AN ABSTRACT OF THE THESIS OF

Gregory D. Wiens for the degree of Doctor of Philosophy in the Department of Microbiology presented on February 7, 1992.

Title: Structural and Functional Analysis of the 57 kDa Protein Produced by the Fish Pathogen, Renibacterium salmoninarum.

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Stephen L. Kaattari

Little is known about the virulence factors of Renibacterium salmoninarum, the causative agent of salmonid bacterial kidney disease. The predominant protein produced by R. salmoninarum in broth culture or during infection is a 57/58 kDa protein (p57) which is associated with strain virulence. In this study monoclonal antibodies (MAbs) to p57 were developed and used as tools to antigenically characterize and quantify the protein. Monoclonal antibodies 4D3 and 2G5 recognize p57 and appear to be species specific as they did not cross-react with proteins produced by bacterial species within the genera Streptococcus, Carnobacterium, Vibrio and Aeromonas, or with fish serum proteins. Further, these MAbs recognize conserved epitopes on p57 shared by 10 isolates from geographically diverse areas.

In vitro activities attributed to p57 include the suppression of antibody production, and the agglutination of rabbit erythrocytes and salmonid spermatocytes. We described a novel in vitro agglutinating activity of p57 toward salmonid leukocytes that was inhibited by two of a panel of eight
MAbs. The location of the putative epitopes recognized by the MAbs were determined by two-dimensional electrophoresis and Western blotting of proteolytic breakdown fragments of p57. Amino acid sequencing of several of the fragments suggested that the antibodies which inhibit agglutinating activity bind proximal to the amino terminus of the protein.

To investigate the mechanism of leukocyte agglutination, p57 was purified to near homogeneity using anion-exchange and size-exclusion fast-pressure liquid chromatography. P57 eluted as a protein monomer and retained leukoagglutinating activity. In addition, results of antibody-capture, enzyme-linked immunosorbent assays suggest that a monomer exists in culture supernatant and infected fish tissue.

Antigenic analysis with MAbs has also been useful for developing immunoassays for detecting and quantifying p57 levels in vivo. Using a quantitative ELISA, the prevalence of salmon with antigen levels above 3 ng/ml of kidney homogenate varied from 12.8 to 36.6% in 740 adult spawning chinook salmon returning to an Oregon hatchery from 1989 to 1991. A rapid, semi-quantitative, Field ELISA was also developed for use under hatchery conditions, in addition to a sensitive chemiluminescent Western blot protocol for confirming ELISA positive samples.
Structural and Functional Analysis of the 57 kDa Protein Produced by the Fish Pathogen, *Renibacterium salmoninarum*

by

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CONTRIBUTION OF AUTHORS

Dr. S.L. Kaattari was an advisor on all manuscripts included in this thesis. Dr. Dan Rockey and L. Gilkey originally devised the Field ELISA assay described in Chapter 6 and participated in collecting and assaying samples. J. Bishop helped develop the protocol for Western blotting described in Chapter 6, and Dr. J. Heidel performed part of the FAT analyses in this chapter. Dr. P. Turaga performed the experiments correlating antigen levels with hematocrits which appears in Appendix A, and assisted with the preparation of Appendix B.
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Bacterial kidney disease (BKD) is one of the most prevalent diseases of cultured salmonids (Fryer and Sanders, 1981), and mortality due to BKD can occur in both the freshwater and saltwater life stages (Earp et al., 1953; Banner et al., 1983). The etiological agent of BKD is Renibacterium salmoninarum (Rs) which causes a chronic bacteremia with focal lesions in the viscera, particularly the kidney (Fryer and Sanders, 1981).

The economic loss due to BKD has not been calculated, however, the disease is generally regarded as one of the most important bacterial diseases affecting resident and anadromous salmonid stocks in the Pacific Northwest. In British Columbia coastal waters it is estimated that 20 to 60% of farmed salmonids may succumb to BKD (Albright et al., 1988). In the Western United States, the effects of BKD on spring/summer chinook salmon (Oncorhynchus tshawytscha) are of particular concern (Bullock and Wolf, 1986; Warren, 1991). For example, a recent survey of out-migrating spring/summer chinook smolts from several sites on the Columbia River, one of the major salmonid production areas of the world, found that 97 to 100% of the smolts harbored detectable levels of Rs antigen (Elliott and Pascho, 1991). The presence of Rs may, therefore, be a major factor contributing to poor smolt survival and the
low percentages of returning adult fish (Raymond, 1988; Elliott and Pascho, 1991).

In spite of the importance of BKD, techniques for its control are limited both in number, and more importantly, efficacy (Elliott et al., 1989; Warren, 1991). Current management strategies include reducing stress to fish, quarantining infected stocks, chemotherapy, culling of infected broodstock, and/or total hatchery depopulation and sterilization. As of yet, no efficacious vaccine is available for widespread use (Munro and Bruno, 1988; Kaattari et al., 1988). Control strategies are primarily limited due to a lack of understanding of the mechanisms of pathogenesis, and the salmonid response to infection. Research on Rs has been hampered by technical difficulties related to the culture of the organism. These difficulties include a very slow generation time of 24 hr, difficult primary isolation, and the long incubation times (1-4 mo) required for experimental challenge of fish (Fryer and Sanders, 1981).

The goal of this research has been to further characterize the host-pathogen relationship between the salmonid and Rs. Preliminary studies demonstrated that Rs produces large quantities of extracellular protein antigen(s) (previously referred to as soluble protein) both in culture and during infection (Turaga et al., 1987b). In vitro studies revealed that the extracellular protein had inhibitory effects on antibody production of coho salmon B-cells (Appendix A). Analysis of the extracellular protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis identified that the predominant component is a 57/58 kDa protein doublet (p57) first described by Getchell et al. (1985). To further characterize the p57 molecule, I produced a battery of monoclonal antibodies to this protein. The first manuscript, Chapter 3, describes the immunochemical characteristics of the p57 molecule that have been
delineated with the monoclonal antibodies. Chapters 4 and 5 describe the use of the monoclonal antibodies, together with physico-chemical and biological assays, to define the structure and functional regions of p57. The analysis of the antigenic regions of p57 also facilitated the development of enhanced assays for the rapid and accurate clinical detection of Rs described in Chapter 6.
CHAPTER 2

LITERATURE REVIEW

This literature review will discuss the characteristics of *Renibacterium salmoninarum* (Rs), methods of detection, pathogenesis, host immune response, putative virulence factors, and the 57 kDa protein. Additional aspects of bacterial kidney disease have been reviewed by Earp et al. (1953); Fryer and Sanders (1981); Klontz (1983); Munro and Bruno (1988); Bullock and Herman (1988); Kaattari et al. (1988); Elliott et al. (1989).

**Identification, Taxonomy, and Phenotypic Characteristics of *Renibacterium salmoninarum*.**

**Early Identification.** Bacterial kidney disease was first identified as Dee disease of Atlantic salmon (Mackie et al., cited in Smith, 1964), and has also been referred to as white boil disease, salmonid kidney disease, and corynebacterial kidney disease. In the United States, Belding and Merrill (1935), observed a gram-positive bacterium associated with necrotic kidney lesions of salmonids, but they were unable to culture the disease agent. Rucker et al. (1954), and Ordal and Earp (1956) were the first to report the completion of Koch's postulates and identify the etiological agent as a slow growing gram-positive bacterium.
Taxonomy. Until recently the classification of the etiological agent of bacterial kidney disease was unresolved. Initially, Ordal and Earp (1956) classified the organism as a Corynebacterium. Smith (1964) concurred with this classification based on specific characteristics of the bacterium such as aerobic growth, lack of endospores, production of metachromatic granules, reproduction by binary fission, and pathogenic capability. Young and Chapman (1978), however, were unable to observe the presence of metachromatic granules or the post-fission snapping process associated with corynebacteria. Further, the absence of mycolic acids (Goodfellow et al., 1976; Fryer and Sanders, 1981), the presence of lysine in the peptidoglycan instead of meso-diaminopimelic acid (Sanders and Fryer, 1980), and the difference in lipid composition (Collins, 1982; Embley et al., 1983) does not support the placement of the bacterium in the genus Corynebacterium or in the genus Listeria as proposed by Bullock et al. (1975). Sanders and Fryer (1980) concluded that the organism belonged to a unique genus and named the organism Renibacterium salmoninarum. Molecular cataloging and sequencing of the 16s ribosomal RNA from Rs (Stackebrandt et al., 1988; Gutenberger et al., 1991b) and recent recalculation of the G + C content (Banner et al., 1991) support the placement of the organism as a member of the high G + C content gram-positive eubacterial subdivision of the actinomycetes. The closest known relatives to Rs include Arthrobacter and Micrococcus. As of yet, no additional species have been included in this genus, nor have subspecies been identified.

Phenotypic Characteristics of Renibacterium salmoninarum. Renibacterium salmoninarum is a small (0.3-1.5 µm by 0.1-1.0 µm), non-motile, non-acid fast, non-sporeforming, strongly gram-positive rod that usually occurs in pairs (Sanders and Fryer, 1980). The biochemical properties and
cell wall composition of Rs isolates appear to be remarkably conserved (Bruno and Munro, 1986c; Fiedler and Draxl, 1986). Biochemical characteristics include an inability to produce acid from sugars (Sanders and Fryer, 1980), a cysteine requirement for growth (Ordal and Earp, 1956; Daly and Stevenson, 1985), the presence of catalase (Ordal and Earp, 1956), and a lack of oxidase. Proteinase (Ordal and Earp, 1956; Smith, 1964; Rockey et al., 1991b), DNase, and beta hemolytic activities against salmonid erythrocytes have also been described (Bruno and Munro, 1986c).

*Renibacterium salmoninarum* possesses a unique peptidoglycan and an unusual cell wall polysaccharide containing N-acetylfucosamine in addition to galactose, rhamnose, and N-acetylglucosamine (Kusser and Fiedler, 1983). Polysaccharide comprises more than 60 percent of the dry weight of trypsinized cell wall preparations (Fiedler and Draxl, 1986) and may constitute the recently described capsule (Dubrieul et al., 1990b). Growth is aerobic and optimal at 15-18°C, and the generation time during the log phase of growth is approximately 24 hr (Fryer and Sanders, 1981).

**Antigenic Characterization.** Antigenically, Rs isolates appear homogenous by analysis with polyclonal rabbit antisera. Bullock et al. (1974) demonstrated serologic homogeneity by agglutination and precipitin reactions with sonic extracts of whole Rs cells from 10 USA isolates. Banowetz (1974) identified two antigens in the saline wash extract from whole cells, both of which had a molecular mass of more than 200 kDa and were composed of protein and carbohydrate. Fiedler and Draxl (1986) identified a homogeneous, polymeric polysaccharide with a subunit weight of approximately 10 kDa which was immunogenic to rabbits. The polysaccharide was similar among 13 Rs isolates from the USA, Canada and Europe. In addition, they identified a predominate trypsin-sensitive protein present on the
cell surface with an approximate molecular weight of 70 kDa. Getchell et al. (1985) characterized Rs antigens using a variety of techniques, including immunodiffusion, immunoelectrophoresis, rocket immunoelectrophoresis, and 2-dimensional immunoelectrophoresis. Seven antigens (A-G) were identified as being shared by seven isolates of Rs. Antigen F (57 kDa) was the major component and found to be heat stable (100°C for 0.5 h). Cross absorption experiments with bacterial isolates confirmed that Antigen F is the predominant cell surface antigen. Monoclonal antibodies have been produced which recognize antigens unique to particular Rs isolates (Arakawa et al., 1987), however, the composition of these antigens has not been determined.

**Epizootiology**

**Host Range.** Bacterial kidney disease has been identified primarily in cultured salmonid stocks, with cases reported from North America, Europe, Japan (Fryer and Sanders, 1981), South America (Sanders and Barros, 1986), and Scandinavia (Guomundsdottir et al., 1988; Ljungberg et al., 1990). However, significant populations of free-ranging fish have also been shown to harbor Rs (Pippy, 1969; Evelyn et al., 1973; Ellis et al., 1978; Mitchum et al., 1979; Paterson et al., 1979; Banner et al., 1986; Souter et al., 1987; Elliott and Pascho, 1991).

Salmonids of the genera *Oncorhynchus*, *Salmo*, and *Salvelinus* appear to be the primary host of Rs (reviewed in Fryer and Sanders, 1981). Recently, natural infections have also been documented in *Hucho hucho* (danube
salmon; Pfeil-Putzien et al., 1985), and *Thymallus thymallus* (greyling; Kettler et al., 1986). Further, experimental infections have been induced in a non-salmonid species, *Anoplopoma fibria* (sablefish; Bell et al., 1990), but not in *Cyprinus carpio* (carp; Sakai et al., 1989b) or *Lampetra tridentata* (lamprey; Bell and Traxler, 1986). Efforts so far have failed to identify Rs in mussels (*Mytilus edulis*) or non-salmonid finfish which grow in and around sea-water net-pen facilities (Paclibare et al., 1988).

**Transmission.** While the transportation of cultured salmonids by humans is believed to have facilitated the dissemination of Rs (Ganzhorn et al., in press), the natural route of infection in salmonids is not fully understood. There is evidence that the disease can be transmitted both horizontally and vertically. Circumstantial evidence for natural horizontal transmission was obtained by Mitchum and Sherman (1981) who stocked rainbow (*O. mykiss*), brook (*S. fontinalis*), and brown trout (*S. trutta*), which had tested negative for Rs by the indirect fluorescent antibody technique (IFAT), into a river system enzootic with BKD. When stocked trout were recovered, Rs was identified in 32-45% of these fish by IFAT. Mortalities attributed to BKD were observed within 9 months of stocking. Unfortunately, a group of stocked fish were not kept as controls to determine if they might have harbored undetectable levels of Rs prior to release. Evidence for horizontal transmission of Rs between sockeye salmon (*O. nerka*) was also obtained by Bell et al. (1984) by experimental cohabitation of infected fish with saline injected controls. Almost complete mortality was observed in tanks of fish challenged using this method.

The mechanism through which horizontal transmission occurs has not been precisely delineated. One route may be via oral ingestion of infected material. Wood and Wallis (1955) found that 100% transmission was achieved by feeding a diet of 50% infected adult viscera for 52 days to
fingerling chinook salmon, however, Snieszko and Griffin (1955) were unable to achieve disease transmission by a one day feeding of infected viscera to brook trout. Daly (1989) was able to infect rainbow trout by a single oral intubation of $4.6 \times 10^{11}$ CFU of Rs. A 43% mortality ($n=30$) was observed within 154 days under these conditions.

Another natural source of infective material may be feces of clinically infected or carrier fish. Viable Rs has been demonstrated in fresh and salt water by the fluorescent antibody technique and bacterial culture (Austin and Rayment, 1985; Balfry et al., 1991; Elliott and Pascho, 1991). Balfry et al. (1991) found that oral intubation of fecal material, but not autoclaved material, resulted in IFAT positive fish and subsequent mortality. They suggested that this mechanism may contribute significantly to the high prevalence of the disease on salmon farms.

In addition to horizontal transmission, Rs may be vertically transmitted to offspring through infected gametes. Allison (1958), and Bullock et al. (1978) were the first to report circumstantial evidence that gametes from infected adults, transferred to historically disease-free locations, resulted in clinically infected progeny. They, however, did not postulate the mechanism of transmission. Subsequently, Evelyn et al. (1984) reported the presence of Rs both outside and inside the eggs of a naturally infected female coho salmon (*O. kisutch*) which had $4 \times 10^9$ CFU Rs/ml of ovarian fluid. Culturable Rs was found in 15.1% of surface disinfected eggs, and tissue sections demonstrated that the bacterium resided within the yoke. Under laboratory conditions, infection of unfertilized steelhead (*O. mykiss*), coho, and chinook salmon eggs by immersion challenge has only been accomplished using high innoculums of Rs ($1.4 \times 10^9$, $1.3 \times 10^{12}$, and $1.7 \times 10^5$ respectively; Evelyn et al., 1986a). Under these conditions, only 1-5.5% of the experimentally challenged eggs
contained viable *R. salmoninarum* (Evelyn et al., 1986a; Lee and Evelyn, 1989). Evelyn et al. (1986a) postulate that under natural circumstances eggs may become infected after ovulation while in contact with coelomic fluid. Bruno and Munro (1986b) observed, however, the presence of Rs in tissue sections of maturing oogonia of experimentally challenged trout suggesting that infection may occur directly from infected ovarian tissue before ovulation.

Lee and Evelyn (1989) were able to demonstrate that smolts which had been reared from eggs of naturally infected adults, but not uninfected adults, had subclinical levels of Rs as determined by the fluorescent antibody test (FAT). Additionally, the prevalence of subclinical infection appeared to be correlated with the experimental challenge dose. Low levels of Rs (23-113 CFU/ml in ovarian fluid) were associated with a 1-2% incidence of smolts with subclinical infections. Interestingly, no mortality was attributed to BKD in subclinically infected offspring even though the prevalence was 44% in the highest challenge group. Unfortunately, bacterial cells from the FAT positive fish were not cultured or enumerated.

**Control of Bacterial Kidney Disease and the Development of Diagnostic Procedures to detect *R. salmoninarum***

Control of BKD is widely recognized as a difficult problem for fish culturists (reviewed by Elliott et al., 1989). Currently, the main method of disease control consists of chemotherapy with erythromycin. Erythromycin phosphate injection (11 mg/kg every 21 to 30 days prior to spawning) was reported to reduce adult pre-spawning mortality and the incidence of BKD
lesions (Fryer and Sanders, 1981; Groman and Klontz, 1983). Adult injection may also reduce levels of Rs within the egg (Evelyn et al., 1986b; Brown et al., 1990). Feeding of erythromycin reduces mortality of eastern brook trout (Wolf and Dunbar, 1959), rainbow trout (Austin, 1985), and chinook salmon experimentally challenged with Rs (Moffit and Bjornn, 1989). However, erythromycin chemotherapy is not completely effective in curing challenged fish or preventing vertical transmission (Austin, 1985; Brown et al., 1990). Additionally, the demonstration in vitro resistance to erythromycin (Bell et al., 1988), the bacteriostatic mechanism of action of erythromycin, and the widespread prophylactic treatment of juvenile salmonids and adult broodstock, all increase the probability that the emergence of resistant strains may be a future concern.

The lack of an efficacious vaccine (Kaattari et al., 1988; Munro and Bruno, 1988) and the potential problems with chemotherapy have stimulated research to develop accurate and rapid methods for diagnosis of Rs. Accurate and rapid diagnosis are important for limiting the transfer of salmonids to areas not already enzootic with Rs and for the segregation of infected gametes. Control of bacterial kidney disease by segregation is based on the premise that progeny from disease-free adults, or perhaps adults with low levels of infection, should have a decreased incidence of BKD, assuming vertical transmission is the predominant mechanism of transmission (Armstrong et al., 1989; Elliott et al., 1989). Reports on the efficacy of segregation appear promising. Pascho et al. (1991b) have reported the segregation and rearing of gametes from spring chinook salmon at Dworshak National Fish Hatchery in Idaho with high and low levels of Rs as determined by the enzyme-linked immunosorbent assay (ELISA) and membrane fluorescent antibody technique (MFAT). Offspring from the highly infected adults had both a higher cumulative
pond mortality (17% vs 5%) and infection incidence at the time of release (85% vs 62%) than offspring from the adults with low antigen levels. The authors have suggested, therefore, that implementation of segregation practices may reduce losses to BKD. Segregation of infected gametes did not completely eliminate the incidence of infection in the progeny from adults with low antigen levels, suggesting that either the segregation of infected adults was not complete or that horizontal transmission from other sources may be an important component under hatchery conditions. In a separate experiment, segregation practices resulted in an increased rate of adult return (Warren, 1991). In this experiment spring chinook gametes were segregated into a high adult antigen group and a low adult antigen group, then reared to smolt stage, marked and released from Warm Springs National Fish Hatchery in Oregon. Two times the number of expected adults from the low antigen group returned, while only 87% of the expected adults from the high antigen group returned.

The accurate and rapid identification of Rs has become increasingly important in attempts to segregate adults and to prevent the transfer of bacterial kidney disease to locations where it is not enzootic. Two general methods of detection have been attempted; first, direct detection of the organism by culture, fluorescent antibody staining, or detection of specific antigens, and second, diagnosis of infection by detection of host antibodies to the bacterium.

Diagnosis of Rs infection by the presence of antibodies has not met with widespread success. While salmonids can produce agglutinating antibodies to Rs (Evelyn, 1971), evidence suggests that antibody levels do not correlate with the level of infection. Banowetz (1974) assayed 207 yearling coho salmon from a population undergoing an epizootic of BKD and found that fish
with high agglutinin titers, of 1:128 or greater, generally did not harbor the bacterium as determined by FAT or culture. Conversely, fish which were positive by culture or FAT had low agglutinin titers. Similar findings have been reported by Bruno (1987) who found that serum agglutinins were not present in heavily infected Atlantic salmon (Salmo salar) smolts, but that the titer of agglutinins increased prior to the end of the epizootic. The variable levels of serum agglutinins documented in apparently healthy fish (Evelyn et al., 1981; Paterson et al., 1981; Bruno, 1987) and the low levels of detectable agglutinins in infected fish suggest that serum agglutination assays may be of limited efficacy in determining active infection (Banowetz, 1974; Bruno, 1987).

Direct detection of Rs is the most widely used method for identifying infected fish. Early efforts in detection of BKD relied on the observation of gram-positive diplobacilli and the presence of clinical signs (Earp, et al., 1953; Bell, 1961; Pippy, 1969). However, the use of the Gram Stain is limited because of its low sensitivity (1 x 10^7-9 bacterial cells/g tissue (Bullock et al., 1980; Sakai et al., 1987a; Pascho et al., 1987), and the presence of melanin granules in kidney tissue which can obscure low numbers of organisms. Bruno and Munro (1982) have used a periodic-acid schiff stain to identify Rs in tissue sections, however, the stain is not specific to the genus Renibacterium.

Culture followed by serological identification is considered the definitive test for Rs (Fryer and Sanders, 1981). Several different media have been used to isolate the bacterium from infected fish. Originally, Ordal and Earp (1956) used Muller-Hinton medium supplemented with cysteine. Evelyn (1977) subsequently devised a kidney disease medium containing peptone, yeast extract, cysteine and 10% serum (KDM-II) that reportedly allows the primary isolation of a single bacterium from fish tissue (Evelyn, 1977). This serum requirement can be replaced by charcoal, which is thought to absorb
toxic substances such as fatty acids present in the medium (Daly and Stevenson, 1985; Daly, 1989). Isolation of Rs is also complicated by growth inhibitory factors which are present in kidney tissues (Evelyn et al., 1981; Daly and Stevenson, 1988). Also different sources of peptone may not be suitable for isolation (Evelyn and Prosperi-Porta, 1989). Primary isolation even under optimal conditions takes from 2 weeks from a highly infected fish to as long 12 weeks from subclinically infected fish (Evelyn, 1977; Fryer and Sanders, 1981; Gudmunsdottir et al., 1991). The fastidious culture requirements, slow growth of Rs, and common overgrowth of petri plates by contaminating organisms generally limit the usefulness of culture techniques for screening large numbers of fish.

A number of serological techniques have been developed for the identification of infected fish. Bullock and Stuckey (1975) first described the direct fluorescent antibody technique (FAT) and concluded that it was more sensitive than the Gram Stain and could detect subclinical infections of Rs. Laidler (1980) found the FAT to be statistically superior to the Gram Stain for the detection of Rs in tissue smears. Several methods to quantify Rs with the FAT have been used, including a subjective 1+ to 4+ fluorescence intensity scale (Bullock et al., 1980) and a membrane FAT procedure where numbers of organisms are directly counted after immobilization onto filter paper grids (Elliott and Barila, 1987). Sakai et al., (1989a) have reported that the indirect FAT is able to detect $10^3$ to $10^4$ bacterial cells/gm of kidney tissue, while Elliott and Barila (1987) have detected less than $10^2$ bacterial cells/ml of coelomic fluid. Lee (1989) has reported a similar sensitivity using kidney tissue. While the FAT can be a very sensitive test for Rs, this method can be both tedious and labor-intensive when large numbers of samples are examined.
An alternate method of diagnosis is the detection of a soluble antigen produced by Rs. The soluble antigen was first detected by the immunodiffusion technique using samples from the kidney, liver, spleen, and blood of infected fish (Chen et al., 1974). The kidney and liver tissue appeared to be the preferred organs for diagnosis. Kimura et al. (1978) found the antigens are heat stable and that their presence was highly correlated with a positive Gram Stain and clinical signs of disease. The major limitation of the immunodiffusion technique is its inability to detect subclinical levels of Rs (Fryer and Sanders, 1981; Cipriano et al., 1985; Sakai et al., 1989a). An increase in sensitivity above immunodiffusion or bacterial culture has been reported with counterimmunoelectrophoresis (Cipriano et al., 1985), however, Pascho et al. (1987) have experienced variable sensitivity with this technique. Detection of subclinically infected fish was accomplished by Kimura and Yoshimizu (1981) with the staphlococcal coagglutination technique. This qualitative test requires 1.5 h to complete and was found to have equal sensitivity with the FAT (Sakai et al., 1987b).

Enzyme-linked immunoassays are currently considered the most efficient and sensitive technique for rapid analysis of large numbers of samples (Sakai et al., 1987a,b; Dixon, 1987; Pascho and Mulcahy, 1987; Turaga et al., 1987b). A dot blot technique has been developed by Sakai et al. (1987a) for qualitative antigen assessment, while enzyme-linked immunosorbent assays (ELISAs) have been developed for the qualitative and quantitative detection of antigen (Dixon 1987; Pascho and Mulcahy, 1987; Turaga et al., 1987b). The ELISA developed by Dixon (1987) was able to rapidly detect antigen in 0.5 h but was unable to detect subclinically infected fish. The assays developed by Pascho and Mulcahy (1987) and Turaga et al. (1987b) take longer to perform but are able to detect antigen concentrations as
low as 2-20 ng and 10 ng per ELISA well respectively. The ELISA has been reported to be more sensitive than either culture or the FAT in a direct comparison of coho salmon tissue and fluid samples (Pascho et al., 1987).

The development of immunodiagnostic assays rely on the specificity of antisera for the accurate identification of Rs. Numerous reports of cross-reactive gram-positive, gram-negative and unidentified organisms have been documented in the literature (Bullock et al., 1980; Evelyn et al., 1981; Austin et al., 1985; Yoshimizu et al., 1987). Fiedler and Draxl (1986) concluded that galactose present in the polysaccharide of Rs accounted for cross-reactions of antisera with *Brevibacterium lyticum* and *Arthrobacter aurescens*. Dixon (1987) was able to reduce cross-reactivity of polyclonal antisera by preabsorption with *Rothia dentocariosa* and *Bacillus sphaericus*. Arakawa et al., (1987) postulate that a monoclonal antibody designated K18/4 identified a determinant on Rs which may be widespread in nature as cross-reactivity was noted by ELISA with three gram-positive organisms. Antigen cross-reactivity has also been documented with putative non-bacterial antigens by Western blot (Turaga et al., 1987b), and by ELISA (Pascho et al., 1991a).

**Disease Pathology and Interaction with the Salmonid Immune System**

**Pathology.** The gross external pathology of bacterial kidney disease is variable and ranges from no clinical signs to a general external darkening in color, distended abdomen, exophthalmia, petechial hemorrhaging, and hemorrhagic areas near fins (Belding and Merrill, 1935). Generally, internal
infection is characterized by a chronic, systemic infiltration of the viscera by the bacteria (Ferguson, 1989). Pathologic changes in the kidney are visually the most obvious symptom and include a general enlargement of the kidney as well as small to large granulomatous lesions (Smith, 1964; Wood and Wallis, 1955). Focal granulomas also occur in the musculature, spleen, liver, heart, swimbladder, pancreas, and subcutaneous tissue behind the eye. Less affected but reported sites of infection include the lamina propria of the gut and meninges of the brain (Ferguson, 1989). Pseudo-membranes composed of thin layers of fibrin and collagen with fibroblasts, histiocytes, degenerating leukocytes, and Rs have also been described which are external to the kidney, liver, and spleen (Smith, 1964). Bruno (1986b) concluded that death may be attributed to several causes including the obliteration of normal kidney and liver structure by Rs lesions, heart failure, and direct damage by toxic products liberated from Rs or disrupted macrophages.

Atypical cases of BKD have been reported in which ocular and skin lesions are the predominant pathological symptoms. Hoffman et al., (1984) identified Rs frequently in skin and ocular lesions but less frequently in the kidney of rainbow trout fingerlings using fluorescent antibody staining. Adult spawning coho salmon have also been identified with external pustules, yet few lesions in kidney tissue (Hoskins and Stone, 1983). This may be related to the "spawning rash" peculiar to rainbow trout in Southern Ontario, Canada (Ferguson, 1989). Small intradermal vesicles and granulomas are detected at spawning time but are not apparent at other times of the year. Hendricks and Leek (1975) postulated that eye and skin abrasions may serve as primary entry points of Rs, and that the entry points may subsequently develop into external lesions followed by systemic infection.
**Process of infection and host response.** To characterize the host-pathogen interaction *in vivo*, Bruno (1986b) followed the histopathological changes which occur after experimental infection. Within 45 minutes of intraperitoneal injection of live or formalin-fixed Rs, bacterial cells were observed within the phagocytes of the kidney and spleen. After 4-6 days scattered extracellular Rs were observed, and by 6-10 days, Rs could be observed in blood monocytes and macrophages where they appeared to multiply. At 14 days, phagocytic cells containing Rs were observed between myocardial bundles in the heart. After 28 days Rs was found intracellularly in endothelial cells lining the glomerular blood vessels and within the lumen of the collecting ducts, but not within the proximal tubules. Colonization of kidney tissue and the cell-mediated reaction to the presence of Rs probably results in development of granulomatous lesions, a common reaction to intracellular pathogens. A number of researchers have observed Rs within phagocytic cells (Young and Chapman, 1978; Bruno, 1986b; Zhuo, 1990). While conclusive evidence of intracellular replication has been difficult to substantiate, Rs appears to be able to escape from the macrophage phagosome into the cytoplasm of the cell (Gutenberger et al., 1991a).

A number of changes in the hematological and serum parameters have been recorded in experimentally and naturally infected fish (Hunn, 1964; Wedemeyer and Ross 1973; Bruno 1986a). Bruno and Munro (1986a) observed that circulating erythrocytes in experimentally infected rainbow trout and Atlantic salmon decreased 59 to 66%. Correlated with these findings was a decrease in erythrocyte diameter from 16.6 μm to 14.5 μm, and an increase in the erythrocyte sedimentation rate. A decrease in hematocrit has also been correlated with increased progression of infection (Bruno and Munro, 1986a; Turaga et al., 1987a). No changes in small and large lymphocyte numbers
have been observed, however, there was a transitory increase in neutrophils, monocytes, and thrombocytes after bacterial injection which subsequently returned to normal (Bruno and Munro, 1986a).

Correlated with the decrease in hematocrit is the increase in the levels of a soluble protein antigen (Turaga et al., 1987a), which may reach concentrations approaching 1 mg/ml in the serum of moribund fish (Turaga et al., 1987b). Turaga (1989) hypothesized that humoral immunity to the 57 kDa protein, the main component of the soluble protein, may lead to immune-complex formation and subsequent hypersensitivity reactions in the glomeruli. In experimentally and naturally infected brook trout and Atlantic salmon Young and Chapman (1978) have observed electron dense subendothelial deposits which resembled immune complexes. Additionally, staining of alternate tissue sections with a monoclonal antibody specific for fish immunoglobulin or the 57 kDa protein, revealed similar distribution profiles, providing evidence of immune complex deposition (Turaga, 1989). However, double-labeling with monoclonal antibodies as well as the isolation of immune complexes is required before this hypothesis can be confirmed.

Limited circumstantial evidence suggests that salmonids, once infected, can mount a protective immune response. Munro and Bruno (1988) describe a natural epizootic of Rs in Atlantic salmon which occurred during smoltification and resulted in 18% cumulative mortality. Up to 69 weeks after transfer to seawater fish were Gram Stain and IFAT negative, however, 100% had an agglutinin response and a resolution of granulomatous lesions suggesting that a protective immune response occurred in the survivors. While agglutinating antibodies can be induced by vaccination, the response is very slow (Evelyn, 1971). From a series of extensive vaccine trials, Evelyn et al., (1988) concluded that salmon in the genus Oncorhynchus are unable to
mount a protective immune response. Turaga (1989) found that the best protection afforded by vaccination of coho salmon was with Freunds complete adjuvant or killed *Mycobacterium cheloni* both of which significantly delayed the mean time to death. It is not known whether a specific immune response to a cross-reactive determinant, or a non-specific cell mediated response resulted in the limited protection observed.

**Putative Virulence Factors of *Renibacterium salmoninarum***

Relatively little is known about virulence factors of Rs, although a number of factors have been described which may contribute to virulence. Catalase, DNase, hemolytic, proteolytic, and exotoxin activities have been described (Bruno and Munro, 1982; Shieh, 1988a). The catalase and DNase enzymes remain uncharacterized, however, a putative Rs hemolysin has been cloned (Evenden et al., 1990). The cloned DNA is approximately 1.6 kb in length and produces a hemolysin which has activity against rainbow trout erythrocytes. The cloned insert hybridized with four isolates of Rs genomic DNA including the type strain ATCC 33209. No DNA hybridization was observed with *Aeromonas salmonicida* or *Yersina ruckeri* strains. In contrast to the findings of Evenden et al. (1990), Bandin et al. (1991) have been unable to detect expression of hemolytic activity from ten Rs isolates. This discrepancy may be explained by the low amount of hemolysin produced, or by Bruno and Munro's (1986c) observation that hemolytic activity decreases with continued passage.
Proteases are important virulence factors for a variety of pathogenic microorganisms (Trust, 1986). Proteolytic activities of Rs have been described by a number of investigators (Smith, 1964; Bruno and Munro, 1986c; Rockey et al., 1991b). Rockey et al. (1991b) identified two proteolytic bands by substrate gel electrophoresis, one larger than 100 kDa and one less than 20 kDa. The higher molecular weight protease has activity against p57 and against denatured bovine serum albumin or hen ovalbumin substrates. The direct role of the protease(s) in virulence has yet to be established.

Several investigators have suggested that pathological evidence may exist for the presence of a toxin in vivo (Bruno and Munro, 1986a; Bell et al., 1990). Bruno and Munro (1986a) observed an accumulation of erythrocytes in the spleen of experimentally infected rainbow trout and suggested that a toxin may be damaging erythrocytes resulting in their sequestration within the spleen. Alternatively, Bruno and Munro (1986a) postulate that the splenomegaly could be caused by decreased erythrocyte membrane stability due to lower levels of serum cholesterol. Bell et al. (1990) observed a meningitis in experimentally infected sablefish but could not histologically detect bacterial cells, and, therefore, suggested that a toxin may be responsible. Shieh (1988a) has putatively identified an exotoxin from Rs culture supernatant which was lethal for fingerling Atlantic salmon. Injection of 160 μg of extracellular product per fish was lethal to 10/10 fingerling (9-12g). The toxin was concentrated by acetone and ammonium sulphate precipitations followed by DEAE cellulose chromatography and was heat labile suggesting it has a proteinaceous nature. These results should be interpreted with caution, however, as a control extract was not prepared to rule out the possibility that the exotoxin was a contaminant of the defined medium used (Shieh, 1988b). In an extensive analysis of 10 different isolates of Rs,
Bandin et al. (1991) were unable to demonstrate any toxicity of the extracellular product (ECP) for fish cell lines or by intraperitoneal injection of rainbow trout fingerlings. No cytotoxicity was observed when 100-200 μg/ml of ECP was added to CHSE-214, RTG-2, FHM, EPC, BF-2 and BB cell lines for 7 days. Similar concentrations of ECP from fish pathogens *Vibrio anguillarum* and *Aeromonas hydrophila* demonstrated positive cytotoxicity.

One of the first steps in identifying virulence factors is the isolation of mutants which have reduced virulence followed by the identification of the characteristic(s) which the mutants lack (Smith, 1989). Bruno (1988) has identified three *R. salmoninarum* isolates which have a reduced virulence by intraperitoneal challenge. Virulent isolates produced a 73-81% mortality with the final mortality after 14-25 days, while isolates with a decreased virulence produced 8-18% total mortality and produced a final mortality after 28-38 days. Gross lesions were observed in both fish injected with virulent and decreased virulence isolates. The reduced virulence isolates were catalase positive and hemolytic against horse or sheep blood. Interestingly, the isolates with reduced virulence no longer autoagglutinated in culture and had a reduced cell surface hydrophobicity as measured by the salt aggregation technique. *Renibacterium salmoninarum* strains isolated from naturally infected rainbow trout and from Atlantic salmon in either fresh or salt water consistently autoaggregate (Bruno, 1988). The loss of autoaggregation and cell surface hydrophobicity phenotypes was thought to be the result of routine subculturing. *Renibacterium salmoninarum* re-isolated from reduced virulence isolates did not revert to the original phenotype and were stable during the 18 months of experiments. Correlated with the reduction in virulence was the lack of a saline extractable 57 kDa protein (Bruno, 1990).
Characterization of the 57 kDa Protein

Getchell et al. (1985) first described the 57 kDa protein as antigen F, a major surface antigen present in a soluble antigen extract from saturated ammonium sulfate precipitated culture supernatant. Turaga et al. (1987b) were able to identify an approximately 60 kDa protein antigen by Western blot in concentrated culture supernatant and infected fish serum. The 60 kDa antigen was first observed in infected fish sera 5 days after challenge and increased in a temporal manner, accumulating until mortality. Further analysis of the antigens present in concentrated culture supernatant and infected fish suggest that the 57 and 60 kDa proteins are identical (Turaga, 1989).

Daly and Stevenson (1990) have demonstrated that the 57 kDa protein has hemagglutinating activity for rabbit erythrocytes. Active hemagglutinin preparation was harvested from *R. salmoninarum* cells grown on biphasic media. Cells were extracted in distilled water with an acidic pH (5.5), which enhanced the release of the hemagglutinin. Active hemagglutinin was purified by elution from 5% native gels. Under SDS-PAGE the hemagglutinin migrates as a doublet consisting of predominant 57 kDa band and a minor 58 kDa band. Addition of β-mercaptoethanol did not change the migration of the 57/58 kDa proteins suggesting that they were not complexed by disulfide bonding. Interestingly, one out of 9 strains lacked the 58 kDa protein yet still retained hemagglutinating activity suggesting that only the 57 kDa protein is required for hemagglutinating activity. Strain MT239, originally described by Bruno (1988), was observed by Daly and Stevenson (1990) to lack both the 57/58 kDa bands and hemagglutinating activities.
The 57 kDa protein also contributes to bacterial surface hydrophobicity. Hydrophobicity could be restored to water washed cells incubated with the hemagglutinin (Daly and Stevenson, 1990). Relative cell surface hydrophobicity was found to be resistant to trypsin and protease K treatment, however, purified hemagglutinin was sensitive to a 20 minute incubation in either enzyme. Daly and Stevenson (1990) hypothesize that when the protein is on the cell surface proteolytic sites are not available, but when the protein is removed from the cell surface proteolytic sites are exposed.

Dubreuil et al. (1990a) have also purified the 57 kDa protein from a water extract of whole cells. Lyophilization of the extract resulted in an insoluble precipitate which could only be solubilized in 6 M urea. Fast protein liquid chromatography (FPLC) was used to purify the protein to homogeneity. The pl of the 57 kDa protein is 4.8 under non-reducing conditions, and the amino acid composition is rich in glycine, asx (aspartic acid and asparagine), valine and alanine, while no methionine was detected. Thirty-three percent of the amino acids are hydrophobic. Electron microscopic studies suggest that the 57 kDa protein may compose short peritrichous fimbriae with a diameter of less than 2 nm. The fimbriae were hypothesized to be broken from the bacterial cell surface by low speed centrifugation. No attached sugar was detected on the 57 kDa protein as determined by Schiff staining.

A number of investigators have recently shown that the 57 kDa protein is unstable and susceptible to proteolytic degradation (Dubrieul et al., 1990a; Griffiths and Lynch, 1991; Rockey et al., 1991b). Dubrieul et al. (1990a) found it was necessary to add phenylmethyl-sulfonyl fluoride (PMSF) to fimbriae extracts to prevent degradation of the protein. Griffiths and Lynch (1991) have found that the 57 kDa protein in the ECP or cell surface extracts is unstable after either freezing or heating. A 53 kDa band appeared after freeze/thaw.
and corresponded to a decrease in the 57 kDa protein. They also found that qualitatively more of the lower molecular weight bands were present in the older culture supernatants. No change was noted when ECP was incubated for 10 hours at 4 or 15°C, however, at 25 and 30°C there was a decrease in the concentration of the 57 kDa antigen over a 10 hour incubation period. Addition of 5 mM PMSF decreased the amount of degradation. Degradation in sample buffer was also decreased at the zero time point, suggesting that the sample buffer increased the antigen susceptibility to degradation prior to electrophoresis. This led Griffiths and Lynch (1990) to propose that the protein may be autolytic or may co-migrate with a protease. Bands at 57 and 33-37 kDa possessed autolytic activity as determined by 2-D electrophoresis.

Rockey et al., (1991b) identified a high molecular weight serine protease which has activity against the 57/58 kDa protein (p57) both at 17 and 37°C and might explain some of the proteolysis observed by Griffiths and Lynch (1991). Digestion of a partially purified p57 yielded a spectrum of breakdown products similar in molecular mass and antigenicity to those seen in ECP. The pattern of digestion suggested that the immunologically related constituents of ECP are generated by the degradation of p57. The protease was inhibited by PMSF, methanol, ethanol, and 10 minutes incubation at temperatures greater than 65°C.

In summary, characterization of the 57/58 kDa protein (p57) has been difficult due to its susceptibility to degradation by an endogenous, Rs serine protease and much needs to be learned about the structure and functional role of this protein in the pathogenesis of BKD. The hydrophobic composition, acidic pl, and filamentous structure suggests that p57 may form fimbriae on the cell surface of Rs. These fimbrial structures and may mediate the in vitro agglutination of several species of mammalian erythrocytes and salmonid
spermatocytes. *In vivo*, p57 is associated with isolate virulence and by virtue of the surface exposed location significantly interact with the salmonid immune system.
CHAPTER 3

Monoclonal Antibody Analysis of Common Surface Protein(s) of *Renibacterium salmoninarum*

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We are reporting the development of two monoclonal antibodies which recognize ten separate isolates of *Renibacterium salmoninarum*. Both monoclonal antibodies recognize a 57 kDa protein doublet and several lower molecular weight antigens present in bacterial lysates. The 57 kDa protein appears to correlate with a 57 kDa surface antigen named Antigen F by Getchell et al. (1985). Antibody capture ELISA analysis indicates that the antibodies recognize different epitopes of the same protein(s). Cross-reactions were not observed with other bacteria or with normal chinook salmon serum proteins.
INTRODUCTION

*Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), causes significant mortality in salmonid populations throughout the world (Fryer and Sanders, 1981). BKD has historically been a difficult disease to control, due to its chronic nature (Fryer and Sanders, 1981), the possibility of vertical transmission (Evelyn et al., 1984), and the lack of a commercial vaccine. A wide variety of serological techniques have been developed for detection of the pathogen, including those employing immunodiffusion (Chen et al., 1974), indirect fluorescent antibody technique (Bullock and Stuckey, 1975), coagglutination (Kimura and Yoshimizu, 1981), counterimmunoelectrophoresis (Cipriano et al., 1985), peroxidase-antiperoxidase (Sakai et al., 1987b), and antibody capture enzyme-linked immunosorbent assays (ELISA), (Turaga et al., 1987b; Pascho and Mulchay, 1987; Dixon, 1987). Monoclonal antibody technology provides the ability to enhance immunological or serological analysis by providing a uniform reagent of potentially unlimited quantity. Arakawa et al. (1987) have developed thirteen different monoclonal antibodies to *R. salmoninarum* cells, however, they found that only one antibody recognized all tested isolates. This antibody also cross-reacted in an ELISA with three other species of gram-positive bacteria.

We are reporting the development of two monoclonal antibodies which recognize different epitopes of Antigen F (Getchell et al., 1985). These monoclonal antibodies appear to be specific for *R. salmoninarum* and do not cross-react with other gram-positive and gram-negative bacteria tested.
MATERIALS AND METHODS

**Bacterial strains.** *Renibacterium salmoninarum* isolates were a generous gift from Dr. J.S. Rohovec and C.R. Banner, Dept. of Microbiology, Oregon State University. The origins of the 10 isolates are listed in Table 1. The bacteria were cultured for 7-12 days in Kidney Disease Medium (KDM-II) with 10% calf serum (Gibco, Buffalo N.Y.) as described by Evelyn (1977). *Streptococcus facium*, *S. fecalis*, and *Carnobacterium piscicola* were grown in Muller-Hinton media (Difco, Detroit, MI), while *Vibrio anguillarum* and *Aeromonas salmonicida* were grown in tryptic soy broth (Difco).

**Soluble protein preparation.** *Renibacterium salmoninarum* isolates ATCC 33209 and D6 were grown for 10 days in two, one liter flasks containing KDM-II without calf serum. The soluble proteins were harvested from the supernatant as described by Turaga et al. (1987b). Briefly, cells were removed by centrifugation (6000 x g for 30 min.) and the supernatant was concentrated to 100 ml (20x) by ultrafiltration with a PTGC-10,000 NMWL filter packet (Millipore, Bedford, MA). The proteins were further concentrated by two 50% saturated ammonium sulfate (SAS) precipitations and dialyzed against three 1 liter changes of 10 mM phosphate buffered saline (PBS), pH 7.2. The solution was filter sterilized with a 0.45 µm filter (Millipore) and stored at 4°C.

**Production of monoclonal antibodies (MAbs).** Balb/c mice were immunized with 0.1 ml of a mixture of 1 O.D. (500 nm) formalin-killed *R. salmoninarum* and 50 µg of soluble protein (ATCC 33209) in Freund's Complete Adjuvant (Difco). After 4 weeks, mice were boosted with a mixture of 25 µg of soluble protein and 10 µg of *E. coli* LPS serotype 026:B6, (Difco).
Table 3.1. Designation and source of *R. salmoninarum* isolates.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Source of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lea-1-74 (ATCC 33209), Type Strain</td>
<td>Leaburg Hatchery, McKenzie River, OR; chinook salmon</td>
</tr>
<tr>
<td>2. 33739 (ATCC)</td>
<td>Brook trout,</td>
</tr>
<tr>
<td>3. D6</td>
<td>Oregon; from coho salmon held in salt water</td>
</tr>
<tr>
<td>4. Little Goose</td>
<td>Little Goose Dam, Idaho; chinook salmon</td>
</tr>
<tr>
<td>5. K-28</td>
<td>France; from the eye of pen reared coho salmon</td>
</tr>
<tr>
<td>6. K-70</td>
<td>England; rainbow trout</td>
</tr>
<tr>
<td>7. 684</td>
<td>Norway; sea trout</td>
</tr>
<tr>
<td>8. K50</td>
<td>Norway; net pen culture Atlantic salmon</td>
</tr>
<tr>
<td>9. Kvilan</td>
<td>Kvilan, Iceland; Atlantic salmon</td>
</tr>
<tr>
<td>10. Grindavik</td>
<td>Grindavik, Iceland; Atlantic salmon</td>
</tr>
</tbody>
</table>
Three days later SP2 myeloma cells were fused with the primed splenocytes after which wells containing antibody-secreting hybridomas (4D3 and 2G5) were cloned twice by limiting dilution and injected into Balb/c mice for preparation of ascitic fluid. Ascitic fluid was harvested after 8-12 days, SAS precipitated, and resuspended in 10 mM PBS, diluted 1:1 with glycerol, and stored at -20°C. Both monoclonal antibodies were of the IgG1 isotype as determined by the ICN Immunobiologicals (Lisle, IL) isotyping kit.

**Biotinylation of antibody.** Antibody was dialyzed in 0.1 M NaHCO₃ and reacted with a ratio of 1.0 mg of protein to 5.7 µl of a 0.1 M solution of BNHS (Biotin-N Hydroxysuccinimide, Calbiochem, CA) dissolved in distilled dimethyl formamide (Kendall et al., 1983). The mixture was reacted for one hour, and dialyzed against three changes of PBS during a 24 hour period. The biotinylated antibody was diluted 1:1 in glycerol and stored at -20°C.

**Preparation of cells for electrophoresis and Western blotting.** Bacterial cells were centrifuged at 6000 x g, for 0.5 hours; 200 ml of the pellet was subsequently washed three times with 1 ml of 10 mM PBS, resuspended in an equal weight to volume of PBS, and frozen at -20°C. Two microliters of cells were resuspended in 48 µl of double distilled H₂O and 50 µl of sample buffer consisting of 120 mM tris base, 4% w/v SDS, 10% v/v 2-mercaptoethanol, 20% v/v glycerol, and 3 mM bromophenol blue. The samples were immediately immersed in boiling water for 3 minutes and were electrophoresed in a 12% SDS-polyacrylamide gel (83 x 103 mm mini-slab gel, Idea Scientific, Corvallis OR) with a constant current of 20 mAmps for approximately 1.5 hours. The proteins were transferred from the gel to nitrocellulose paper by electrophoresis at 60 V for 3.5 hrs., at 4°C (Bio-Rad Trans-Blot apparatus), according to a modified method of Towbin et al. (1979). The nitrocellulose was subsequently blocked for 1 hour, at 37°C, with 3%
bovine serum albumin (BSA, Sigma) diluted in Tris-buffered saline (TBS), pH 8.0. MAb 4D3 was diluted 1:500 in TBS and allowed to react with the blots for 1 hour at room temperature. Excess antibody was removed by three, ten minute rinses with 0.1% Tween(20)-TBS (T-TBS). Peroxidase-conjugated, goat anti-mouse Ig (Hyclone, Logan UT) was diluted 1:200 in T-TBS and applied to the blot for 1 hr after which it was rinsed 3 times as described above. Visualization of the protein bands, was achieved using a substrate solution consisting of 2 ml of 4-chloro-1-napthol (3 mg/ml in methanol; Bio-Rad), 10 µl of 30% H₂O₂ (Fisher Scientific, NJ), and 10 ml of 10 mM PBS. The other half of the blot was stained for total protein with 10 ml of Aurodye colloidal gold solution used as recommended by the manufacturer (Janssen, Belgium).

**Collection of sera from experimentally infected salmon.** Sera was collected from three 20-30 gm chinook salmon which had been experimentally infected with 0.1 ml of the D6 isolate of *R. salmoninarum* for twenty days. Equal amounts of sera from the three infected fish or three noninfected fish were pooled, diluted 1:5 in double distilled water, mixed with an equal amount of sample buffer, and subjected to electrophoresis and Western blotting as described above.

**Indirect fluorescent antibody techniques (IFAT).** Kidney smears of fish infected with the D6 isolate of *R. salmoninarum* were made on pre-cleaned micro slides (VWR, CA) and fixed for 15 seconds with a 1:1 acetone:xylene solution. MAb 4D3 or 2G5 ascites was diluted to 1.5 µg/ml in 10 mM PBS, and 100 ml of the solution was added to the slide for 30 minutes. After a 5 minute wash with PBS, 50 ml of biotinylated goat anti-mouse (Hyclone) diluted 1:100 in PBS was added for 15 minutes, after which 50 ml of Av-FITC (HyClone) diluted 1:250 was added for 15 minutes and washed for 5
minutes. A 1% methylene green solution was used as a counter stain. Slides were observed under a standard microscope (Zeiss) utilizing an IV F1 epi-fluorescent condenser and a 12 V, 100 watt halogen tungsten light source. All ten of the *R. salmoninarum* isolates and three gram-positive control bacteria were also analyzed by indirect FAT. Bacterial colonies from 15 day plate cultures were picked and dried on 3% gelatin coated slides which were subsequently heat fixed. Fluorescent staining was performed as described above.

**Epitope analysis utilizing 4D3 and 2G5 antibodies.** Epitope analysis was performed using a double antibody capture ELISA as described by Berzofsky (1984), and Kohno et al. (1982). Briefly, one hundred μl of a 5 μg/ml solution of either monoclonal antibody, or soluble protein (in carbonate-bicarbonate coating buffer, pH 9.6) was coated overnight at 17°C (Voller et al., 1976) onto EIA flatbottom plates (Costar, Cambridge, MA). The plates were inverted, with shaking, to remove excess protein. All wells were subsequently blocked for 1 hr with 150 ml of 1% BSA (Fraction V, Sigma) diluted in T-TBS. After washing three times with T-TBS and TBS, 100 μl of a 5 μg/ml solution of soluble protein diluted in T-TBS or T-TBS alone (negative control) were added to appropriate wells for one hour. After rinsing, 100 μl of 5 μg/ml biotinylated antibody was added for 1.5 hours. A 1:250 dilution of streptavidin-horseradish peroxidase (Sigma) was added for 45 minutes. After the last rinse, 100 μl of substrate solution was added and color development was monitored spectrophotometrically at 405 nm on an EIA autoreader (Model 310, Biotek Instruments, Burlington, VT). The substrate solution was a mixture of 10 ml of citrate buffer (pH 4.0), 10 μl of H₂O₂, and 75 μl of a 10 mg/ml solution of 2,2'-Azinobis, 3-ethyl benzathiazoline sulfonic acid (ABTS) in distilled water.
RESULTS

*R. salmoninarum* isolate characterization by Western blotting. Cellular proteins of ten geographically distinct isolates (Table 3.1) were electrophoresed and tested by Western blotting with MAb 4D3. The antibody reacted with one major protein (Mr 57 kDa) and four minor proteins (Mr 58, 50, 45, 43 kDa) present in all isolates (Figure 3.1). No cross-reactive proteins were detected in the gram-positive control bacteria (Figure 3.3). Additionally, no cross-reactive proteins were detected in Western blots of the gram-negative salmonid pathogens *Vibrio anguillarum* or *Aeromonas salmonicida* (data not shown). Protein profiles of the ten isolates of bacteria also suggest that the isolates are very homogeneous (Figure 3.2).

Assessment of *R. salmoninarum* proteins in infected fish sera. *R. salmoninarum* 57 and 58 kd proteins were detected in experimentally infected fish sera by Western blot analysis using MAb 4D3 (Figure 3.4, lane 2). There was no cross-reactivity with normal serum proteins (lane 1).

Indirect fluorescent antibody technique. An indirect fluorescent antibody assay was used to detect unique surface epitopes on the *R. salmoninarum* cells. Experimentally infected chinook salmon kidney smears and colony smears of the ten isolates all tested positive, while none of the other gram-positive control bacteria fluoresced.

ELISA epitope analysis. To determine if the two monoclonal antibodies recognize different epitopes, a double antibody capture ELISA was used. In this assay antibody was bound to the wells, soluble protein added, and a biotinylated second antibody added to determine if any epitopes were
Figure 3.1. Western blot analysis of ten isolates of *Renibacterium salmoninarum* using monoclonal antibody 4D3. Lanes are as follows: 1, 5, and 9) Type strain ATCC 33209, 2) ATCC 33739, 3) D6 isolate, 4) Little Goose isolate, 6) K-28 isolate, 7) K-70 isolate, 8) 684 isolate, 10) K-50 isolate, 11) Kvilan isolate, 12) Grindavik isolate. The weights of Sigma pre-stained molecular weight markers (MW) are listed on the left.

Figure 3.2. Total protein profile as detected by Aurodye colloidal gold staining of duplicate nitrocellulose strips from Fig. 3.1.
Figure 3.1

Figure 3.2
Figure 3.3. Western blot analysis of cellular antigens of *Renibacterium salmoninarum* and gram-positive control bacteria using monoclonal antibody 4D3 (panel A), and total protein profile as detected by colloidal gold staining (panel B). Lanes are as follows; 1) Type strain ATCC 33209, 2) *Carnobacterium piscicola*, 3) *Streptococcus faecium*, and 4) *Streptococcus fecalis*.

Figure 3.4. Western blot of infected chinook sera probed with MAb 4D3. Lanes are as follows: 1) Pooled sera from three control fish (5 μl), 2) Pooled sera from three experimentally infected fish (5 μl), 3) *R. salmoninarum* soluble proteins (3 μg).
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Figure 3.3

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Figure 3.4
Figure 3.5. Epitope analysis by ELISA. Triplicate wells were coated with either Monoclonal antibody 4D3 (panel A), 2G5 (panel B), or *R. salmoninarum* soluble proteins (panel C) used as a positive control. Soluble protein was then added, incubated and removed. Biotinylated antibody (4D3, 2G5, or polyvalent rabbit (RB) anti-soluble protein) was used to assay apparent soluble protein in each well. Data is expressed as a percentage of the maximum OD (panel A, OD=.713; B, OD=.319; C, OD=.740), after subtraction of the background.
still available. No increase in optical density was observed when either 4D3 or 2G5 were coated onto the plate and the homologous biotinylated second MAb was used (Figure 3.5, A and B). Use of a heterologous monoclonal or polyclonal antibody indicated that epitopes were available (80-100% of the maximal OD). As a positive control, to determine that the biotinylated antibodies were equally functional, soluble protein was coated directly onto the ELISA well and the binding of each biotinylated antibody assessed. Figure 3.5 C shows that the optical densities of the wells were all within 10% of one another.

**DISCUSSION**

This study demonstrates the presence of at least two common epitopes on the cell surface of ten separate isolates of *R. salmoninarum*. These epitopes were defined by two murine monoclonal antibodies, 4D3 and 2G5. Both monoclonal antibodies recognize a 57/58 kd protein doublet and several lower molecular weight antigens present in bacterial preparations. ELISA epitope analysis suggests that each monoclonal antibody recognizes a single epitope. In addition, the two recognized epitopes are different (Figure 3.5, A and B). Within the complex of antigens recognized by the two monoclonal antibodies is a 57 kDa band which correlates with a 57 kDa surface protein named Antigen F by Getchell et al. (1985). Turaga et al. (1987b) have also observed that this is the predominant protein found in experimentally infected fish sera and is also seen in Fig. 3.4. The electrophoretic profiles of Aurodye stained nitrocellulose blots of the 10 isolates appear homogenous, supporting
the observed uniformity in other characteristics such as biochemical properties, cell wall carbohydrates, and hydrophobicity (Bruno and Munro, 1986c; Fiedler and Draxl, 1986; Daly and Stevenson, 1987).

Arakawa et al. (1987) have produced monoclonal antibodies to a heat stable antigen and found antigenic diversity among isolates. They speculate that the antibodies might recognize Antigen F epitopes which are isolate specific. Antigenic variability of epitopes on one protein is not uncommon; Buchanan et al. (1987) characterized 14 separate epitopes on a 65 kDd *Mycobacterium leprae* protein. Thirteen epitopes were common to other Mycobacterial species while one monoclonal antibody recognized an epitope which was species specific. Monoclonal antibodies 4D3 and 2G5 bind two separate epitopes which are common to all tested isolates of *R. salmoninarum* and yet are species specific. The apparent pan-specificity of the monoclonal antibodies and their lack of cross-reactivity with other bacterial species and normal chinook serum proteins indicates that the two antibodies may be useful diagnostic reagents.
CHAPTER 4

Monoclonal Antibody Characterization of a Leukoagglutinin
Produced by Renibacterium salmoninarum

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ABSTRACT

*Renibacterium salmoninarum* causes a chronic disease of salmonid fish known as bacterial kidney disease. High concentrations of bacterially produced extracellular protein (ECP) are present in plasma, kidney, and spleen tissue of naturally and experimentally infected fish. ECP agglutinated salmonid leukocytes *in vitro* at concentrations which correspond to levels found in highly infected fish. Association of biological activity with the structure of the major protein constituent of ECP, p57, was accomplished by monoclonal antibody (MAb) analysis. Location of the antigenic binding sites recognized by the MAbs was determined by two-dimensional electrophoresis and Western immunoblotting of the proteolytic breakdown fragments of p57. Eight MAbs have been classified into three groups on the basis of their differential recognition of these proteolytic breakdown products. Group I MAbs bound a region proximal to the amino terminus of the protein. Two of these MAbs were also able to block leukoagglutinating activity. Group III MAbs bound to a region associated with the bacterial cell surface, while group II MAbs bound a region between group I and group III. These analyses have allowed the identification of potential structural and functional regions of p57.
INTRODUCTION

*Renibacterium salmoninarum* is the etiologic agent of bacterial kidney disease in salmonid fish (Sanders and Fryer, 1980). This chronic disease causes important economic losses of cultured salmon worldwide (Fryer and Sanders, 1981), however, control of the disease has been limited because of the absence of an effective vaccine (Munro and Bruno, 1988) and because of vertical (intraovum) transmission (Evelyn et al., 1984).

Little is known about the virulence factors of *R. salmoninarum*, which is a gram-positive, slowly growing, facultative intracellular bacterium (Fryer and Sanders, 1981; Young and Chapman, 1978). Isolate virulence has been correlated with cell surface hydrophobicity, autoaggregation, and the production of a 57 kDa protein (Bruno, 1988; Bruno, 1990). High concentrations of a 57/58 kDa protein doublet, (also referred to as antigen F; Getchell et al., 1985), hemagglutinin (Daly and Stevenson, 1987; Daly and Stevenson, 1990) or p57 (this paper) can be found in experimentally and naturally infected fish tissues and sera (Turaga et al., 1987b; Wiens and Kaattari, 1989). Additionally, p57 is the predominant protein antigen found on the bacterial cell surface, and is the major component of the soluble protein, hereafter referred to as extracellular protein (ECP), isolated from bacterial culture supernatants (Getchell et al., 1985; Wiens and Kaattari, 1989). The function of this protein *in vivo* is unknown. However, a diverse number of *in vitro* activities have been attributed to p57, including hemagglutination of rabbit and other mammalian erythrocytes (Daly and Stevenson, 1987), agglutination of salmonid spermatozoa (Daly and Stevenson, 1989),
restoration of cell surface hydrophobicity (Daly and Stevenson, 1990), and suppression of antibody production (Turaga et al., 1987a; Rockey et al., 1991b).

In order to further characterize this important protein of *R. salmoninarum* we have produced a panel of monoclonal antibodies which recognize p57. Several of these MAbs inhibit hemagglutinating activity and a novel leukoagglutinating activity of p57. Two-dimensional (2-D) electrophoresis and N-terminal amino acid sequencing have facilitated the correlation of p57 structure with biological function.

**MATERIALS AND METHODS**

**Animals.** Coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), cutthroat trout (*Oncorhynchus clarkii*), and rainbow trout (*Oncorhynchus mykiss*) were held in 12°C well water at the Oregon State University Fish Disease Laboratory. The fish weighed between 400 and 600 g and were fed Oregon Moist Pellets (Bioproducts, Warrington, OR).

Balb/c mice (Simonsen Laboratory Inc., Gilroy, CA) were maintained by the Laboratory Animal Resource Center at Oregon State University.

**ECP preparation.** *R. salmoninarum* ATCC 33209 was cultured in 2.8-liter flasks containing 1 liter of medium. Kidney disease medium (KDM-II) was prepared according to the method of Evelyn (1977), excluding the addition of serum, and was subsequently ultrafiltered with a PTGC-10,000 filter packet (Millipore Corp., Bedford, MA) to remove molecules larger than 10 kDa.
Cultures were incubated at 17°C with constant rotational shaking for 10 days. ECP was harvested by the method of Turaga et al. (1987b). Briefly, bacterial cells were removed by centrifugation at 6,000 x g for 30 min (4°C), and the supernatant was concentrated 10x by ultrafiltration. ECP was further concentrated by two 50% saturated ammonium sulfate precipitations and dialyzed against three 1-liter changes of 10 mM phosphate-buffered saline (PBS; pH 7.2). Total protein was assessed by the method of Lowry et al. (1951) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard. ECP for the agglutination assays was prepared by dialysis against one liter of RPMI 1640 (pH 7.4; Gibco Laboratories, Grand Island, NY). The ECP was filter-sterilized (pore size, 0.45 μm) and stored at -70°C until needed or at 4°C for immediate use.

Control preparations of chicken egg ovalbumin (grade V; Sigma) or KDM-II medium were precipitated by 50% saturated ammonium sulfate fractionation and dialyzed against PBS followed by RPMI 1640 as described above.

**Production and purification of MAbs.** Female Balb/c mice were injected interperitoneally with a mixture of 10 mg of PBS-washed *R. salmoninarum* cells mixed 1:1 with Freund complete adjuvant. Two months postimmunization, the mice were injected with 1 mg of wet, packed cells and 10 μg of *Escherichia coli* lipopolysaccharide (LPS) Serotype 026:B6 (Difco Laboratories, Detroit, MI). Mice were sacrificed 3 days postchallenge, and the spleens removed aseptically. Splenocytes were then fused with SP2/0 cells using the polyethylene glycol method (Campbell, 1984). Hybridomas were screened by enzyme-linked immunosorbent assay (ELISA) for antibodies against ECP (see below). Five hybridomas of the immunoglobulin G (IgG) isotype were identified and subcloned twice by limiting dilution. Isotyping was
performed with an ICN Immunobiologics kit (Lisle, IL). The production of MAbs 2G5, 4D3 (Wiens and Kaattari, 1989) and 3H1 (Rockey et al., 1991a) has been previously described.

Cell-free ascites fluid was separately prepared from each hybridoma. Each antibody was purified by protein A chromatography using the MAPS system following the manufacturer's instruction (Bio-Rad Laboratories, Richmond, CA). Protein content was determined by the method of Lowry et al. (1951). A commercial hybridoma ascites (MOPC 21; Sigma) was purified as described above and used as a control. Antibodies were dialyzed in RPMI 1640 for use in agglutination assays.

ELISA. ECP at a concentration of 1 µg/ml was diluted in 24 mM carbonate-bicarbonate buffer, pH 9.6, and coated onto enzyme immunoassay flat-bottom plates (Costar, Cambridge, MA) overnight at 17°C. Wells were blocked with 1% bovine serum albumin (BSA; fraction V, Sigma) diluted in 0.1% Tween-Tris-buffered saline (50mM Tris, 1 mM EDTA, 8.7% NaCl, [pH 8.0]). Hybridoma tissue culture supernatant or protein A-purified MAb was added for 1 h, and after washing, a 1:4,000 dilution of peroxidase-labeled goat anti-mouse immunoglobulin (Hyclone, Logan, UT) secondary antibody was added. Substrate (0.2 mg of 2,2'-azinobis[3-ethylbenzthiazoline sulfonic acid diammonium salt]) per ml in 9.5 mM citrate buffer (pH 4.0), and 0.05% of 30% H₂O₂ was applied for 30 min, and the A₄₀₅ was determined on an enzyme immunoassay autoreader (Biotek Instruments, Burlington, VT).

Gel Electrophoresis and Western immunoblot analysis of anti-p57 MAbs. ECP prepared as described above consistently contains p57 and a number of lower-molecular-mass proteolytic breakdown fragments due to the activity of an endogenous serine protease (Rockey et al., 1991b). Preliminary localization of antigenic binding sites recognized by each MAb
was determined by 2-D electrophoresis (O'Farrell, 1975) of ECP followed by Western blotting. *R. salmoninarum* ECP (3 μg) was mixed 1:1 with 1-D sample buffer (9.5 M urea, 2.0% Triton X-100, 5.0% β-mercaptoethanol, 1.0% pH 3-5 ampholyte, and 1.0% pH 4-6 ampholyte; Bio-Rad). Proteins were focused at 500 V for 10 min and then at 750 V for 3.5 h. Two-dimensional gels (1.0 mm, sodium dodecyl sulfate [SDS]-10% polyacrylamide) were run for 1.5 h at 100 V. Proteins were transferred to nitrocellulose either for 14 h at 30V, or 1.5 h at 100 V (Towbin et al., 1979). Blots were then stained with a colloidal-gold total protein stain (Bio-Rad) or probed with approximately 5 μg of the appropriate MAb per ml as described previously (Wiens and Kaattari, 1989).

**Microsequencing.** *R. salmoninarum* ECP was separated by 1-D or 2-D electrophoresis and transferred (80 V, 10-15 min) to Immobilon-PVDF (Millipore) in transblotting buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 20% methanol, [pH 11.0]; Matsudaira, 1987). Membranes were stained either with Coomassie blue or Ponceau red for 1 min and destained with 50% methanol-10% acetic acid. Bands were excised, rinsed with nanopure water, dried and frozen at -20°C until sequencing. Amino terminus sequencing was performed at Oregon State University Center for Gene Research with a gas-phase microsequencer (model 475A; Applied Biosystems, Foster City, CA) and was followed by high-performance liquid chromatography.

**Peripheral blood leukocyte separation and in vitro tissue culture.** Peripheral blood (2 to 8 ml) was collected in a sterile heparinized VACUTAINER system from the caudal vein of anesthetized fish (Kaattari and Irwin, 1985). Peripheral blood leukocytes were separated by Histopaque 1077 (Sigma) gradient centrifugation as described (Kaattari and Holland, 1990). Leukocytes were resuspended in tissue culture medium (10% fetal calf
serum [Hyclone], 50 µg of gentamicin sulfate per ml, and 2% sodium bicarbonate in RPMI 1640 [Gibco]). Cultures were incubated in 96-well flat-bottom plates (Corning, Corning, NY) at 17°C under a blood-gas mixture.

**Agglutination assays. (i) Leukoagglutination.** Peripheral blood leukocytes (10⁷ cells per ml) were coincubated with dilutions of ECP, control protein (chicken egg ovalbumin), or concentrated KDM-II medium in a total volume of 100 µl. All incubations were done in triplicate. Agglutination was assessed by microscopic examination of wells with a CK Olympus inverted microscope (Boyle Instruments, Gig Harbor, WA). Cells were photographed after 24 h.

Determination of MAb inhibition of agglutination was assessed by preincubation of 5, 10, 25, 50, 100, 250, 500 or 1,000 µg of each MAb per ml for 1 h with 50 µg per ml of ECP at 17°C before addition of leukocytes.

(ii) **Hemagglutination of rabbit erythrocytes.** Blood from New Zealand white rabbits was mixed 1:1 with Alsever's solution and centrifuged at 500 x g for 10 min. Erythrocytes were resuspended in tissue culture medium (1.5 x 10⁷ cells per ml) and incubated with 50 µg of ECP per ml. Inhibition of agglutination was determined as described above.

**Analysis of leukocyte-adherent ECP.** Peripheral blood leukocytes (2 x 10⁶) in tissue culture medium were mixed with 200 µg of ECP per ml, a control protein (MAb 2G5, 200 µg/ml), or RPMI 1640 alone in a total volume of 200 µl. This preparation was suspended at 17°C for 3 h with agitation every 15 min. Unbound ECP was removed by five 1-ml washes with RPMI 1640 followed by centrifugation at 500 x g for 10 min after each wash. Following the final wash, the cell pellet was lysed by the addition of 10 µl of extraction buffer (10 mM Tris-hydrochloride [pH 7.2], 0.15 M NaCl, 0.02% NaN₃, 0.5% [wt/vol] Nonidet P-40; Jones, 1980). After 15 min on ice, insoluble
cellular constituents were removed by a 10 min centrifugation in a microfuge E (Beckman Instruments Inc., Palo Alto, CA). The supernatant was removed and frozen at -70°C until electrophoretic analysis (Laemmli, 1970).

**Analysis of bacterial cell-associated protein. (i) Bacterial cells.** *R. salmoninarum* was cultured as described above, and the bacterial cells were washed three times (1 [wet weight]:100 [volume of PBS]) and pelleted by a subsequent 2-min centrifugation in a microfuge. Cells were finally resuspended in an equal (wt/vol) amount of PBS and 2 µl was mixed with 48 µl of double distilled water and 50 µl of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. Samples were boiled for 3 min and subjected to electrophoresis.

(ii) **Fluorescent Antibody Analysis.** Tissue smears of infected coho salmon kidneys were made on precleaned glass slides (American Scientific Products, McGraw Park, IL). After being air dried, slides were blocked by incubation in 0.25% BSA in PBS for 0.5 hr. Slides were subsequently probed with 10 µg of protein A-purified MAbs per ml for 0.5 hr. Unbound antibody was removed by washing with 0.25% BSA in PBS, and 1:100 goat anti-mouse immunoglobulin fluorescein isothiocyanate-labeled secondary antibody (Hyclone) was applied for 0.5 h. Slides were observed using a standard microscope (Zeiss) utilizing a IV F1 epifluorescent condenser and a 12-V, 100-W halogen tungsten light source.
RESULTS

Characterization of monoclonal antibodies (MAbs). All MAbs used in this study were of the IgG isotype. ELISA and Western blots were used to determine the relative activities and specificities of the MAbs. All eight MAbs bound to ECP in the ELISA and recognized the 57/58 kDa protein (p57) on Western blots (Table 4.1). A control MAb, MOPC 21, had no reactivity against ECP in either of the assays.

Antigenic binding site analysis of p57 with MAbs. The relative locations of antigenic binding sites on p57 were established by determining the pattern of MAb binding to proteolytic fragments of p57 present in ECP. Five major proteolytic fragments with approximate molecular masses of 45, 36, 34, 25, and 20 kDa were resolved from ECP by 2-D electrophoresis (Figure 4.1, A). The pls of p57 and proteolytic fragments were acidic. Different isoelectric forms were present as well as minor amounts of higher-molecular-mass aggregates. Replicate blots were probed with each MAb, and the MAbs were divided into three groups on the basis of the patterns of immunoreactivity. Group I MAbs, 4H8, 4C11 and 4D3, bound to p34 and p20, which have a more acidic pl than p57 (Figure 4.1, B). MAb 4D3 also recognized p45 which was not recognized by MAbs 4H8 or 4C11, indicating a subgroup within this group (not shown). Group III MAbs, 1A1, 4D10 and 2G9A, bound proteolytic fragment p45, and two fragments p36 and p25, which have a more basic pl than p57 (Figure 4.1, D). Group II MAbs, 2G5 and 3H1, recognized both p36 and p34 (Figure 4.1, C). These data suggests that group II MAbs recognize a determinant located between those recognized by group I and III MAbs, since
Table 4.1. MAb isotype characterization and activity against *R. salmoninarum* ECP.

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<td>IgG1(k)</td>
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<td>4C11</td>
<td>IgG2b(k)</td>
<td>1.46(0.04)</td>
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<td>4D3</td>
<td>IgG1(k)</td>
<td>1.36(0.04)</td>
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<td>2G5</td>
<td>IgG1(k)</td>
<td>1.16(0.05)</td>
<td>+</td>
</tr>
<tr>
<td>3H1</td>
<td>IgG1(k)</td>
<td>1.17(0.04)</td>
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</tr>
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<td>1A1</td>
<td>IgG1(k)</td>
<td>1.12(0.10)</td>
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</tr>
<tr>
<td>4D10</td>
<td>IgG1(k)</td>
<td>1.33(0.15)</td>
<td>+</td>
</tr>
<tr>
<td>2G9A</td>
<td>IgG1(k)</td>
<td>1.18(0.04)</td>
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</tr>
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<td>MOPC 21</td>
<td>IgG1(k)</td>
<td>0.06(0.03)</td>
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$^aA_{405}$ activity of 1 µg of protein A-purified MAb per ml tested in triplicate by ELISA. Plates were coated with 1 µg of ECP per ml.

$^b$Immunoreactivity with the 57/58-kDa protein (p57). +, Reactivity; -, no reactivity.
Figure 4.1. Total protein stain and Western blots of *R. salmoninarum* ECP separated by 2-D electrophoresis. Proteins were transferred to nitrocellulose and stained with colloidal gold (A) or probed with MAbs 4H8 (B), 3H1 (C), and 1A1 (D), representative of groups I through III, respectively. Mwt, Molecular mass markers (in kilodaltons).
Figure 4.1
group II MAbs bound proteolytic fragments recognized by both of the other groups.

**Identification of the amino terminus of p57.** Both p20 and p34 had the same amino-terminal sequence as p57, suggesting that they are proteolytic fragments from the amino terminus of p57 (Table 4.2). Residue eight was consistently unidentifiable even after carboxymethylation, suggesting possible posttranslational modification or an artifact of the sequencing methodology.

**Inhibition of p57 biological activity with group I MAbs.** *R. salmoninarum* ECP agglutinated coho salmon leukocytes in a dose-dependent manner with a lower threshold of 10 µg/ml. Agglutination of coho salmon leukocytes occurs on the addition of 50 µg per ml (Figure 4.2, A). Leukocytes were not agglutinated in tissue culture medium alone (Figure 4.2, B) or when incubated with similar concentrations of control protein, chicken egg albumin, or concentrated KDM-II medium proteins (not shown). Agglutination was not salmonid species specific, since leukocytes from chinook and coho salmon and rainbow and cutthroat trout were also agglutinated. Fish erythrocytes and mouse splenocytes were not affected. The component of the ECP responsible for leukocyte agglutination was determined by cellular absorption analysis of leukocyte-adherent ECP. The p57 component of the ECP was detected in the leukocyte extract (Figure 4.3 A, lane 3), but none of the lower-molecular mass components were found to be present (Figure 4.3 A, lane 2). Binding was deemed specific because the goat anti-mouse horseradish peroxidase conjugate did not detect the control protein (MAb 2G5) in the membrane extracts (Figure 4.3 A, lane 5). Approximately equal amounts of leukocyte extracts were run in each lane, as determined by the total protein stained blot (Figure 4.3, B). These results
Table 4.2. N-terminal amino acid sequences of various proteins.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino acid\textsuperscript{b} at residue no.:</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>p57</td>
<td>NH\textsubscript{2}-</td>
<td>S</td>
<td>Q</td>
<td>G</td>
<td>E</td>
<td>G</td>
<td>N</td>
<td>S</td>
<td>T</td>
<td>X</td>
</tr>
<tr>
<td>p34</td>
<td>NH\textsubscript{2}-</td>
<td>S</td>
<td>Q</td>
<td>G</td>
<td>E</td>
<td>G</td>
<td>N</td>
<td>S</td>
<td>X</td>
<td>T</td>
</tr>
<tr>
<td>p20\textsuperscript{c}</td>
<td>NH\textsubscript{2}-</td>
<td>S</td>
<td>Q</td>
<td>G/F</td>
<td>E/D</td>
<td>G</td>
<td>N</td>
<td>S/Q</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Proteins were sequenced after SDS-PAGE and transferred to PVDF membranes.

\textsuperscript{b}X, Unidentified residues.

\textsuperscript{c}Sequenced from 2-D gel.
Figure 4.2. Light micrographs of cultured peripheral blood leukocytes from coho salmon. Leukocytes (1 x 10^6) were incubated with 50 μg ECP per ml (A) or tissue culture media alone (B). Agglutination was inhibited by preincubation of ECP with 50 μg of MAb 4H8 per ml (C) but not with 1000 μg of MOPC 21 per ml (D). All incubations were performed in a total volume of 100 μl at 17°C for 24 h. Magnification, x160.
suggest that the p57 component of ECP was binding to and agglutinating leukocytes. Further, agglutinating activity of the ECP was sensitive to incubation at 37°C, a temperature which enhances the activity of the endogenous serine protease.

MAbs were tested for their ability to inhibit agglutinating activity of ECP. Inhibition profiles are given in Table 4.3. Only MAbs 4H8 (Figure 4.2, C) and 4C11 inhibited agglutination of coho salmon peripheral blood leukocytes cultured with 50 µg of ECP per ml. None of the other MAbs or the control MAb MOPC 21 (Figure 4.2, D) inhibited agglutination at the highest concentration tested, 1000 µg/ml. MAbs 4H8, 4C11, and 4D3 inhibited agglutination of rabbit erythrocytes. The minimum concentration of MAb 4C11 (50 µg/ml) required for complete inhibition was lower than those of MAbs 4H8 and 4D3 (100 and 250 µg/ml, respectively).

Identification of p57 bacterial cell associated determinant by using group III MAbs. Two observations suggest that p36 and p25 contain a determinant of p57 which is bound to the bacterial cell surface. First, p57, p36 and p25 are present in washed R. salmoninarum cell extracts separated by SDS-PAGE (Figure 4.4 A, lane 3) and were identified by MAb 1A1 (Figure 4.4, B). Second, group III MAbs, which bind p36 and p25, were unable to immunofluoresce bacterial cells present in infected coho salmon kidney tissue. Since equal concentrations of MAbs had comparable binding activity against ECP (Table 4.1), the inability of group III MAbs to immunofluoresce bacterial cells suggests that the antigenic binding site is sterically unavailable when p57 is bound to the bacterial cell surface.
Table 4.3. Minimum concentration of MAbs required for complete inhibition of leukoagglutination or hemagglutination mediated by *R. salmoninarum* ECP.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Group</th>
<th>Minimum concentration (µg/ml) required for complete inhibition of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leukoagglutination</td>
</tr>
<tr>
<td>4H8</td>
<td>la</td>
<td>50</td>
</tr>
<tr>
<td>4C11</td>
<td>la</td>
<td>50</td>
</tr>
<tr>
<td>4D3</td>
<td>lb</td>
<td>NI(^a)</td>
</tr>
<tr>
<td>2G5</td>
<td>II</td>
<td>NI</td>
</tr>
<tr>
<td>3H1</td>
<td>II</td>
<td>NI</td>
</tr>
<tr>
<td>1A1</td>
<td>III</td>
<td>NI</td>
</tr>
<tr>
<td>4D10</td>
<td>III</td>
<td>NI</td>
</tr>
<tr>
<td>2G9A</td>
<td>III</td>
<td>NI</td>
</tr>
<tr>
<td>MOPC 21</td>
<td>Control</td>
<td>NI</td>
</tr>
</tbody>
</table>

\(^a\)NI, No inhibition at a MAb concentration of 1,000 µg/ml, except for MAb 2G9A, which was only tested up to a concentration of 250 µg/ml.
Figure 4.3. Western blot (A) and total protein stain (B) of coho salmon leukocyte protein extracts obtained from leukocytes incubated with \textit{R. salmoninarum} ECP or a control protein (protein A purified MAb 2G5). Lanes: 1, pre-stained molecular weight markers (Sigma); 2, 2 \(\mu\)g ECP; 3, leukocytes incubated with 200 \(\mu\)g of ECP per ml; 4, leukocytes incubated in tissue culture media alone; 5, leukocytes incubated with 200 \(\mu\)g MAb 2G5 per ml; 6, 2 \(\mu\)g 2G5. The Western blot was probed with 5 \(\mu\)g (each) of MAbs 4D3 and 3H1 followed by a 1:500 dilution of second antibody, goat anti-mouse horseradish peroxidase.

Figure 4.4. Total protein stain (A) and Western blot (B) of washed \textit{R. salmoninarum} cells. Lanes: 1, pre-stained molecular weight markers (Sigma); 2, 3 \(\mu\)g of \textit{R. salmoninarum} ECP; 3, 2.5 \(\mu\)l of \textit{R. salmoninarum} cells washed three times. The Western blot was probed with 5 \(\mu\)g of purified MAb 1A1 per ml.
Figure 4.3

Figure 4.4
In this study MAbs were used to investigate the structure and function of p57, which is the predominant extracellular and cell surface protein produced by the fish pathogen *R. salmoninarum*. Analysis was facilitated by an autologous serine protease which cleaves p57 in broth culture, resulting in fragments with molecular masses of 45, 36, 34, 25, and 20 kDa (Rockey et al., 1991b). Resolution of ECP by 2-D electrophoresis followed by Western blotting allowed the MAbs to be divided into three groups on the basis of their differential recognition of these proteolytic fragments. Group I MAbs recognize p34 and p20, which have an identical amino-terminal sequence to p57. Therefore, unless a repetitious sequence exists, group I MAbs bind proximal to the amino terminus of the protein (Figure 4.5). Since agglutinating activity was inhibited by group I MAbs, the agglutinating domain(s) may be near the amino terminus. Antigenic binding sites recognized by group I and II MAbs are exposed on the surface of the bacterial cell, as demonstrated by immunofluorescence experiments. However, group III MAbs recognize an antigenic binding site which is sterically unavailable when p57 is attached to the bacterial cell surface. The fragments p36 and p25, recognized by the group III MAbs, are also present in cell extracts from washed bacterial cells. These observations suggest that p36 and p25 may contain a determinant of p57 which is important for the attachment of the protein to the bacterial cell surface. This determinant may be responsible for the *in vitro* reassembly of the protein onto a *R. salmoninarum* strain lacking p57 described by Daly and Stevenson (1990).
Figure 4.5. Proposed structural model of p57. Protein has been depicted in a linear manner. Lower molecular mass proteolytic products with a minimum number of proteolytic sites are indicated (I).
Previously, p57 was demonstrated to possess agglutinating activity against salmonid spermatazoa and a number of mammalian erythrocytes but not fish erythrocytes (Daly and Stevenson, 1987 and 1989). We have described a novel activity of p57, the agglutination of salmonid leukocytes. In this study, ECP was used to agglutinate the leukocytes, and two experiments demonstrate that p57 is associated with this activity. First, by using leukocytes as an ECP absorbent, p57 was the only component found in membrane extracts. Second, two MAbs which recognize p57, 4H8 and 4C11, were able to block agglutinating activity. These MAbs also blocked hemagglutination of rabbit erythrocytes by ECP, suggesting that these activities are mediated by the same site on p57. Assuming that p57 binds in a receptor-specific manner, there may be a similar receptor determinant on both the rabbit erythrocyte and the salmonid leukocyte. Differences must exist, however, because another MAb, 4D3, was able to inhibit hemagglutination but not leukoagglutination.

The physiological significance of leukocyte agglutination by p57 is still unclear. As yet there is no evidence that leukocytes are agglutinated \textit{in vivo} during infection, even though high concentrations, approaching 1 mg/ml are present in moribund fish (Turaga et al., 1987b). It is interesting to note that p57 possesses characteristics resembling a number of proteinaceous adhesins (Arp, 1988; Jones and Isaacson, 1983). These include acidic pl, hemagglutinating activity, and contribution to bacterial cell surface hydrophobicity. A fimbrial structure composed of p57 has recently been described on \textit{R. salmoninarum} (Dubreuil et al., 1990a), therefore, p57 may function as an adhesin for the bacterial attachment to cellular receptors allowing intracellular invasion by the bacteria, as proposed by Daly and Stevenson (1987 and 1990). Cloning and sequencing of the p57 gene and the development of isogenic mutants of \textit{R. salmoninarum} will be useful for the
further confirmation of the proposed model and the role of this protein in bacterial virulence and adhesion.
CHAPTER 5

Analysis of the Mechanism of Action of the Renibacterium salmoninarum Leukoagglutinin by Partial Purification and Epitope Mapping

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**ABSTRACT**

*Renibacterium salmoninarum* produces high concentrations a 57/58 kDa protein (p57) in broth culture and during infection of salmonid fish. The presence of the protein has been correlated with the virulency of *R. salmoninarum* isolates, however its function in pathogenesis remains unknown. *In vitro* activities of p57 include suppression of antibody production, agglutination of salmonid leukocytes and spermatocytes, and the agglutination of rabbit erythrocytes. To investigate the mechanism of cellular agglutination, p57 was partially purified from culture supernatant using fast pressure liquid anion exchange and size exclusion chromatography. P57 eluted as a protein monomer and retained agglutinating activity. Epitope analysis by ELISA also indicated that a monomer exists in culture supernatant and in infected fish tissue. Models of the possible agglutination mechanisms are discussed.
INTRODUCTION

*Renibacterium salmoninarum*, a bacterial pathogen of salmonids, produces a 57/58 kDa protein doublet (p57). P57 is localized on the cell surface and is also the major component of extracellular protein (ECP) concentrated from bacterial culture medium. High concentrations of p57, up to 200 µg/ml, have been found in naturally infected adult salmon and in moribund fish (Rockey et al., 1991a; Turaga et al., 1987b). The presence of p57 has been correlated with isolate virulence (Bruno, 1990), and suppression of antibody production (Turaga et al., 1987a; Rockey et al., 1991b), however, the exact role of p57 in pathogenesis remains unknown.

*In vitro* ECP, containing p57, agglutinated a number of cell types, including rabbit, pigeon, horse, and rat erythrocytes (Daly and Stevenson, 1987 and 1990), salmon spermatocytes (Daly and Stevenson, 1989) and salmon leukocytes (Wiens and Kaattari, 1991). This activity has been attributed to p57 because gel-purified p57 agglutinates rabbit erythrocytes. Additionally, our previous research demonstrated that antibodies to the amino terminal region of p57 blocked agglutination, providing further evidence that p57 has agglutinating activity.

Recently, Dubreuil et al. (1990a) have observed short, flexible fimbriae extending beyond the capsule of *R. salmoninarum*. The fimbriae have a diameter of less than 2 nm and are composed of a 57 kDa subunit, however, whether this protein exists as an aggregate of two or more subunits was not determined.

In this study we used two techniques to determine whether the protein exists as a dimer or multimer and if this conformation is required for
agglutinating activity. Partial purification of p57 and capture ELISA analysis using monoclonal antibodies to p57 both indicate that p57 exists as a monomer in the ECP and in infected salmonid tissue, and, thus, does not agglutinate cells by an aggregate dependent mechanism.

MATERIALS AND METHODS

Animals. Rainbow trout (Oncorhynchus mykiss) were held in 12°C well water at the Oregon State University Salmon Disease Laboratory and were fed Oregon Moist Pellets (Bioproducts, Warrington, OR). Samples of infected kidney tissue were obtained from spawning spring chinook salmon at Marion Forks Hatchery, OR., and Carson Hatchery, WA. and homogenized as previously described (Rockey et al., 1991a). Rabbits were maintained by the Laboratory Animal Resource Center at Oregon State University. Rabbit blood was collected in Alsevers solution and stored at 4°C.

Extracellular protein (ECP) preparation and partial purification of p57. Renibacterium salmoninarum ATCC 33209 was cultured in 2.8 liter flasks containing 1 liter of kidney disease medium (Evelyn, 1977), excluding serum. Cultures were incubated at 17°C with rotational shaking for 10 days. Extracellular protein (ECP) was harvested by the method of Turaga et al. (1987b). Briefly, bacterial cells were removed by centrifugation at 6000 x g for 30 min (4°C). The culture supernatant was concentrated 10x by ultrafiltration using a PTGC-10,000 NMWL filter packet (Millipore Corp., Bedford, MA) to remove molecules larger than 10 kDa. ECP was further concentrated by two 50% saturated ammonium sulfate precipitations and
dialyzed against three, 1 liter changes of 10 mM phosphate buffered saline (PBS; pH 7.2). Total protein was assessed by the method of Lowry et al. (1951) using bovine serum albumin (Sigma, St. Louis, MO) as a standard. The ECP was filter-sterilized (0.45 μm) and stored at -70°C or kept at 4°C for immediate use.

Fast pressure liquid chromatography (FPLC) was used for further purification of P57 from ECP as this technique was previously found to be efficacious (R.P. Levine, Wash. Univ. Med. Cent., St. Louis, MO; personal communication). Prior to FPLC, ECP was dialyzed in 50 mM phosphate buffer (pH 7.2) and filtered through a 0.2 μm low protein binding filter (Acrodisc 13, Gelman Sciences, Ann Arbor, MI). Approximately 3.9 mg of protein was loaded onto a HR 5/5 Mono Q anion exchange column and eluted with a biphasic salt gradient of 0-0.6 M NaCl, followed by 0.6-1.0 M NaCl in 50 mM phosphate buffer (pH 7.2). One ml fractions from the anion exchange column were stored at -70°C until analyses were performed.

To determine the native molecular weight of p57, fraction 6 (75 μg total protein) from the anion exchange column was run on a HR 10/30 Superose 12 FPLC gel filtration column (10 x 30 cm, Pharmacia) using a buffer of 50 mM NaCl and 50 mM phosphate (pH 7.2). The peak absorbing fraction (15) from the gel filtration column was concentrated 12-fold by ultrafiltration using a Centricon-3 (3000 MW cutoff, Amicon Division, Grace and Co., CN).

**Protease assay.** A protease assay was adapted from Sarath et al. (1989), using a substrate of 2% azoalbumin (Sigma) in a 0.1% sodium doedcyl sulfate, 10 mM phosphate, 7.5% NaCl buffer (pH 7.2). Substrate solution was filter sterilized and stored at -20°C. Protease activity was determined by mixing 30 μl of each column fraction with 50 μl of substrate at an incubation temperature of 37°C for 15 h. Intact substrate was precipitated
with 200 µl of 10% TCA for 15 min at room temperature. Samples were microfuged for 5 min, and 100 µl of supernatant was transferred to ELISA wells to which 100 µl 1.0 M NaOH was added. The $A_{450}$ was determined using a EIA autoreader (Biotek Instruments, Burlington, VT). Units of activity were calculated from a log-log plot using trypsin (#T2271, Bovine Pancreas, Sigma) as a standard.

**Leukoagglutination and hemagglutination assays.** Rabbit erythrocytes and rainbow trout leukocytes were prepared as previously described (Wiens and Kaattari, 1991), except that leukocytes were resuspended in a final concentration of 2% autologous plasma (DeKoning and Kaattari, 1991) instead of 10% fetal bovine serum. Agglutination reactions worked equally well in either serum source. A total of $1.0 \times 10^6$ leukocytes/well or $1.5 \times 10^6$ erythrocytes/well were mixed with 50 µl of each column fraction or 25 µl of the concentrated fraction 15 from the gel filtration column. Agglutination was assessed after 24 h by microscopic examination of wells with a CK Olympus inverted microscope (Boyle Instruments, Gig Harbor, WA).

**Purification of MAbs.** Cell free ascites fluid was prepared and antibody was purified by protein A chromatography using the MAPS system following the manufacturer's instruction (Bio-Rad Laboratories, Richmond, CA). Protein content was determined by the method of Lowry et al. (1951).

**Enzyme linked immunosorbent assays (ELISA).** A capture enzyme-linked immunosorbent assay was performed as described previously (Wiens and Kaattari, 1989) to determine if a dimer or a multimer of p57 could be detected. Formalin-fixed *R. salmoninarum* cells were used as a positive control to ascertain that aggregates of p57 could be detected by the ELISA. *R. salmoninarum* cells were fixed for 12h with 0.3% formalin, washed 3x in
phosphate buffered saline, and resuspended at a concentration of 50 μg wet weight/ml of 1% bovine serum albumin in 0.1 % Tween-Tris-buffered saline (50 mM Tris, 1 mM EDTA, 8.7% NaCl, pH 8).

**Gel electrophoresis.** Standard 10% SDS-PAGE and Western blotting were performed as previously described using the Mini-PROTEAN II system (Bio-Rad) (Wiens et al., 1990). Polyacrylamide gels (0.75 mm, 10% SDS-polyacrylamide) were run for 1.5hr at 100 V.

**RESULTS**

**Partial purification of p57 from culture supernatant.** To determine the native molecular weight of the *R. salmoninarum* p57, ECP was first concentrated from culture supernatant by ultrafiltration followed by precipitation with 50% saturated ammonium sulfate. P57 was then partially purified from ECP by FPLC anion-exchange chromatography. Figure 5.1 shows the A₂₈₀ absorbing components eluted from the column using a biphasic salt gradient. Approximately 70% of the total protein loaded onto the column was recovered in fractions 1-30. P57 was identified as the major component eluting in fractions 5 through 14 as determined by total protein staining of polyacrylamide gels (Figure 5.2, A). The identity of p57 in these fractions was further confirmed by Western blotting using MAb 1A1 (Figure 5.2 B, lanes 4-8). Fractions 5 through 15 possessed hemagglutinating activity. ECP also contains a high molecular weight serine protease capable of degrading p57.
Figure 5.1. FPLC anion-exchange chromatography of concentrated culture supernatant (ECP) from *R. salmoninarum* ATCC 33209. ECP (protein content 4.6 mg) was applied to a Mono-Q column (HR 5/5) and eluted with a biphasic NaCl gradient in a 50 mM phosphate buffer (pH 7.2; flow rate 1 ml min⁻¹). The protein content of the eluent was detected photometrically at 280 nm with a UV monitor. A linear salt gradient of 10 to 600 mM was used to elute proteins from fraction 8 to 28. A 600 to 1000 mM salt gradient was used from fraction 28 to 32. ECP was originally dialyzed in 50 mM phosphate pH 7.2 and 10 mM NaCl before application to the column.
Figure 5.2. Western blot and total protein stain of replicate blots confirming the presence of p57 in FPLC anion exchange column fractions 5-15. Total protein stain (A) and Western blot (B) of FPLC mono-Q fractions. A total of 2.5 μl of each fraction (fx) was electrophoresed through a 10% SDS polyacrylamide gel. Lanes: 1) Molecular weight markers (Bio-Rad low in blot A; Sigma pre-stained mwt markers in blot B), 2) ECP, 3) fx 2, 4) fx 5, 5) fx 7, 6) fx 9, 7) fx 12, 8) fx 14, 9) fx 16, 10) fx 18, 11) fx 19, 12) fx 20, 13) fx 22, 14) fx 24, 15) fx 26.
Figure 5.2
A peak of protease activity was identified in fraction 3 while there was not detectable activity after fraction 5 (data not shown).

**Determination of native molecular weight.** To determine the native molecular weight of p57, 75 μg of fraction 6 from the anion exchange column were subjected to size exclusion chromatography on a Superose 12 column. Dextran blue (30 μg) was included as an internal marker. Two peaks eluted, the first peak was dextran blue at 7.5 min and the second peak, confirmed to be p57 by Western blotting, eluted at 14.3 min (Figure 5.3, A). Calculations of relative molecular mass reveal that the protein eluted as a monomer and not as a dimer or multimer (Figure 5.3, B). P57 was purified to near homogeneity (Figure 5.4, lane 3), excluding a small amount of p45, as compared to the starting material (Figure 5.4, lane 1). Functionally, this purified protein possessed hemagglutinating and leukoagglutinating activity.

**ELISA epitope analysis.** Capture enzyme-linked immunosorbent assay analysis indicated that p57 agglutination is not mediated by a dimeric or multiaggregate form of the protein, providing further evidence that p57 exists as a monomer in the ECP and infected fish tissue. In the capture assay three N-terminus reactive monoclonal antibodies 4D3, 4H8 and 4C11, and control polyclonal rabbit antisera and monoclonal antibody MOPC 21 were used to probe available epitopes. Using these antibodies in a capture ELISA, homologous antibody was unable to bind while the heterologous antibodies were able to do so (Figure 5.5 A, B, and C). Capture antibodies had equivalent activity when ECP was coated directly onto the plate (Figure 5.5, D). Monoclonal 4C11 showed some reactivity when biotinylated 4C11 was added, however, the A405 was not greater than the non-specific antibody control, MOPC 21. The lack of homologous antibody binding suggests that repetitive determinants were not available, as would be the case with a dimer.
Figure 5.3. A. FPLC Superose 12 chromatography of the peak fraction (75 µg) from the Mono-Q column. Dextran blue (30 µg) was included as an internal standard. B. Relative migration of molecular weight markers using a FPLC superose 12, size exclusion column. The column was equilibrated with 50 mM phosphate and 50 mM NaCl, pH 7.2, and Sigma standards were used to calibrate the column. Standards included dextran blue (2,000,000), thyroglobulin (669,000), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (29,000) and cytochrome C (12,400). The dashed line represents the calculated molecular weight for p57.
Figure 5.4. Western blot of column purified p57. The Western blot was probed with 1 µg/ml of a pool of purified MAbs 4D3, 3H1, and 1A1. Primary MAb was detected by a 1:4000 goat anti-mouse antiserum linked to horseradish peroxidase. The blot was developed with a chemiluminescent substrate (Amersham) and the blot was exposed to film for 1 min. Lanes: 1) ECP, 1 µg, 2) FPLC Mono-Q fraction 5, 3) Superose 12 fraction 15.
Figure 5.4
Figure 5.5. ELISA epitope analysis using combinations of group I MAbs, 4D3, 4H8, and 4C11. Antibody was coated onto the ELISA plate at 5 µg/ml followed by 5 µg/ml ECP diluted in T-TBS. Four different biotinylated capture antibodies (5 µg/ml) were used to determine the steric availability of epitopes. Rabbit antisera which recognizes p57 was used as a positive control, and monoclonal antibody MOPC 21 was used as a negative control to determine nonspecific binding. After substrate was added plates were incubated for 30 min before $A_{405}$ was measured.
Figure 5.6. ELISA epitope analysis using infected fish tissue from four infected adult chinook salmon which were previously found to have antigen levels above 1000 ng/ml. Plates were coated with 4D3, blocked, and a 1:100 dilution of tissue, 5 µg/ml ECP, or *R. salmoninarum* cells (50 µg/ml) were added. ELISA plates were then probed with 1 µg/ml of biotinylated MAb 4D3 or 3H1 followed by a 1:400 dilution of Av-HRPO. After substrate was added plates were incubated for 10 min before *A_405* was measured.
or multimer. Analysis of kidney homogenates from infected fish also revealed that the homologous antibody (e.g. 4D3) could not bind, suggesting that p57 exists as a monomer in naturally infected fish (Figure 5.6). Formalin-fixed bacteria, used as a positive control, demonstrated that detection of a multimeric protein was possible by ELISA.

**DISCUSSION**

The agglutination of erythrocytes is a common property of a number of bacterial adhesins (reviewed in Jones and Isaacson, 1983; Jann and Hoschutzky, 1990). Bacterial adhesins have been classified as either fimbrial or non-fimbrial in structure. Recently, Dubreuil et al. (1990a) described a previously unidentified fimbriae on the cell surface of *R. salmoninarum* using immunogold labeled anti-57 kDa polyclonal antisera. The diameter of the fimbriae were less than 2 nm, and length of the fimbriae were at least 50-60 nm. The authors noted that length estimations of the fimbriae are uncertain due to possible shearing of the fimbriae by mechanical forces. Biochemical properties such as the amino acid composition (33% hydrophobic amino acids) and acidic pl of 4.8 resemble those of known fimbriae.

In this manuscript we investigate the mechanism of the functional, agglutinating activity of p57 to determine if the activity is correlated with dimeric or multimeric characteristics expected of fimbriae structures. Partial purification p57 from culture supernatant was achieved using ammonium sulfate precipitation followed by FPLC Mono-Q anion exchange chromatography and Superose 12 size exclusion chromatography. The native
size of the protein, as determined by size exclusion chromatography was smaller than a predicted multimer or dimer. Superose 12 column fraction 15 also possessed agglutinating activity suggesting that activity resides with a monomer of the protein. Additionally, ELISA antibody capture analysis confirmed these results when homologous antibodies were unable to bind to available epitopes. Similar results using naturally infected tissues were observed suggesting that the protein also appears as a monomer in vivo. However, it is also possible that p57 exists as a dimer or multimer on the bacterial cell surface and that proteolysis, purification, or processing procedures may result in a monomeric form.

Since we found no evidence which suggests that agglutination requires the presence of a dimer or multimer (Figure 5.7, A and B), other models are proposed to explain biological activity, assuming that p57 agglutinates cells in a receptor specific manner. In model C (Figure 5.7, C), p57 may exist as a monomer in solution but upon binding to cellular targets effect a change in the charge or hydrophobicity of the cell resulting in agglutination. *Vibrio cholerae* and *Pseudomonas aeruginosa* both produce zinc/calcium dependent proteases which are able to modify erythrocytes and induce hemagglutination (Hase and Finkelstein, 1990). However, we have been unable to detect proteolytic activity by p57, using a number of substrates (Rockey et al., 1991b). Also, agglutinating activity did not elute with the known serine protease produced by *R. salmoninarum*, nor is the agglutinating activity effected by EDTA (unpublished data). Alternatively, p57 may exist as a bifunctional molecule which recognizes separate cellular receptors (Figure 5.7, D). Capture ELISA epitope mapping provides potential evidence for this model as MAbs 4C11 and 4H8, which are able to inhibit leukoagglutinating activity, bind
Figure 5.7. Models of possible mechanisms of cellular agglutination by p57. A) A p57 dimer agglutinates cells by binding to a common leukocyte receptor via identical binding domains. B) A p57 aggregate agglutinates leukocytes in a similar manner to model B. C) A p57 monomer causes agglutination by altering the cell surface properties of the leukocyte. D) A p57 monomer agglutinates leukocytes via two different binding domains for separate cell surface receptors.
Figure 5.7

A. Protein Dimer

B. Protein Multiaggregate

C. Protein Monomer

D. Bifunctional Protein

Figure 5.7
to separate epitopes within a 20 kDa region proximal to the amino terminus of p57. These epitopes may represent two separate agglutinating domains. However, it is also possible that these MAbs bind to different sites of the same agglutinating domain, or they bind outside of the agglutinating domain but sterically inhibit agglutination.

A number of gram-positive bacteria produce bifunctional molecules which are able to bind separate cellular receptors. Superantigens produced by gram-positive bacteria have bifunctional properties as they are able to bind major histocompatibility class II molecules and stimulate families of T cells through specific Vβ receptors (reviewed in Herman et al., 1991; Johnson et al., 1991). While the complete structure and function of superantigens are still being determined, interesting similarities exist between a number of superantigens and p57. Staphylococcal enterotoxins and p57 are both able to suppress antibody production to unrelated antigens in vitro without causing apparent cellular toxicity (Smith and Johnson, 1975). Further, both superantigens and p57 are able to bind to leukocytes and affect mitogenesis (unpublished data). The size of the described p57 fimbrial structures observed on R. salmoninarum resemble that of the M protein fimbriae on Streptococci which has recently been found to have superantigenic activity (Tomai et al., 1990 and 1991).

If p57 functions as a superantigen it could have an important affect on the development of the immune repertoire in infected fish. One mechanism of transmission of R. salmoninarum is thought to be from parent to offspring through the egg (Evelyn et al., 1984). If p57 is expressed in early development, clonal deletion or tolerance, may occur. Recently, superantigens have been discovered to dramatically alter the repertoire of inbred or wild mice (White et al., 1989; Pullen et al., 1990). Elucidation of the
cellular receptors on lymphocytes for p57, further characterization of the
binding domain(s) on p57, and the demonstration of effects on the lymphocyte
repertoire need to be determined before a bifunctional mechanism of
superantigenic activity can be determined.
Quantitative and Qualitative Analysis of P57 in Kidney Tissue and Ovarian Fluid of Spawning Chinook and Coho Salmon Using Monoclonal Antibody-Based Immunoassays

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ABSTRACT

Bacterial kidney disease causes significant losses of cultured salmonids, however, there are limited methods for control of this disease. One proposed method for control is the segregation and elimination of adult infected gametes thereby preventing vertical transmission of *Renibacterium salmoninarum*. A sensitive indicator of infection is the presence of a 57/58 kDa protein (p57) produced by *R. salmoninarum*. Monoclonal antibodies have been useful tools for the specific identification and quantitation of p57 in spawning salmonids. To establish baseline data on the prevalence and concentrations of p57 in adult salmon, antigen levels in 740 spawning spring chinook salmon (*Oncorhynchus tshawytscha*) were determined by a monoclonal antibody-based, quantitative enzyme linked immunosorbent assay (QELISA). The percentage of fish with detectable antigen levels, above 3 ng/ml of kidney homogenate, varied from 12.8% in 1989 to 36.6% in 1991. In addition to the QELISA, a prototype Field ELISA (FELISA) was developed to qualitatively identify infected fish under hatchery conditions. The sensitivity and specificity of the FELISA was compared to the QELISA and the fluorescent antibody test (FAT). The FELISA had a minimal sensitivity of approximately 20 ng/ml as determined by the QELISA, and a 100% correlation with FAT values of 2+ or greater when kidney samples were assayed. Ovarian fluid samples from kidney positive and negative fish were also tested by the QELISA and FELISA. Only 25% (n=20) of the kidney-positive fish had detectable antigen levels in ovarian fluid as determined by the QELISA and FELISA. A sensitive western blot technique was used to confirm the presence of p57 in the ovarian fluid and kidney samples. In summary, the Field ELISA may be useful for the
identification of *R. salmoninarum* antigen in the kidney tissue of highly infected spawning salmon.
INTRODUCTION

Bacterial kidney disease (BKD) is a major obstacle to the successful culture of Pacific and Atlantic salmon (Fryer and Sanders, 1981; Albright et al., 1988). The causative agent of bacterial kidney disease is *Renibacterium salmoninarum*, a slow-growing, gram-positive bacterium (Sanders and Fryer, 1980). Currently, the means of control of BKD are chemotherapy, chemoprophylaxis, and dietary modification (Elliott et al., 1989). Antibiotic therapy has been effective in reducing mortality (DeCew, 1972; Wolf and Dunbar, 1959; Moffitt and Bjørn, 1989), however, treatment does not completely eliminate the presence of *R. salmoninarum* (Wolf and Dunbar, 1959; Austin, 1985; Brown et al., 1990). Unlike most other salmonid pathogens, *R. salmoninarum* is thought to be vertically transmitted to the eggs prior to fertilization (Evelyn et al., 1984, 1986a; Lee and Evelyn, 1989). Therefore, segregation and elimination of gametes from infected female adults may be an effective means to establish disease-free stocks (Elliott et al., 1989; Armstrong et al., 1989). The efficacy of gamete segregation appears promising. In two recent field trials, segregation resulted in a decrease in juvenile mortality and an increased adult return (Pascho et al., 1991b; Warren, 1991). The accurate and rapid identification of *R. salmoninarum* is necessary in order to segregate infected gametes and to prevent the transfer of bacterial kidney disease to locations where it is not enzootic. A number of enzyme-linked immunosorbent assays have been developed to detect *R. salmoninarum* antigens (Dixon, 1987; Pascho and Mulcahy, 1987; Turaga et
However, these assays all rely upon the use of polyclonal antisera which have inherent disadvantages including variability due to the production of different lots of antisera and the increased observed cross-reactivity with non-*R. salmoninarum* antigens (Austin et al., 1985; Fiedler and Draxl, 1986; Dixon, 1987; Yoshimizu et al., 1987; Turaga et al., 1987b). Monoclonal antibodies have been developed which are specific to the 57/58 kDa protein antigen (p57) of *R. salmoninarum* (Wiens and Kaattari, 1989). These monoclonal antibodies have been incorporated into an antigen-capture, quantitative ELISA (QELISA) which is capable of detecting antigen concentrations as low as 3 ng/ml of sample (Rockey et al., 1991a). The correlation between this QELISA and the direct fluorescent antibody test (FAT) was 99%, proving the efficacy of this detection method. While the quantitative ELISA is useful for the accurate determination of antigen levels in a large number of fish, its use in the field is limited due to the extensive sample preparation required and the need for laboratory equipment. This limited practical utility of the QELISA prompted the development of a simple and rapid Field ELISA (FELISA).

Implementation of segregation practices would benefit from a knowledge of the expected prevalence and concentrations of antigen in adult broodstock and the development of a rapid detection method which can be performed at the hatchery. The purpose of this study was three fold: 1) to establish of baseline data on the prevalence and concentrations of antigen present in adult broodstock over a three year period at one hatchery, 2) to determine the sensitivity and specificity of a prototype Field ELISA (FELISA), 3) investigate the use of ovarian fluid as an alternative to kidney tissue as an indicator of the infection status.
MATERIALS AND METHODS

Sample collection. Baseline data on the prevalence and concentration of antigen levels was determined by the quantitative ELISA (QELISA) analysis of kidney samples from 740 spawning chinook (Oncorhynchus tshawytscha). All samples were from fish returning to Marion Forks Hatchery (Marion County, OR) during a three year period from 1989 to 1991. Comparison of a newly developed Field ELISA (FELISA) with the QELISA and fluorescent antibody technique (FAT) was performed with a total of 522 kidney samples. Samples were collected from chinook at South Santiam Hatchery (Linn County, OR; n=88), Bonneville Hatchery (Multnomah County, OR; n=60), Marion Forks Hatchery (n=58) or from coho salmon (O. kisutch) returning to Fall Creek Hatchery (Lincoln County, OR; n=240) and Bandon Hatchery (Coos County, OR; n=76) during the 1990 spawning season. After salmon were spawned, kidney tissue was sampled with cotton tipped applicators (Puritan, Guilford, ME) for FAT and the FELISA analysis. Between 2 and 10 g of tissue was removed for analysis by the QELISA as previously described (Rockey et al., 1991a). Salmon returning to Bonneville, Fall Creek and Bandon hatcheries had been prophylactically injected with erythromycin while chinook returning to South Santiam hatchery had been injected with both erythromycin and oxytetracycline according to individual hatchery protocols. None of the chinook returning to Marion Forks hatchery had been injected with antibiotics.

Ovarian fluid samples were obtained from 20 QELISA kidney-positive, female chinook salmon and 20 QELISA kidney-negative female chinook from
Marion Forks Hatchery in 1991 as part of a broodstock segregation field trial which will be described elsewhere. Eggs were held overnight in zip-lock bags (Dow Brands Inc., Indianapolis, IN) until the QELISA kidney analysis was completed, at which time ovarian fluid samples were removed with a 3 ml syringe. Ovarian fluid samples were centrifuged at 500 x g to remove cellular components and assayed by the quantitative and Field ELISAs. Samples were subsequently frozen at -20°C for Western blot analysis.

**Enzyme linked immunosorbent assays (ELISAs).** The quantitative ELISA (QELISA) was performed as described by Rockey et al. (1991a). Briefly, wells of a 96-well immunoassay plate (Costar) were coated with 50 µl of purified MAb 4D3 (3.2 µg/ml in PBS). Plates were blocked with 1% bovine serum albumin (BSA; fraction V, Sigma, St. Louis, MO) diluted in 0.1% Tween-Tris-buffered saline (TBS; 50 mM Tris, 1 mM EDTA, 8.7% NaCl [pH 8.0]) for 1 h at room temperature. Dilutions of a *R. salmoninarum* extracellular protein standard (1.6-25 ng/ml), or clinical samples were added to the plate (50 µl/well) for 2 h at 17°C. Antigen was detected with biotinylated MAb 3H1 (1 µg/ml in T-TBS) for 1 h, followed by a 1:400 dilution of horseradish peroxidase-conjugated streptavidin (Sigma). Wells were washed after each step with a Titertek Microplate Washer 120 (Flow Laboratories). Substrate was prepared by mixing 200 µl of ABTS (2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid], Sigma; 10 mg/ml in water) and 5 µl of 30% H₂O₂ with 10 ml of 10 mM citrate buffer pH 4. Substrate was added to the plate for 10 min and the absorbance at 405 nm was measured on a Titertek Multiskan Plus plate reader (Flow Laboratories, McLean, VI). The concentration of p57 in samples was calculated from a linear portion of a standard curve. Standards were present on each assay plate.
A rapid and qualitative, Field ELISA (FELISA) was developed and optimized (Gilkey et al., in preparation) which could be performed on site at hatchery locations. The reagents and concentrations were the same as the QELISA, however, assays were performed in 12 x 75 mm polystyrene test tubes (VWR Scientific) in 1990, and Maxisorp NuncImmuno tubes (Nunc, Roskilde, Denmark) in 1991. Briefly, tubes were precoated with MAb 4D3 in a total volume of 500 µl overnight at 17°C, and were blocked with 1% BSA-T-TBS. Kidney tissue was collected by swabbing kidney tissue with a cotton Q-tip and immediately inserting the swab into the 500 µl of 1% BSA-T-TBS blocking solution. The Q-tip was incubated in the blocking solution for 0.5 h, removed, and tubes were washed 5 times with T-TBS. Antigen was detected with biotinylated MAb 3H1 (500 µl; 1 µg/ml in T-TBS), followed by a 1:400 dilution (500 µl) of horseradish peroxidase-conjugated streptavidin (Sigma). A final wash was performed prior to addition of 0.5 ml substrate. After 10 min tubes were visually compared to positive and negative controls. Independent confirmation was performed by 3 observers. Results are reported as positive if one or more observer identified a color change as compared to the negative control. The total assay time was approximately 3 h.

**Fluorescent antibody test (FAT).** Smears of kidney tissue were made on precleaned glass slides and dried. Slides were stained using a 1:25 dilution of anti-*R. salmoninarum* antisera (Kirkegaard and Perry, Gaithersburg, MD). The samples were scored on a 0-4+ scale currently used by the Oregon Department of Fish and Wildlife (Bullock et al., 1980). In these analyses 1+ equaled one bacteria to 10 bacteria per field at 400x magnification, 2+ equaled between 10-100 per field, 3+ equaled 100-1000, and 4+ equaled greater than 1000 per field. One to two hundred fields were observed on each slide.
Western blot analysis of ovarian fluid and kidney samples.

Conditions for pouring, running, and transblotting gels have been previously described (Wiens et al., 1990). Briefly, samples were diluted 1:1 (v:v) with sample buffer, boiled for 3 min, and insoluble precipitates were removed by centrifugation at 15,000 x g for 0.5 min. A total volume of 7 μl of sample was electrophoresed at 100V on 10% SDS-polyacrylamide gels. Proteins were electroblotted onto PVDF membranes (Millipore Corp, Bedford, MA) for 1 h with 100V, and nonspecific sites were blocked with 5% nonfat dry milk-T-TBS for 1 h at 37°C. Blots were washed for 15 min and probed with a combination of 1 μg/ml in 1% BSA-T-TBS of 3 MAbs 4D3, 3H1, and 1A1 whose specificities have been previously described (Wiens and Kaattari, 1991). Excess primary antibody was removed by rapidly washing twice with T-TBS, followed by one wash for 15 min and one for 5 min. A secondary sheep anti-mouse HRPO conjugate (Amersham Corp., Arlington Heights, IL) was diluted 1:1500 in 1% BSA-T-TBS and used to probe the blots. The secondary antisera was preabsorbed with 0.08% ELISA-negative chinook kidney tissue supernatant to reduce background staining. After 45 min, blots were washed followed by a final rinse in TBS. Enhanced chemiluminescent substrate was applied and ECL-Hyperfilm was exposed for 1-10 min, according to the manufacture's directions (Amersham Corp.). Blots which were to be exposed for longer than 10 min were washed for an additional 1 h to reduce background luminescence. The sensitivity of the assay was established using dilutions of *R. salmoninarum* extracellular protein standard in 0.1% BSA-T-TBS.
RESULTS

Prevalence of spring chinook salmon with detectable antigen determined by the quantitative ELISA. The concentration of *R. salmoninarum* extracellular antigen (p57) in the kidney tissue of 740 spawning adult chinook returning to Marion Forks Hatchery was determined by the QELISA. The majority of chinook sampled (> 60%) had p57 levels below the previously established baseline cut-off of 3 ng/ml. The cumulative incidence of all fish sampled in 1989, 1990 and 1991 with antigen levels above 3 ng/ml was 24.2% (n=740). Variation in the percentage of fish with detectable antigen concentrations was observed when the data was analyzed on a year by year basis (Table 6.1). Chinook sampled in 1989 had a 12.8% prevalence, the lowest, while fish sampled in 1991 had a 36.6% prevalence, the highest. The variation was not evenly distributed over antigen levels. The percentage of fish with p57 concentrations between 3-10 ng/ml increased almost four fold from 5.9% in 1989 to 21.4% in 1991, while there was a two fold increase in the percentage of salmon with antigen concentrations above 10 ng/ml from 7.9% in 1989 to 15.2% in 1991. The cumulative prevalence of female fish with antigen levels above 3 ng/ml for female chinook was 21.8% (n=398), while for male chinook it was 24.8%, n=342.

Comparison of a Field ELISA with the QELISA and FAT. A Field ELISA was developed for rapid screening of fish at spawning facilities. A total of 522 kidney samples were tested by the FELISA and results were compared to the QELISA and FAT. Ten fish were identified as having a FAT score of 2+ or greater, all were positive by the Field ELISA (Table 6.2 A). The agreement between the FELISA the FAT was 94.2%. Disagreement with the
Table 6.1. Percentage of adult spring chinook salmon sampled at Marion Forks hatchery in 1989, 1990, and 1991 with antigen levels above 3 ng/ml. Antigen levels were determined by the QELISA.

<table>
<thead>
<tr>
<th>Antigen (ng/ml)</th>
<th>1989 (n=265)</th>
<th>1990 (n=237)</th>
<th>1991 (n=238)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3</td>
<td>87.2</td>
<td>78.5</td>
<td>63.4</td>
</tr>
<tr>
<td>3-9.9</td>
<td>5.9</td>
<td>11.9</td>
<td>21.4</td>
</tr>
<tr>
<td>10-99.9</td>
<td>1.9</td>
<td>3.0</td>
<td>5.5</td>
</tr>
<tr>
<td>100-999.9</td>
<td>2.3</td>
<td>3.0</td>
<td>4.6</td>
</tr>
<tr>
<td>1000-9999.9</td>
<td>2.6</td>
<td>2.1</td>
<td>1.7</td>
</tr>
<tr>
<td>&gt;10,000</td>
<td>1.1</td>
<td>1.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Total &gt; 3</td>
<td>12.8</td>
<td>21.7</td>
<td>36.6</td>
</tr>
</tbody>
</table>
Table 6.2. Comparison of the FELISA with the QELISA and the FAT. A. Comparison of FELISA to the FAT, B. Comparison of FELISA to the QELISA.

Numbers indicate adult fish sampled.

A.

<table>
<thead>
<tr>
<th>FELISA</th>
<th>FAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>475</td>
</tr>
<tr>
<td>+</td>
<td>10</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>FELISA</th>
<th>QELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;3</td>
</tr>
<tr>
<td>-</td>
<td>448</td>
</tr>
<tr>
<td>+</td>
<td>7</td>
</tr>
</tbody>
</table>
FELISA and FAT occurred at low FAT levels of 1+. Only 25% of the 1+ fish were identified by the FELISA. The percent agreement between the FELISA and the QELISA was 89.7% (Table 6.2 B). The FELISA was generally unable to identify kidney tissue with antigen levels less than 20 ng/ml. There was a high correlation of FELISA with the QELISA when p57 levels were above 20 ng/ml with the exception of one fish which had a mean antigen concentration of 115 ng/ml. Seven fish were identified as being presumably false positive by the FELISA as these salmon did not have detectable antigen levels as determined by the QELISA.

**Correlation of p57 levels in kidney tissue with those of ovarian fluid.** The levels of antigen in ovarian fluid from 20 kidney-positive and negative chinook were determined. Antigen levels in the kidney were 20 to 1500 times higher than levels in the ovarian fluid from the same fish (Table 6.3). Seventy-five percent (n=20) of the fish with antigen levels above 3 ng/ml in the kidney had antigen levels below 3 ng/ml in the ovarian fluid. The FELISA was able to identify all four fish which had ovarian fluid antigen concentrations above 20 ng/ml and 11 out of 12 fish with kidney antigen levels above 20 ng/ml. None of the twenty ELISA kidney-negative fish had antigen levels in ovarian fluid above 3 ng/ml as determined by the QELISA (data not shown).

The low levels of antigen present in the ovarian fluid prompted investigation of the possibility that p57 may be masked or partially degraded. To verify the presence of intact p57 in ovarian fluid, a sensitive western blot technique was developed using a substrate which produces a chemiluminescent product upon cleavage with the enzyme, horse-radish peroxidase. Diluted p57 could be visualized down to concentrations between 6.25 and 3.12 ng/ml, or approximately 20 to 10 pg/lane (Figure 6.1, lanes 7-8).
Table 6.3. Comparison of p57 concentrations in kidney tissue and ovarian fluids from spawning chinook salmon returning to Marion Forks Hatchery. Kidney tissue was collected and antigen levels determined by the QELISA. Based on the QELISA results, ovarian fluid samples were taken the following day and assayed by the field and quantitative ELISA. The Western blot was used for the confirmation of antigen levels in samples.

<table>
<thead>
<tr>
<th>Fish #</th>
<th>QELISA (ng/ml ± SEM)</th>
<th>Field ELISA +</th>
<th>Western Blot +</th>
<th>QELISA (ng/ml ± SEM)</th>
<th>Field ELISA +</th>
<th>Western Blot +</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>120,350 ± 28,043</td>
<td>+</td>
<td>+</td>
<td>88 ± 22</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>27</td>
<td>13,938 ± 1,750</td>
<td>+</td>
<td>+</td>
<td>10 ± 2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>47</td>
<td>13,215 ± 1,703</td>
<td>+</td>
<td>+</td>
<td>593 ± 177</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>10,123 ± 859</td>
<td>+</td>
<td>+</td>
<td>&lt;3&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>170</td>
<td>4,484 ± 737</td>
<td>+</td>
<td>+</td>
<td>150 ± 28</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>79</td>
<td>4,102 ± 908</td>
<td>+</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>99</td>
<td>1,287 ± 128</td>
<td>n.t.&lt;sup&gt;1&lt;/sup&gt;</td>
<td>+</td>
<td>32 ± 13</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>106</td>
<td>635 ± 88</td>
<td>n.t.</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>80</td>
<td>227 ± 64</td>
<td>+</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>117</td>
<td>187 ± 16</td>
<td>n.t.</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>114</td>
<td>63 ± 9</td>
<td>n.t.</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>60 ± 6</td>
<td>+</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>91</td>
<td>53 ± 8</td>
<td>+</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>144</td>
<td>51 ± 10</td>
<td>+</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>97</td>
<td>46 ± 7</td>
<td>n.t.</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>142</td>
<td>31 ± 8</td>
<td>+</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>23 ± 6</td>
<td>-</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>19 ± 2</td>
<td>-</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>141</td>
<td>13 ± 1</td>
<td>n.t.</td>
<td>+</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>70</td>
<td>4 ± 1</td>
<td>-</td>
<td>-</td>
<td>&lt;3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>1</sup>n.t. = not tested  
<sup>2</sup><3 = antigen level was below the sensitivity of the QELISA.
This represents approximately a 50-100 fold increase in the detection level as compared to the conventional peroxidase substrate, 4-chloro-1-naphthol.

Analysis of ovarian fluid samples revealed a strongly immunoreactive band in fish samples with concentrations of 593 ng/ml, 150 ng/ml, and 88 ng/ml (Figure 6.2, lanes 2,3,4) at the same molecular weight as the p57 standard (Figure 6.2, lane 1). Weak bands were detected in samples from ovarian fluid of fish # 99 with 32 ng/ml and from fish #27 with a concentration of 10 ng/ml (Figure 6.2, lanes 5 and 6). No degradation was apparent by Western blot, nor was any antigen detected in ovarian fluid samples which were QELISA negative (Figure 6.2, lane 7; and Table 6.3). Western blot analysis was also able to confirm the presence of p57 in 19 of the 20 QELISA kidney positive samples.
Figure 6.1. Determination of the sensitivity of the Western blot using a chemiluminescent developing reagent. Serial dilutions of ECP were made in 0.05% bovine serum albumin T-TBS. The blot was probed with a combination of 1 μg/ml of MAbs 4D3, 3H1 and 1A1. Lanes: 1) 500 ng/ml; 2) 250 ng/ml, 3) 125 ng/ml 4) 50 ng/ml, 5) 25 ng/ml, 6) 12.5 ng/ml, 7) 6.25 ng/ml, 8) 3.12 ng/ml, 9) diluent alone. Film was exposed for 10 min.

Figure 6.2. Western blot of ovarian fluid samples. Ovarian fluid was mixed 1:1 with sample buffer and 7 μl/lane was loaded. The blot was probed with a combination of 1 μg/ml MAbs 4D3, 3H1 and 1A1. Lanes: 1) 50 ng/ml standard, 2) Fish #47, 593 ng/ml, 3) Fish #170, 150 ng/ml, 4) Fish #44, 88 ng/ml, 5) Fish #99, 32 ng/ml, 6) Fish #27, 10 ng/ml, 7) Fish #12, < 3 ng/ml. Film was exposed for 30 min.
DISCUSSION

Spring chinook salmon are considered to be especially sensitive to bacterial kidney disease (Bullock and Wolf, 1986; Warren, 1991), and the prevalence of BKD among these stocks may contribute to the low percentage of returning adults to the Columbia river system (Raymond, 1988). The lack of an efficacious vaccine (Kaattari et al., 1988; Munro and Bruno, 1988), and the possibility of antibiotic resistance (Bell et al., 1988) suggest that novel strategies are needed for the control of this disease. Precise knowledge of antigen levels would facilitate the implementation of disease control strategies such as the segregation of infected gametes. Accurate determination of the incidence of *R. salmoninarum* infection has been difficult as most fish may be infected with subclinical levels (Fryer and Sanders, 1981; Pascho et al., 1987b). Previously, we have found that the quantitation of a soluble protein antigen, of which the main component is a 57/58 kDa protein (p57), is a good indicator of the magnitude of infection by *R. salmoninarum* (Turaga et al., 1987b; Rockey et al., 1991a). We have used a quantitative monoclonal antibody-capture ELISA (QELISA) to assay adult spawning spring chinook salmon over a three year period returning to one Hatchery in Oregon. The QELISA was also used to verify the results of a newly developed Field ELISA which was designed for use by hatchery personnel or pathologists in the field and can be completed without laboratory ELISA equipment.

Previous estimates of the prevalence and levels of antigen in chinook by the monoclonal-based ELISA varied with the stock tested but ranged from 5 to 25% (Rockey et al., 1991a). In this study, baseline data on the year to year
variation was obtained from uninjected chinook returning to Marion Forks Hatchery. The prevalence of antigen-positive adult chinook salmon varied from 12.8% in 1989 to 36.6% in 1991. Sampling of this same stock by the FAT revealed a similar variation in the prevalence of 26.7% (n=60) in 1988 to 8.3% (n=60) in 1989 (A. Amandi, Oregon Department of Fish and Wildlife, unpublished data). Pascho et al. (1991b) have reported that the prevalence of erythromycin-injected antigen-positive chinook at Dworshak National Fish Hatchery was 37% (n=302) in female fish, and 21% (n=271) in male fish (Pascho et al., 1991b). Since the majority of chinook tested (>60%) have kidney antigen levels below detectable limits, segregation of spring chinook gametes may only require a removal of 13-37% of the gametes. The efficacy of segregation for the control of bacterial kidney disease has recently been demonstrated at two hatcheries (Pascho et al., 1991b; Warren, 1991).

Segregation of large numbers of adult salmon gametes would benefit from a rapid assay which can be performed at the hatchery, optimally without the use of laboratory ELISA equipment. A rapid staphylococcal coagglutination assay has been previously developed by Kimura and Yoshimizu (1981), however, the test requires homogenization and heating of kidney samples. The prototype Field ELISA described only required the kidney to be swabbed as is performed for the FAT. The FELISA was able to identify subclinically infected fish which had antigen levels above 20 ng/ml. The FELISA had a 100% correlation with fluorescent antibody technique values equal to or greater than 2+. A possible limitation of the assay was the low sensitivity compared to the QELISA or the FAT.

The level of sensitivity which is needed to detect *R. salmoninarum* and to effectively reduce vertical transmission is unknown. Experimental infection of chinook salmon eggs has been accomplished by egg exposure to $1.7 \times 10^5$
*R. salmoninarum* cells/ml ovarian fluid (Lee and Evelyn, 1989). Smolts exposed as eggs to $1.7 \times 10^3$ *R. salmoninarum* cells/ml were found to have a 8 to 10% infection rate as determined by the FAT. Lee and Evelyn (1989) have reported a 1-2% infection rate in three lots of smolts which were raised from the eggs of females which had natural levels of *R. salmoninarum* between 28 and 113 bacteria/ml of ovarian fluid. Infection was not present in two lots of smolts which were obtained from FAT-negative females. While low levels of *R. salmoninarum* may result in vertical transmission, recent results by Pascho et al. (1991b) suggest that complete elimination of adults with low levels of *R. salmoninarum* may not be necessary to significantly reduce the mortality of hatchery-reared spring chinook salmon due to bacterial kidney disease.

A number of investigators have advocated caution in the interpretation of the prevalence of *R. salmoninarum* using immunodiagnostic methods such as the FAT due to potential cross-reactivity problems (Fryer and Sanders, 1981, Evelyn et al., 1981). Confirmation of infection is ideally performed by the culture of *R. salmoninarum* (Fryer and Sanders, 1981). Due to the lengthy incubation time and the technical difficulty of avoiding contamination (Armstrong et al., 1989) other methods of confirmation are desirable. Western blotting is an ideal technique for the confirmation of p57 in ELISA positive samples as both the molecular weight and antigenic identity can be determined (Sakai et al., 1990; Wiens et al., 1990). A previous limitation of the Western blot was the lack of sensitivity (Rockey et al., 1991a). Using a substrate which can be cleaved to produce a chemiluminescent product, the sensitivity was increased 50-100x over the conventional peroxidase substrate. The presence of p57 in samples was confirmed as low as 13 ng/ml in kidney tissue and 10 ng/ml in ovarian fluid. Difficulty still exists with low level detection due to the lengthy exposure times required and the subsequent
increase in background luminescence. Further effort will be targeted to developing a western blot protocol which is as sensitive as the QELISA.

The antigen levels in ovarian fluid from QELISA-positive females was quantified to determine if ovarian fluid might serve as an alternate sampling source for the detection of bacterial kidney disease. Low levels of p57 were observed in ovarian fluid samples as compared to kidney tissues from the same fish. Inconsistencies have also been reported by Pascho et al. (1991b) who were unable to detect antigen in ovarian fluid samples with bacterial counts below $1 \times 10^5$/ml. The authors speculate that undefined ovarian fluid factors may influence the detection of antigen. Armstrong et al. (1989) have reported the presence of agglutinin titers to heat killed *R. salmoninarum* as high as 1:36 in chinook salmon ovarian fluid. In a preliminary report, Griffiths and Lynch (1990) identified *R. salmoninarum* reactive antibodies in the ovarian fluid. Assuming that the low level of antigen was not due to an artefact in the collection process, one possible explanation might be that the antigen was being masked by ovarian fluid components. Western blotting was used in an attempt to identify antigen as it was assumed that the denaturing and heating treatment of the samples prior to electrophoresis would liberate any antigen non-covalently complexed to antibody or any other protein. Western blot analysis confirmed that QELISA-positive ovarian fluid samples contained p57, however, p57 was not detected in QELISA-negative ovarian fluid samples even though fish had kidney antigen concentrations greater than 10,000 ng/ml. A second possible explanation for the low quantities of p57 observed in the ovarian fluid may be the proteolysis of antigen. Little degradation of p57 was observed in ovarian fluid or kidney samples confirming the earlier findings of Rockey et al., (1991a). A third possibility may be that p57 was bound by components present on the surface of the egg.
Little is known about the function of p57 in vivo or the tissue binding capacity of p57. P57 has been shown to bind fish leukocytes (Wiens and Kaattari, 1991) and spermatocytes in vitro (Daly and Stevenson, 1989). A final explanation may be that the synthesis of p57 may be down-regulated by the bacteria after contact with egg components. Regardless, these experiments suggest that the detection of antigen in ovarian fluid may be of a limited utility for the diagnosis of *R. salmoninarum* infection. Further analysis of the function and the genetic regulation of p57 expression is required to understand the host-pathogen interactions and will be useful for the development of enhanced techniques for the detection of *R. salmoninarum*. 


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APPENDICES
APPENDIX A

Bacterial Kidney Disease: The Potential Role of Soluble Protein Antigen(s)

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ABSTRACT

Soluble protein antigens, isolated from the supernatants of *Renibacterium salmoninarum* cultures, have been found to suppress the *in vitro* antibody responses of coho salmon, *Oncorhynchus kisutch*, lymphocytes and to be associated with decreasing hematocrit values *in vivo*.

INTRODUCTION

*Renibacterium salmoninarum* is responsible for one of the most important salmonid bacterial diseases in the world (Fryer and Sanders, 1981), bacterial kidney disease (BKD). Of major epizootiologic importance is the fact that this disease is not only a problem in hatchery-reared salmonids, but also in wild populations (Mitchum et al., 1979). Unfortunately, little information has been forthcoming as to the possible molecular mechanisms of the pathogenesis of BKD. This study, therefore, focuses on the possible toxigenic role(s) that *R. salmoninarum* soluble proteins (SP) may have in BKD pathology.
MATERIALS AND METHODS

*Renibacterium salmoninarum* SP were prepared as described by Getchell et al. (1985). The effects of SP on the *in vitro* antibody responses of normal and infected coho salmon to trinitrophenylated-lipopolysaccharide (TNP-LPS; Jacobs and Morrison, 1975) were examined using *in vitro* tissue culture medium (TCM) and techniques (Kaattari and Yui, 1987). Prior to culture addition, SP was diluted in TCM and filter sterilized (0.45 μm). Hematocrit values were assessed using heparinized blood samples taken from 5 fish at 10-day intervals after intraperitoneal injection of 0.1 ml of 1 O.D. (500 nm) live *R. salmoninarum*. The serum portions of these samples were analyzed for the concentration of SP by use of an ELISA procedure (Turaga et al., 1987b).

RESULTS

*In vitro* cultures of anterior kidney lymphocytes, stimulated with an optimal concentration of TNP-LPS, were suppressed by 10 and 100 μg/ml SP (Figure A.1). Comparable concentrations of the control protein, chicken ovalbumin, were not suppressive. This suppression was not due to a toxic effect, since control and suppressed cutures expressed equivalent cellular viability as assessed by trypan blue exclusion staining.

Anterior kidney lymphocytes from normal and infected fish were cultured with TNP-LPS (Figure A.2). Cultures of lymphocytes from infected fish
Figure A.1. Plaque forming cell response of coho salmon anterior kidney lymphocytes cultured with TNP-LPS in the presence of 1, 10, 100 μg/ml SP, or ovalbumin (OVA). The control response (without protein addition) was equal to 431 ± 71 antibody-producing cells per 10^6 lymphocytes. Each point represents the mean of triplicate cultures and bars ± 1 S.E.
Figure A.2. Plaque forming cell response of coho salmon anterior kidney lymphocytes from normal or infected salmon cultured with 0.04, 0.4, and 4.0 µg ml\(^{-1}\) of TNP-LPS, or tissue culture medium alone (TCM). A portion of the normal lymphocytes were also cultured with 100 µg ml\(^{-1}\) SP (a concentration comparable to that seen in infected fish). Each histogram represents the mean of triplicate culture and ± 1 S.E. are indicated.
(possessing 3-80 µg/ml serum SP) demonstrated a marked suppression as compared to lymphocytes from normal fish. This suppression was comparable to the suppression seen when normal lymphocytes are co-cultured with 100 µg/ml SP.

Examination of experimentally infected salmon at various times post-injection revealed a distinct association of decreasing hematocrit with increasing levels of SP in the serum (Figure A.3).

**DISCUSSION**

These studies demonstrated that extracellular soluble proteins produced by *R. salmoninarum* are capable of suppressing the *in vitro* antibody response, and are associated *in vivo* with decreasing hematocrit values.

Studies concerning the mechanisms of pathogenesis for *R. salmoninarum* have primarily been limited to the analysis of the histopathology (Wood and Yasutake, 1956; Hendricks and Leek, 1975; Lester and Budd, 1979; Young and Chapman, 1978) and the appearance of abnormal clinical indices (Hunn, 1964). Although the initial focus of the infection appears to be the kidney (Wood and Yasutake, 1956) with subsequent hematological dysfunction (Hunn, 1964), the disease eventually becomes systemic, with lesions occurring in many organs and tissues.

The identification of *R. salmoninarum* toxins has not been forthcoming, except for the detection of an hemolysin-like activity found in formalinized cells (Bruno and Munro, 1986a). Those authors have suggested that a putative toxin may be responsible for the decreases in hematocrit values and for
Figure A.3. Hematocrit values, expressed as percentage packed red cell volume, versus the corresponding SP serum concentration for individuals sampled at various stages of infections.
indices of splenomegaly associated with the disease. Our observations of increasing serum SP levels associated with decreasing hematocrits lend support for that pathogenic mechanism.

The *in vitro* antibody assay revealed that a non-cytotoxic antigen(s) was capable of suppressing the production of the antibody response. Of particular interest was the observation in the present study of a decrease in the number of adherent (e.g. macrophage) cells upon culture with SP. Recent studies with catfish (Miller et al., 1985) and with coho salmon lymphocytes (Tripp and Kaattari, in prep.) reveal that antibody responses to TNP-LPS require adherent cell function. Since antibody responses from lymphocytes from infected fish appear suppressed (Figure A.2), it may be possible that immune dysfunction *in vivo* could be mediated by the elaboration of these antigens. Further studies, however, will be required to determine the role of alternative *in vivo* mechanisms (e.g. bacterial destruction of tissues, or contamination due to live *R. salmoninarum*) involved in the reduction of antibody-producing cells from infected fish.
APPENDIX B

Western Blot Analysis of a Fish Pathogen

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INTRODUCTION

Western blotting is a widely used procedure by which a mixture of molecules can be simultaneously characterized electrophoretically and antigenically. This is accomplished by coupling the technique of electrophoresis with that of the enzyme-linked immunoassay. Initially, a sample is subjected to electrophoresis for separation of antigens according to their charge and size, or size alone. A second electrophoretic step is conducted to transfer the antigens from the gel to an immobilizing surface, such as nitrocellulose paper as originally described by Towbin et al. (1983). After this transfer the antigen components can easily be probed with a specific enzyme-conjugated antibody. A chromogenic substrate is then added to determine which electrophoretic band is bound by the antibody. Western blotting is useful for a number of purposes including characterization of unknown antigens or antibody specificities (Campbell, 1984), confirmation of the presence of bacterial antigens in sera or tissues (Turaga et al., 1987a; Wiens and Kaattari, 1989), and detection of seropositive individuals which have been exposed to a pathogen (Sarngandhan et al., 1984). While protein antigens are typically analysed in Western blots, a number of other macromolecules can be separated and transferred to nitrocellulose including glycoproteins, lipoproteins, glycolipids and lipopolysaccharides (Bers and Garfin, 1985). The primary advantage of Western blotting, as opposed to other immunoassays, is the high degree of specificity in resolving distinct antigens. Clinically, this technique has been useful in the identification of false positives, when used in conjunction with other immunoassays such as an ELISA (Weiss et al., 1985). There are, however, two disadvantages associated with Western
blot analysis. First, the Western blot is mainly a qualitative assay and quantification of antibody or antigen is difficult (Bjerrum and Heegaard, 1988b). Second, if the antigen sample must be denatured (such as in SDS-PAGE), antigenic activity may be reduced or destroyed (Bers and Garfin, 1985; Dunbar, 1987).

As the investigator begins to develop a Western blot protocol for detection of a new antigen, a number of parameters will require optimization. An initial consideration for successful Western blotting should be the determination of the optimal electrophoretic parameters for resolution of the antigens of interest. This primarily requires the identification of the percentage of acrylamide that yields optimal separation of the electrophoretic bands. Next, it must be empirically determined whether the antigens are capable of binding to the nitrocellulose. Finally, the antigen detection procedure must be highly specific and sensitive (Oberfelder, 1989). For more detailed information a number of reviews on the theoretical and technical aspects of polyacrylamide gel electrophoresis (Hames et al., 1981; Dunbar, 1987), and Western blotting (Gershoni and Palade, 1983; Bers and Garfin, 1985; Oberfelder, 1989; Bjerrum and Heegaard, 1988a and b) have been published.

In the following protocol, a specific procedure is described which has been used to detect the presence of a 57 kd protein produced by *Renibacterium salmoninarum* (a gram-positive salmonid fish pathogen) during an infection. Additionally, Western blotting has been useful for characterizing the specificities of polyclonal antisera (rabbit and salmonid) and murine monoclonal antibodies to extracellular and cell surface antigens of *R. salmoninarum*. 
MATERIALS

Equipment

1. Electrophoretic gel apparatus (Mini Protean System, Bio-Rad)
2. Power supply (EC 600, E-C Apparatus Corporation)
3. Microliter syringes, 50 μl capacity (Hamilton Company)
4. Shaker
5. Water bath
6. 37°C incubator
7. Gloves
8. Disposable, polystyrene petri dishes, 100 x 15 mm style

Reagents and Stock Solutions

1. Antigen: Soluble protein (SP) from concentrated culture supernatant of *Renibacterium salmoninarum* (Turaga et al., 1987b)
2. Antibodies:
   a) Monoclonal antibody 4D3 (Wiens and Kaattari, 1989)
   b) Goat anti-mouse antibody (polyvalent Igs) peroxidase labeled (Hyclone)
   c) Biotinylated rabbit anti-SP antibody (Turaga et al., 1987b)
4. Aurodye protein staining kit (Jansen Chemicals)
5. Substrate, 4-chloro-1-naphthol (Bio-Rad).
6. Nanopure water
7. Pre-stained molecular weight markers (Sigma).
8. Whatman #1 filter paper.
9. Hydrogen peroxide 30%.
10. Sample Dye (2) Stock
    0.15 gm Tris Base
    0.4 gm Sodium Dodecyl Sulfate
    1.0 ml 2-Mercaptoethanol
    2.0 ml Glycerol
    7.0 ml Nanopure water
    0.02 gm Bromophenol Blue
11. Gel Buffer, pH 8.1-8.4
   14.4 gm Glycine
   3.03 gm Tris Base
   1.0 gm SDS
   Bring to one liter with nanopure water.
   Do not adjust the pH with acid or base.

12. Transblot Buffer, pH 8.1-8.4
   14.4 gm Glycine
   3.03 gm Tris Base
   200.0 ml Methanol
   Bring to one liter with nanopure water.
   Do not adjust the pH with acid or base.

13. Phosphate Buffered Saline 10 mM, pH 7.2
   8.0 gm NaCl
   0.2 gm KCl
   1.44 gm Na$_2$HPO$_4$ 2H$_2$O
   0.2 gm KH$_2$PO$_4$
   Bring to one liter with nanopure water.

14. Stock Substrate Solution
   0.15 gm 4-cloro-1-naphthol
   50.0 ml Methanol
   Store in the dark at -20°C

15. Water Saturated Isobutanol
   30.0 ml dd H$_2$O
   10.0 ml Isobutanol

16. Tris Buffered Saline (TBS), pH 8.0
   6.07 gm Tris base
   0.41 gm Disodium EDTA (Fw 372)
   8.7 gm NaCl
   Bring to 1 liter with ddH$_2$O.

17. Tween - Tris Buffered Saline (TTBS), pH 8.0
   1.0 ml Polyoxyethylene Sorbitan Monolaurate (Tween-20)
   Bring to 1 liter with TBS.

18. Separating gel recipe for a 12% gel (Two mini-gels, modified from Laemeli, 1970)
   4.6 ml Nanopure H$_2$O
   1.25 ml 3 M Tris HCl pH 8.8
   4.0 ml 30% Acrylamide
   0.8% Bisacrylamide, deaerate 10 min.
   0.1 ml 10% SDS in ddH$_2$O
   0.05 ml Ammonium persulfate (AMPS), 0.1 gm/ml in ddH$_2$O
(made just prior to use)
0.005 ml N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)

METHODS

The following Western blot protocol is designed for use with the Mini-
Protean II electrophoresis system supplied by Bio-Rad. The gel assembly,
quantity of acrylamide used per gel, power settings, time of electrophoresis,
and conditions for transblotting may vary if equipment from other
manufacturers is used.

1. Assemble the gel holder by sandwiching two spacers between the outer
definition of two pre-cleaned glass plates (102 x 83 cm glass plates and 0.75 mm
spacers). Tightly clamp the plates together along the edges so that no leaks
are possible. It is important to wear gloves at all times to prevent protein
contamination of the glass plates, and contact with acrylamide, which is a
potent neurotoxin.

2. Prepare the separating gel mixture and deaerate under a vacuum for 10
minutes. Insert a well forming comb in between the glass plates, and using a
marker pen, make a mark 1 cm below the bottom teeth of the fully inserted
comb. Remove the comb. Add the SDS, AMPS and TEMED to the gel
mixture, gently swirl, and use a pasteur pipette to quickly fill the gel holder to
the premade mark. Pour the solution along one of the side spacers with a constant flow so as not to introduce bubbles into the gel. Approximately 200 ml of water saturated isobutanol should then be slowly layered on top of the liquid gel. The isobutanol serves to isolate the gel from atmospheric oxygen which impedes polymerization, and also eliminates irregularities on the surface of the gel. Allow the gel to polymerize for 1 h before rinsing off the isobutanol with nanopure water. At this point the gel can be stored for up to two weeks if it is kept refrigerated (4°C) in a moist, airtight chamber.

3. Prepare stacking gel and deaerate for 10 minutes. Set the well-forming comb into the top portion of the glass sandwich and pour the stacking gel solution until the teeth of the comb are covered. Allow polymerization to occur for 30-40 min.

4. Insert the gel sandwich into the gel apparatus according to the manufacturer's suggestions. Add sufficient gel buffer to the upper and lower chambers to submerge the top and bottom edges of the gel to a depth of approximately 1 cm. Remove the well forming comb and use a Pasture pipette to gently rinse out each well.

5. Mix the samples 1:1 with the 2X sample buffer and heat in a boiling water bath for 2-3 minutes. Typically, a total of 0.5 to 5 mg of protein in a total of 5 ml can be loaded into a 0.5 wide by 1 cm deep well. Samples are loaded most accurately with a microliter syringe. The syringe should be rinsed with gel buffer at least ten times before a different sample is loaded. Introduce pre-stained molecular weight markers into wells at either end of the gel for subsequent comparison with test samples. These markers are also useful for monitoring the progress of electrophoresis and the efficiency of transblotting.

6. Electrophorese with a constant voltage (100 V), until the dye front reaches the bottom edge of the gel (approx. 1.5 h).
7. After electrophoresis, disconnect the power supply, and remove the gel sandwich. Gently pry the glass plates apart by twisting on of the gel spacers. The gel should stick to one of the glass plates.

8. Carefully slide the gel off the glass plate and into transfer buffer and incubate approximately 10 min. Equilibrate the nitrocellulose paper, fiber pads, and filter papers in the transfer buffer at this time. The nitrocellulose paper should be slowly wetted from one corner to prevent air entrapment.

9. Partially submerge the gel holder cassette in transblot buffer and layer the blotting materials in the order shown in Figure B.1. First, lay one presoaked fiber pad and at least three filter papers onto the cassette which will be closest to the cathode (- or black lead). Remove trapped air bubbles after each step by rolling a pastuer pipette or a test tube over each layer. If the air bubbles are not completely removed the electrophoretic transfer will not be uniform. Next, carefully lay the equilibrated gel on the filter paper. Place a piece of nitrocellulose paper (onto which the protein bands will be transferred) on top of the gel, making sure that there is even contact throughout. Place another set of filter papers on top of the nitrocellulose, followed by the other fiber pad, to complete the sandwich. Clamp the cassette together.

10. Place the cassette into the transblot module with the gel side oriented toward the cathode (- or black lead).

11. Fill the chamber with the transblot buffer, connect the leads to the power supply and select an appropriate voltage for the length of the transfer. Either 100 volts for 1.5 h, or 30 volts for 14 h are suggested. Both operations should be performed in a cold room with constant buffer mixing using a stir bar and stir plate. If the transfer is conducted at 100 volts, the chamber should also be cooled with an additional cooling unit (usually supplied with the apparatus).
Figure B.1. Organization of materials inside of the gel cassette holder for transfer of proteins from the gel to nitrocellulose paper.
12. After the transfer, turn off the power supply and remove the cassette from the buffer chamber. Tweezers are used to remove the nitrocellulose paper and place it in an appropriate blocking solution.

**Immunostaining of the blot**

1. Block the remaining free protein binding sites on the nitrocellulose (NC) paper with a 1% BSA-TTBS solution at 37°C for 1 h.
2. It is advised to also process the gel for protein staining (Coomassie blue, silver staining etc.) to assess whether the complete transfer of the proteins has occurred.
3. After blocking, wash the NC paper with three, 5 min TTBS changes while continually shaking, on a reciprocating shaker.
4. Place the NC paper in the primary antibody (either the described monoclonal antibody, or rabbit anti-SP biotinylated conjugate) solution (about 5 µg/ml in T-TBS). The blot should be completely covered with the solution and at no point should it be allowed to dry. Incubate for 1 h at room temperature, with gentle shaking.
5. Repeat step 3.
6. Add a sufficient volume of the secondary antibody reagent (goat anti-mouse peroxidase labeled and diluted 1:500 in T-TBS for the monoclonal antibody system, or streptavidin-peroxidase conjugate at a 1:200 dilution in T-TBS for the biotinylated rabbit anti-SP system) at room temperature with gentle shaking. Incubate the goat anti-mouse for 1 h and the streptavidin-peroxidase for 0.5 h.
7. Repeat step 3. Rinse two times for 1 min with TBS alone to remove the tween.
8. Prepare the substrate solution immediately prior to the addition to the NC paper. Mix 2 mls of 4-chloro-1-naphthol stock substrate solution with 10 mls of
PBS and 10 μl of hydrogen peroxide. Add the substrate to the NC paper and incubate at 25-37°C until dark purple bands appear. Stop the development of the blot by rinsing the NC paper in distilled water for a minimum of 5 min. Blot the NC paper dry and store in the dark. If 4-chloro-1-naphthol is used the blot will begin to fade after 3 weeks. The blot should be photographed soon after development if a precise record is required for later analysis or publication.

**Protein staining of the blot**

1. Block the NC paper by incubating in PBS, containing 0.3% tween-20, at 37°C for 1.0 h. The blocking solution used in the immunostaining cannot be used here, since it contains the protein BSA.

2. Wash the NC paper with three, 10 min. rinses with the same blocking buffer while continuously shaking.

3. Rinse the NC paper with nanopure water for 1 min, and add approximately 10 ml of Aurodye staining reagent. Shake for approximately 3 h and dark red bands should appear. There is no over-staining problem associated with this reagent.

**DISCUSSION**

This protocol describes the technical aspects of performing a Western blot. Figure B.2a shows a Western blot which was developed with a mouse monoclonal antibody (4D3) and a goat anti-mouse horseradish peroxidase second antibody. The monoclonal antibody recognizes a 57/58 kd protein present in concentrated culture supernatant (lane 1) and a number of breakdown products. The 57/58 kDa protein is also associated with the
Figure B.2. An example of a Western blot (A) and a total protein stain (B) of two replica 12% SDS polyacrylamide gels. The nitrocellulose in A was probed with an anti *R. salmoninarum* monoclonal antibody and a goat anti-mouse horseradish peroxidase linked second antibody. The nitrocellulose in B was stained for total protein with colloidal gold. Lanes are as follows, (Mw) Sigma pre-stained molecular weight markers, 1) *R. salmoninarum* soluble protein, 2) Double distilled water wash of *R. salmoninarum* cells, 3) *R. salmoninarum* whole cells, 4) Coho salmon sera from three experimentally infected fish, 5) Control coho salmon sera from three fish, (Mw) Bio-Rad low molecular weight markers.
bacterial cell (lanes 2 and 3) and present in experimentally infected coho salmon sera (lane 4). No antigen was detected in control chinook salmon sera (lane 5). A duplicate of this blot (Figure B.2b) was stained for total protein and illustrates the specificity of the monoclonal antibody.