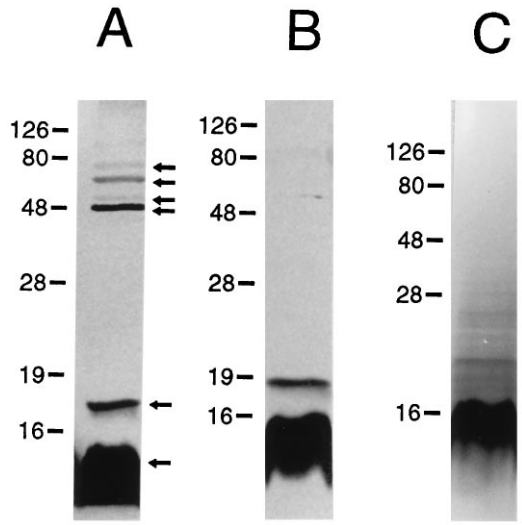

FIG. 2. Western blot analysis of *P. salmonis*. A whole-cell lysate of *P. salmonis* (A) and proteinase K digests of *P. salmonis* (B) were separated by SDS-PAGE (12% polyacrylamide) and reacted with anti-*P. salmonis* rabbit serum followed by immunochemical staining. (C) 12 to 17% gradient SDS-PAGE of PK-digested *P. salmonis*. PK-digested *P. salmonis* was silver stained for LPS.

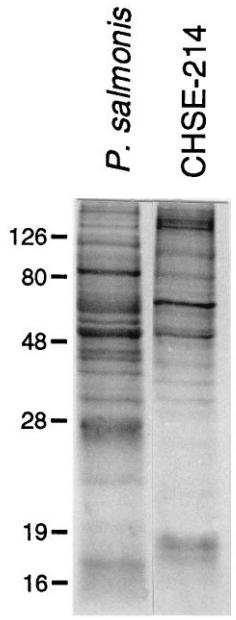

FIG. 3. Analysis of surface-exposed proteins of *P. salmonis*. Biotinylated *P. salmonis* (lane 1) and CHSE-214 proteins (lane 2) were separated by SDS-PAGE (12% polyacrylamide) and detected by streptavidin-conjugated alkaline phosphatase.

Control nonbiotinylated samples of *P. salmonis* and CHSE-214 did not demonstrate any streptavidin binding (results not shown).

Immunogold TEM of CHSE-214 cells infected with *P. salmonis* was conducted with rabbit anti-*P. salmonis* serum to determine if the antibodies reacted with antigens localized to the surface of *P. salmonis* cells. Figure 4 demonstrates that the rabbit antiserum generated against purified *P. salmonis* specifically labeled the surface region of *P. salmonis* with gold particles. Gold labeling of surrounding host cell membrane and cytoplasmic material was negligible.

Analysis of *P. salmonis* carbohydrate antigens. PK-digested

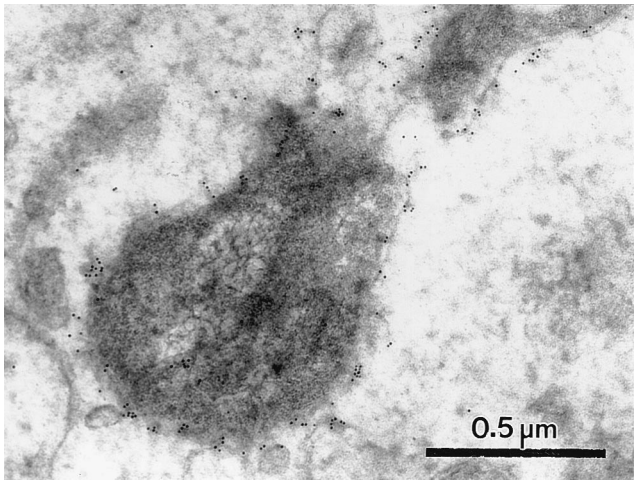

FIG. 4. Immunogold labeling of *P. salmonis*. Thin sections of CHSE-214 cells infected with *P. salmonis* were reacted with rabbit anti-*P. salmonis* serum and labeled with goat anti-rabbit IgG conjugated to 5-nm gold particles. Magnification, ×47,100 (bar = 0.5 μm).

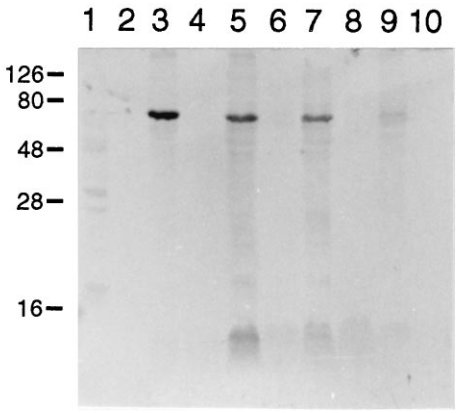

FIG. 5. Western blot analysis of four *P. salmonis* isolates. Undigested and PK-digested samples of CHSE-214 (lanes 1 and 2), *P. salmonis* BC-95 (lanes 3 and 4), *P. salmonis* Atlantic (lanes 5 and 6), *P. salmonis* coho (lanes 7 and 8), and *P. salmonis* LF-89 (lanes 9 and 10) were separated by SDS-PAGE (12% polyacrylamide). Samples were reacted with convalescent-phase rainbow trout anti-*P. salmonis* serum followed by immunochemical staining.

P. salmonis was silver stained to determine if *P. salmonis* has a lipopolysaccharide (LPS)-like component and what its characteristics are (Fig. 2C). PK-digested *P. salmonis* was analyzed by SDS-PAGE with a 12 to 17% polyacrylamide gradient. When silver stained for LPS, PK-digested *P. salmonis* revealed a very faint, ladder-like banding pattern of carbohydrates ranging from 16 to 35 kDa in size with a discrete band around 20 kDa and an intensely stained major band around 11 kDa (Fig. 2C).

Reactivity of convalescent salmonid antiserum with *P. salmonis*. Western blot analysis of four *P. salmonis* isolates was carried out with convalescent rainbow trout antiserum (Fig. 5). PK digests of these isolates were also analyzed to determine if any antigens recognized by the salmonid immune system were likely carbohydrate. A single protein of around 72 kDa that reacted in all four samples was shown to be an artifact that reacted with the mouse anti-salmon IgM second antibody (Fig. 4). Minor immunoreactive bands specific to *P. salmonis*, between 10 and 70 kDa in size, reacted with convalescent salmonid serum and did not react with control serum from uninfected salmonids (Fig. 4, lanes 3, 5, 7, and 9). A PK-resistant immunoreactive antigen was once again observed at ~11 kDa in all four *P. salmonis* isolates (Fig. 5, lanes 2, 4, 6, and 8) but was less pronounced than that seen with rabbit antiserum (Fig. 2A).

DISCUSSION

This report describes a purification protocol for *P. salmonis* from cell line CHSE-214 and the preliminary identification of likely surface-exposed *P. salmonis* antigens. No previous reports have appeared describing the antigenic composition of *P. salmonis* since it was first isolated (13). Past efforts to purify *P. salmonis* did not produce pure *P. salmonis* (21); thus, previous attempts to generate high-specificity polyclonal antiserum for antigen identification and the screening of genomic expression libraries have likely been unsuccessful.

Obtaining any rickettsial cells in amounts large enough to allow purification of proteins or preparation of vaccines has traditionally proven to be extremely difficult and expensive. Inherent difficulties associated with the investigation of rickettsiae made the establishment of an effective purification protocol for *P. salmonis* essential.

Differential sedimentation and density gradient centrifugation were used to successfully separate *P. salmonis* from CHSE-214 host cell material. Immunofluorescence of the purified *P. salmonis* preparation showed that the preparation only contained material consistent with the size and morphology of *P. salmonis* and some vesicular material. Vesicular immunoreactive material most likely originated from *P. salmonis* because Western blot analysis with rabbit anti-*P. salmonis* serum demonstrated that the serum did not cross-react with CHSE-214 proteins. Rickettsiae are generally unstable when separated from their host cells (31); therefore, vesicular material might have been generated by plasmolysis and outer membrane blebbing of *P. salmonis* during the homogenization step of the purification. SDS-PAGE analysis of biotinylated proteins from purified *P. salmonis* cells also suggests that the purified cells are free of membranous CHSE-214 material. If the vesicular material in the purified *P. salmonis* preparation was of CHSE-214 origin, some major biotinylated proteins would be common to both the *P. salmonis* and CHSE-214 samples. The observed banding patterns of major biotinylated proteins from *P. salmonis* and CHSE-214 differed significantly. However, the multiplicity of minor biotinylated proteins suggests that some *P. salmonis* damage occurred during purification.

Rabbit antiserum generated against whole-cell *P. salmonis* reacted with six predominant antigens. Silver staining of PK-digested *P. salmonis* confirmed that the ~11- and 16-kDa immunoreactive antigens were carbohydrate while the remainder were protein. The 11-kDa carbohydrate antigen is most likely the lipooligosaccharide (LOS) component of LPS. LPS is a primary component of the outer lipid leaflet of outer membranes of rickettsiae and other gram-negative bacteria (1). The chemical composition of spotted fever group rickettsiae LPS produces an enterobacterium-like banding pattern (1). Also present within the silver-stained PK-digested sample of *P. salmonis* was a repeating pattern of a high-molecular-weight carbohydrate species that decreased in abundance as its mass increased. The high-molecular-weight carbohydrates most likely represent core region LOS plus O antigen with a diminishing number of repeat units (1). A carbohydrate banding pattern in which carbohydrate becomes less abundant as molecular weight increases is characteristic of random polymerization of O-antigen repeating units (30). The 16-kDa immunoreactive carbohydrate antigen may represent core region LOS with one unit of O polysaccharide antigen, which would be the most abundant species containing O antigen if polymerization of O antigen is random.

To determine the antigens to which natural hosts of *P. salmonis* generate a humoral response, immunoblot analysis was conducted with convalescent salmonid antiserum. The possibility of antigenic variation in *P. salmonis* was also investigated by analyzing two isolates of *P. salmonis* LF-89 that had been experimentally passaged through different salmonid species as well as a new isolate from British Columbia, *P. salmonis* BC-95. No noticeable differences between the immunoreactive bands of the four isolates were observed. A PK-resistant antigen with a relative molecular size of ~11 kDa and minor protein antigens with relative molecular sizes ranging between 10 and 70 kDa were observed. The infected salmonids did not elicit a strong humoral response against this intracellular pathogen. Once again, the ~11-kDa antigen is likely LOS because it was not digested by PK and its relative molecular size was consistent with that observed for *P. salmonis* LOS. LOS was the only noticeably common immunoreactive antigen recognized by both rabbit and salmonid antisera, but the reaction with rabbit antisera was far more intense. Rabbit antiserum recognized a greater number of major immunoreactive *P. salmonis* proteins than convalescent salmonid serum. The absence of a significant

humoral response by salmonids against *P. salmonis* infection may be indicative of the intracellular nature of this bacterium and of the eventual requirement for cell-mediated immunity.

Molecules involved in bacterial adhesion and invasion and evasion and activation of the host immune response are generally surface exposed and contribute to the overall virulence of an organism (28). Characterization of the roles of surface-exposed virulence factors in the molecular pathogenesis of *P. salmonis* may eventually allow development of an inexpensive recombinant vaccine against *P. salmonis*. To identify surface-exposed *P. salmonis* antigens, rabbits were immunized with purified whole-cell *P. salmonis*. The generated antiserum appears to recognize only surface-exposed antigens. Immunofluorescence of purified *P. salmonis* with rabbit anti-*P. salmonis* serum showed a large amount of fluorescence over the entire cell with noticeably brighter fluorescence around the cell perimeter, which is characteristic of surface labeling. Surface biotinylation of purified *P. salmonis* labeled major surface proteins around the 16- and 50-kDa ranges, which correlate well with the molecular sizes of the two major immunoreactive protein antigens recognized by rabbit antiserum at 16 and 51 kDa. Immunogold TEM of a *P. salmonis*-infected cell line clearly demonstrated the high specificity of the anti-*P. salmonis* serum for surface localized antigens.

Recombinant DNA technologies have made the characterization of major surface proteins and antigens of various rickettsial pathogens possible (6, 10, 17–19, 31). Cloning and characterization of *P. salmonis* genes rely upon the ability to isolate genomic *P. salmonis* DNA free of host cell DNA. Screening of *P. salmonis* genomic expression libraries will also require highly specific antiserum against *P. salmonis* for the identification of relative antigens. *P. salmonis* was highly purified from CHSE-214 host cells by using differential sedimentation and Percoll density gradient centrifugation. Rabbit antiserum generated against purified *P. salmonis* recognized LOS, LPS, and four surface-exposed proteins with relative molecular sizes of 65, 60, 54, and 51 kDa. Salmonids generated antibodies against LOS and several minor immunoreactive proteins but appeared to elicit a low humoral response during infection, indicating that activation of innate and cell-mediated immune defense mechanisms may play important roles in combating *P. salmonis* infection. *P. salmonis* LOS and LPS were observed to be of predominantly low molecular weight, but less abundant high-molecular-weight species containing O antigen were present. Further studies will help determine whether these identified immunoreactive cell surface components are required for virulence of *P. salmonis*. It is hoped that protection studies will determine whether or not rabbit anti-*P. salmonis* serum can passively protect salmonids from lethal *P. salmonis* challenge. Such studies will help identify immunoreactive proteins for further immunological characterization and will serve as an important prelude to recombinant vaccine development.

ACKNOWLEDGMENTS

This work was supported in part by the Canadian Bacterial Diseases Network. M.K. was funded by the Science Council of British Columbia through the assistance of a GREAT award.

We thank S. C. Clouthier and S. K. Collinson for helpful discussions concerning experimental design. We are also grateful to B. Magnadóttir for supplying the anti-Atlantic salmon IgM polyclonal antibody. Thanks are also extended to A. White, S. Carlos, D. Harrison, and R. D'arcy for helpful suggestions.

REFERENCES

1. **Amano, K.-I., M. Fujita, and T. Suto.** 1993. Chemical properties of lipopolysaccharides from spotted fever group rickettsiae and their common antigenicity with lipopolysaccharides from *Proteus* species. Infect. Immun. **61:**4350–4355.
2. **Ames, G. F.-L.** 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. **249:**634–644.
3. **Bravo, S. S., and M. L. Campos.** 1989. Coho salmon syndrome in Chile. FHS/AFS Newsl. **17:**3.
4. **Brocklebank, J. R., T. P. T. Evelyn, D. J. Speare, and R. D. Armstrong.** 1993. Rickettsial septicemia in farmed Atlantic and chinook salmon in British Columbia: clinical presentation and experimental transmission. Can. Vet. J. **34:**745–748.
5. **Collinson, S. K., L. Emödy, K.-H. Müller, T. J. Trust, and W. W. Kay.** 1991. Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. J. Bacteriol. **173:**4773–4781.
6. **Crocquet-Valdes, P. A., K. Weiss, and D. H. Walker.** 1994. Sequence analysis of the 190-kDa antigen-encoding gene of *Rickettsia conorii* (Malish 7 strain). Gene **140:**115–119.
7. **Cvitanich, J. D., O. N. Garate, and C. E. Smith.** 1991. The isolation of a rickettsia-like organism causing disease and mortality in Chilean salmonids and its confirmation by Koch's postulates. J. Fish Dis. **14:**121–145.
8. **Dooley, J. S. G., and T. J. Trust.** 1988. Surface protein composition of *Aeromonas hydrophila* strains virulent for fish: identification of a surface array protein. J. Bacteriol. **170:**499–506.
9. **Drancourt, M., and D. Raoult.** 1994. Taxonomic position of the Rickettsiae: current knowledge. FEMS Microbiol. Rev. **13:**13–24.
10. **Emelyanov, V. V.** 1993. Molecular cloning and expression of *Rickettsia prowazekii* genes for three outer membrane proteins in *Escherichia coli*. Microb. Pathog. **15:**7–16.
11. **Engval, E., and P. Perlmann.** 1972. Enzyme-linked immunosorbent assay, ELISA. J. Immunol. **109:**129–135.
12. **Fling, S. P., and D. S. Gregerson.** 1986. Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. Anal. Biochem. **155:**83–88.
13. **Fryer, J. L., C. N. Lannan, J. H. Garcés, J. J. Larenas, and P. A. Smith.** 1990. Isolation of a rickettsiales-like organism from diseased coho salmon (*Oncorhynchus kisutch*) in Chile. Fish Pathol. **25:**107–114.
14. **Fryer, J. L., C. N. Lannan, S. J. Giovannoni, and N. D. Wood.** 1992. *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. Int. J. Syst. Bacteriol. **42:**120–126.
15. **Gaggero, A., H. Castro, and A. M. Sandino.** 1995. First isolation of *Piscirickettsia salmonis* from coho salmon, *Oncorhynchus kisutch* (Walbaum), and rainbow trout, *Oncorhynchus mykiss*, during the freshwater stage of their life cycle. J. Fish Dis. **18:**277–279.
16. **Gilmore, R. D., N. Joste, and G. A. McDonald.** 1989. Cloning, expression and sequence analysis of the gene encoding the 120kD surface-exposed protein of *Rickettsia rickettsii*. Mol. Microbiol. **3:**1579–1586.
17. **Hendrix, L. R., L. P. Mallavia, and J. E. Samuel.** 1993. Cloning and sequencing of *Coxiella burnetii* outer membrane protein gene *com1*. Infect. Immun. **61:**470–477.
18. **Hickman, C. J., C. K. Stover, S. W. Joseph, and E. V. Oaks.** 1991. Molecular cloning and sequence analysis of a *Rickettsia tsutsugamushi* 22 kDa antigen containing B- and T-cell epitopes. Microb. Pathog. **11:**19–31.
19. **Kim, I.-S., S.-Y. Seong, S.-G. Woo, M.-S. Choi, and W.-H. Chang.** 1993. High-level expression of a 56-kilodalton protein gene (*bor56*) of *Rickettsia tsutsugamushi* Boryong and its application to enzyme-linked immunosorbent assays. J. Clin. Microbiol. **31:**598–605.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**154–159.
21. **Lannan, C. N., S. A. Ewing, and J. L. Fryer.** 1991. A fluorescent antibody test for the detection of the rickettsia causing disease in Chilean salmonids. J. Aquat. Anim. Health **3:**229–234.
22. **Lannan, C. N., and J. L. Fryer.** 1991. Recommended methods for the inspection of fish for the salmonid rickettsia. Bull. Eur. Assoc. Fish Pathol. **11:**135–136.
23. **Lannan, C. N., and J. L. Fryer.** 1993. *Piscirickettsia salmonis*, a major pathogen of salmonid fish in Chile. Fish. Res. **17:**115–121.
24. **Pang, H., and H. H. Winkler.** 1994. Analysis of the peptidoglycan of *Rickettsia prowazekii*. J. Bacteriol. **176:**923–926.
25. **Press, C. L., and A. Lillehaug.** 1995. Vaccination in European salmonid aquaculture: a review of practices and prospects. Br. Vet. J. **151:**45–69.
26. **Rikihisa, Y.** 1991. The tribe *Ehrlichieae* and ehrlichial diseases. Clin. Microbiol. Rev. **4:**286–308.
27. **Rodger, H. D., and E. M. Drinan.** 1993. Observation of a rickettsia-like organism in Atlantic salmon, *Salmo salar* L., in Ireland. J. Fish Dis. **16:**361–369.
28. **Roth, J. A.** 1988. Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Schnaitman, C. A., and J. D. Klena.** 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. **57:**655–682.
31. **Schuenke, K. W., and D. H. Walker.** 1994. Cloning, sequencing, and expression of the gene coding for an antigenic 120-kilodalton protein of *Rickettsia conorii*. Infect. Immun. **62:**904–909.

32. **Tamura, A., H. Urakami, and T. Tsuruhara.** 1982. Purification of *Rickettsia tsutsugamushi* by Percoll density gradient centrifugation. Microbiol. Immunol. **26:**321–328.
33. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Anal. Biochem. **119:**115–119.
34. **van Vliet, A. H. M., F. Jongejan, M. van Kleef, and B. A. M. van der Zeijst.** 1994. Molecular cloning, sequence analysis, and expression of the gene encoding the immunodominant 32-kilodalton protein of *Cowdria ruminantium*. Infect. Immun. **62:**1451–1456.
35. **Vishwanath, S., G. A. McDonald, and N. G. Watkins.** 1990. A recombinant *Rickettsia conorii* vaccine protects guinea pigs from experimental boutonneuse fever and Rocky Mountain spotted fever. Infect. Immun. **58:**646–653.
36. **Weiss, E., J. C. Coolbaugh, and J. C. Williams.** 1975. Separation of viable *Rickettsia typhi* from yolk sac and L cell host components by Renografin density gradient centrifugation. Appl. Microbiol. **30:**456–463.

Editor: P. E. Orndorff