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Little is known about the specific nature of the humoral immune response to *Renibacterium salmoninarum,* the causative agent of bacterial kidney disease. Better characterization of the identity, activity and immunogenicity of components present on the surface of this salmonid fish pathogen is needed to promote the selection of well-defined antigens for the development of protective vaccines. In this study, a whole cell enzyme-linked immunosorbent assay (WCE) was optimized and used as a tool to quantify the antibody activity present in hyperimmune serum from chinook salmon injected with native cells, including the predominant protein p57 ($p57^+$) known to suppress antibody production *in vitro*, and cells lacking a majority of the p57 molecule after exposure to autoproteolytic activity by incubation at 37°C ($p57^-$). After $p57^-$ WCE analysis, the average antibody activity from serum samples against heat treated $p57^-$ cells was significantly higher at 32.5 weeks versus $p57^+$ serum. Further, the enhanced antibody activity of $p57^-$ serum was greatly reduced after carbohydrate disruption of $p57^-$ cells with periodate.

The p57 molecule has been postulated to be a source of cross-reactivity after a common, 57-60 kilodalton (kDa) protein was detected by immunoblotting

in other gram-positive bacteria probed with anti-*Renibacterium salmoninarum* polyclonal rabbit sera. We describe the use of previously developed monoclonal antibodies to p57 to antigenically characterize proteins from other selected gram-positive and gram-negative bacteria which migrate at a similar molecular weight. Immunoblot analysis suggests that these selected epitopes of p57 do not exist in any of the tested bacterial species. In addition, results from immunoblots probed with 60 kDa heat shock protein sera from a gram-negative species suggests that a 60 kDa protein distinct from p57 may be a source of cross-reactivity.

Characterization of the Humoral Immune Response to *Renibacterium salmoninarum* in Chinook Salmon (*Oncorhynchus tshawytscha*)

by

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Patricia A. Wood, Author

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CONTRIBUTION OF AUTHORS

Dr. Steve Kaattari was involved in the design and analysis of the manuscript in chapter 3. Dr. Greg Wiens originated the design and assisted in the writing of the manuscript in chapter 4. He also developed the mAbs and produced the anti-*R. salmoninarum* rabbit serum used in this study. Dr. Dan Rockey was also involved in the design, analysis, and writing of chapter 4, and responsible for the immunoblotting work in Figure 4.3. Dr. John Rohovec assisted with minor edits of chapter 4.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Characterization of the Humoral Immune Response to *Renibacterium salmoninarum* in Chinook Salmon (*Oncorhynchus tshawytscha*)

1. INTRODUCTION

Renibacterium salmoninarum (Rs), the causative agent of bacterial kidney disease (BKD), is one of the most prevalent bacterial pathogens of wild and cultured salmonid fish (Fryer and Sanders 1981; Evenden et al. 1993). Mortality from BKD occurs in fresh and saltwater environments (Earp et al. 1953; Banner et al. 1983). The disease is widespread in the Pacific Northwest, as Rs was detected in more than 20% of the wild and hatchery outmigrating juvenile salmonids sampled from the Columbia River basin between 1983 and 1984 (Sanders et al. 1992). Elliot and Pascho (1991) have reported 97-100% of outmigrating spring and summer chinook smolts in the Columbia River had detectable levels of the Rs antigen. Substantial economic loss has been reported by Beacham and Evelyn (1992) who cite BKD as the major cause of death for Pacific salmon in aquaculture operations in British Columbia.

Prophylactic measures including chemotherapy, early diagnosis, selection of resistant stocks, or segregation of infected stocks remain relatively ineffective (Elliot et al. 1989), and no efficacious vaccine is available (Munro and Bruno 1988). Many factors are important to consider if a vaccine is to provide adequate protection. The development of an effective piscine immune response is based on factors such as the form of the antigen, route of administration, dosage, number of serotypes, age of the animal, overall health such as diet, stress, the presence of antibiotics or pollutants, environmental temperature, and long-term stability of the preparation (Ellis 1988). Munro and Bruno (1988) speculate on the failure of past experimental BKD vaccines based on the slow to develop,

nonprotective agglutinating antibody response. Preparations may contain antigens which are not immunogenic, do not have enough of the right antigens to elicit protection, are weakly immunogenic, or contain antigens which suppress the immune response. Also, the intracelluar nature of Rs may allow the bacterium to bypass the humoral arm of the host immune system (Piganelli 1994).

Another problem involves a lack of understanding of the mechanisms of Rs pathogenesis and the salmonid immune response to infection. Therefore, better characterization of the immunogenicity and identity of potential experimental vaccine components may provide immunoprophylactic preparations which elicit protection against BKD.

In spite of the importance of BKD, the nature of the humoral response in salmonids to this pathogen remains limited. There is evidence of a specific antibody response against Rs (Evelyn 1971; Paterson et al. 1979; Weber and Zwicker 1979; Paterson et al. 1981a; Bruno 1987; Turaga et al. 1987; Bartholomew et al. 1991; Sakai et al. 1991). In addition, some level of protection has been reported in instances where agglutinating antibody titers were high (Paterson et al. 1981a, b), however others report that the presence of agglutinating antibodies do not correlate with protection (Bruno 1987; Sakai et al. 1989; Hsu et al. 1991). Humoral responses are known to play an important role in fighting infection by other intracellular mammalian pathogens. For example, David (1990) tested sera from humans recently infected with *Mycobacterium tuberculosis* for immunoglobulin and specific antigen reactivity by ELISA. The significant titers detected early after insult in most cases indicated a state of balance between humoral and cell-mediated immunity. Disease begins as an acute inflammatory reaction with a predominent humoral response that is replaced by the cellular response as the disease becomes chronic. Perhaps an

unsuccessful B-cell response may trigger the T-cell response. The specific antibody response has been reported to revert macrophage phagosome-lysosome inhibition *in vitro* and form complexes which may slow or modify macrophage processing or alter signals to T-lymphocytes.

The purpose of this research has been to further characterize the specific humoral immune response of chinook salmon to Rs. Preliminary studies demonstrated that an endogenous serine protease cleaves p57 into its characteristic breakdown products within 10 h after incubation of the cells at 37°C (Rockey et al. 1991). The predominant component of these extracellular protein antigens (ECP), is a 57/58 kDa protein doublet (p57) (Wiens and Kaattari 1989) first described by Getchell et al. (1985). *In vitro* studies revealed that ECP inhibits the antibody production of coho salmon B-cells (Turaga et al. 1987b), and that this immunosuppression is lost after exposure to the protease (Rockey et al. 1991b). This protease activity has been utilized to remove p57 ($p57^-$ cells), an immunosuppressive, putative virulence factor, from the surface of Rs cells to study the efficacy of the $p57^-$ cell antigen in vaccine trials. Although Piganelli et al. (unpublished; b) observed a delay in mean day to death in fish injected IP with $p57^-$ cells versus controls, these authors have since learned only orally fed $p57^-$ cells afforded protection after water born challenge. This work analyzed whether the experimental $p57^-$ vaccine antigen had enhanced immunogenicity in fish compared to the $p57^+$ antigen. A whole cell enzyme-linked immunosorbent assay (WCE) was optimized to quantitate differences in serum titers from fish hyper-immunized with native $p57^+$ or heat treated $p57^-$ Rs cells. Analysis of $p57^+$ and $p57^-$ cells after enzymatic and chemical disruption by WCE , identified the crude composition of antigens being predominantly recognized by anti-Rs serum. The latter portion of this work described the use of a battery of monoclonal antibodies to p57 produced by Wiens and Kaattari (1989;1991),

together with anti-60 kDa heat shock protein sera, to demonstrate the absence of p57 antigenic determinants in selected gram-positive and gram-negative bacterial species. Preliminary reports claimed that anti-Rs polyclonal sera cross-reacts with proteins migrating at approximately 57-60 kDa in some gram-positive bacteria (Bandin et al. 1993; Toranzo et al. 1993). Further, researchers concluded that p57 is present in these bacterial species. In addition, we provide evidence that may implicate a 60 kDa protein distinct from p57, as the source of previously reported cross-reactivity in immunodiagnostic assays.

2. LITERATURE REVIEW

Selected Characteristics of *Renibacterium salmoninarum*

Phenotypic Characteristics

Renibacterium salmoninarum (Rs), the causative agent of bacterial kidney disease (BKD) in salmonid fish (trout, salmon and charr), is a gram-positive, unaggressive, obligate intracellular diplobacillus (Fryer and Sanders 1981; Evenden et al. 1993). Cultivation of the bacterium requires an unusually lengthy growth period of several weeks at 17°C (Fryer and Sanders 1981), with an absolute requirement for cysteine (Ordal and Earp 1956; Daly and Stevenson 1985).

The cell wall components of Rs isolates appear to be highly conserved (Bruno and Munro 1986c; Fiedler and Draxl 1986). A unique peptidoglycan amino acid sequence composed of the peptides D-Glu-D-Ala and Ala-D-Glu-D-Ala and their quantitative composition was deduced by partial acid hydrolysis and two-dimensional paper chromatography. In addition, the bacterium possesses an unusual cell wall polysaccharide component N-acetylfucosamine with galactose, rhamnose, and N-acetlyglucosamine (Kusser and Fiedler 1983). More than 60% of the dry weight of the cell is composed of polysaccharide (Fiedler and Draxl 1986) and may comprise the recently described capsule (Dubrieul et al. 1990b).

Taxonomy

These traits coupled with the absence of mycolic acids (Fryer and Sanders 1981), the difference in lipid composition (Collins 1982; Embley et al. 1983), and

the presence of lysine in the peptidoglycan instead of meso-diaminopimelic acid (Sanders and Fryer 1980) did not warrent the placement of this bacterial species in the genus *Corynebacterium* as suggested by (Ordal and Earp 1956; Smith 1964). Instead, a unique genus and species for this organism ,called *Renibacterium salmoninarum* , was established by Fryer and Sanders (1980). Further, 16s ribosomal RNA analysis and G+C content calculations (Stackebrandt et al. 1988; Banner et al. 1991; Gutenberger et al. 1991) have more accurately assigned this organism to the high G+C gram-positive eubacterial subdivision of the actinomycetes. *Micrococcus* and the soil bacterium *Arthrobacter* are the closest known relatives.

Host Range

Reports of bacterial kidney disease originated from Scotland in 1930 and a Massachusettes hatchery on the east coast of the United States (Belding and Merrill 1935). The incidence of Rs infection in cultured salmonid stocks is found over many areas of the world, including North America (Fryer and Sanders 1981; Bell et al. 1990; Sanders et al. 1992), Europe (Fryer and Lannan 1993), Asia (Japan; Kimura and Awakura 1977), South America (Chile; Sanders and Barros 1986) and Scandinavia (Iceland; Helgason and Benediktsdottir 1987, cited in Gudmundsdottir et al. 1993). This bacterium has also been isolated in the wild (Pippy 1969; Evelyn et al. 1973; Ellis et al. 1978; Paterson et al. 1979; Banner et al. 1986; Souter et al. 1987; Elliot and Pascho 1991).

Transmission

The ease with which Rs can be transmitted in these environments has led to difficulty in developing effective and reliable control measures. While there is

evidence to support horizontal transmission (Mitchum and Sherman 1981; Bell et al. 1984), the precise mechanism(s) have yet to be delineated. Wood and Wallis (1955) found that feeding fingerling chinook salmon a diet of infected adult viscera for two months caused infection. Hendricks and Leek (1975) postulate eye and skin abrasions may allow entry of Rs. Infected feces may also harbor the bacterium (Bullock et al. 1980; Austin and Rayment 1985) which is known to remain viable in fresh and salt water (Austin and Rayment 1985; Elliot and Pascho 1991).

Rs may also be vertically transmitted to offspring through infected gametes (Allison 1958; Bullock et al. 1978; Lee and Evelyn 1989). The first evidence for a mechanism was reported by Evelyn and co-workers (1984), where Rs was found outside and internally in the yoke of naturally infected coho salmon. Evelyn et al. (1986a) postulate that infection occurs after ovulation when the egg is in contact with the coelomic fluid *in vivo*. However, Bruno and Munro (1986b) suggest that eggs may become infected from ovarian tissue where Rs is present prior to ovulation.

Control and Immunodiagnostic Detection of Bacterial Kidney Disease

The use of chemotherapy with erythromycin has become widely adopted by the aquaculture industry to treat BKD (Wolf and Dunbar 1959; Groman and Klontz 1983; Evelyn et al. 1986b; Moffit and Bjornn 1989; Brown et al. 1990). Other means used to prevent and contol the spread of BKD include segregation of infected gametes (Pascho et al. 1991b), dietary modifications (Wedemeyer 1973; Bell et al. 1984), selective breeding of genetically Rs-resistant fish stocks (reviewed by Fryer and Lannan 1993), vaccine development (Paterson et al. 1981a,b; McCarthy et al. 1984; Shieh 1989), and immunodiagnostics (Bullock

and Stuckey 1975; Pascho and Mulcahy 1987; Turaga et al. 1987b; Rockey et al. 1991).

Early efforts to directly detect the organism relied on observing the gram-positive diplobacillus by Gram stain and any clinical signs (Bell 1961; Pippy 1969). The definitive test for Rs was established by Fryer and Sanders (1981) incorporating culture with serological identification. Cultivation may be accomplished using Muller-Hinton medium supplemented with cysteine (Ordal and Earp 1956), kidney disease medium containing peptone, yeast extract, cysteine and 10% serum (KDM II) (Evelyn 1977), or replacing the serum component with charcoal (Daly and Stevenson 1985). However, growth conditions and incubation periods continue to be optimized (Daly and Stevenson 1988; Evelyn and Prosperi-Porta 1989; Evelyn et al. 1989; Gudmundsdottir et al. 1991; Benediktsdottir et al. 1991; Olsen et al. 1992). A number of more sensitive immunodiagnostic techniques have been developed for the identification of infected fish. These include the direct and indirect fluorescent antibody test (FAT) (Bullock and Stuckey 1975; Bullock et al. 1980; Laidler 1980; Elliot and Barilla 1987; Lee 1989; Sakai et al. 1989a), and the diagnosis of BKD by detection of a soluble antigen in tissue and blood by immunodiffusion (Chen et al. 1974), and counterimmunoelectrophoresis (Cipriano et al. 1985). Staphylococcal coagglutination (Kimura and Yoshimizu 1981) detects subclinical infection and is as sensitive as the FAT (Sakai et al. 1987b). Enzyme-linked immunosorbent assays (ELISA) are the most sensitive techniques for rapid analysis of a large number of samples (Sakai et al. 1987a,b; Dixon 1987; Pascho and Mulcahy 1987; Turaga et al. 1987b). Monoclonal antibody-based ELISAs enhance the specificity of polyclonal anti-sera based ELISAs (Hsu et al. 1991; Rockey et al. 1991) by avoiding the problem of false-positive reactions with ELISAs (Arakawa et al. 1987; Pascho et al. 1991a) or with other methods (Bullock et al. 1980;

Evelyn et al. 1981; Austin et al. 1985; Turaga et al. 1987; Yoshimizu et al. 1987; Barbash 1992; Foott et al. 1992; Gudmonsdottir et al. 1993), using monospecific antibodies to the 57 kDa major cell surface protein of Rs (p57) developed by Wiens and Kaattari (1989; 1991).

Currently, progress in diagnostics is being made by researchers utilizing molecular techniques to enhance sensitivity and specificity. DNA fragments have been isolated which may be used as probes to detect the gene sequence specific for Rs when designing asymptomatic diagnostic tests for cells and ova. Common fragments were eliminated by hybridization to *A. salmonicida* and *Yersinia ruckeri*. Another potentially unique fragment did not hybridize with a battery of different bacterial species, but did have some common homology with *Corynebacterium striatum*. Etchegaray et al. (1991) continue to search for specific probes. Leon et al. (1994) used PCR to identify Rs in infected kidney tissue using a unique 149 base pair sequence which detected as few as 22 Rs cells. *Corynebacterium striatum* and other selected fish pathogens did not hybridize with this probe. Brown et al. (1994) improved on the likelihood of detecting egg infection after looking for Rs in female tissue. Instead, PCR detected as few as two Rs cells directly within an individual egg. Not only is the technique rapid making it feasible for screening broodstock, but can eliminate false-positive reactions seen when using the immunodiagnotic FAT by bypassing the use of polyclonal antisera.

Internal and External Pathology

When preventative measures fail, variable gross external pathology from bacterial kidney disease can result ranging from no clinical signs to darkening in color, petechial hemorrhaging, exopthalmia and distended abdomen (Belding and

Merrill 1935). Internally, the most obvious symptom from visual inspection of the kidney includes enlargement as well as granulomatus lesions (Wood and Wallis 1955; Smith 1964). Invasion of phagocytic cells by Rs has been noted by Young and Chapman (1978) and reported to occur within 45 min of interperitoneal (IP) injection (Bruno 1986b). In addition, Gutenberger (1993) has shown Rs escaping from the phagosome via lysis of the membrane and entering into the cytoplasm of mononuclear phagocytes and lymphocytes. Hematological and serum parameters change as well (Hunn 1964; Wedemeyer and Ross 1973; Bruno 1986a). Erythrocyte number and diameter decrease (Bruno 1986a) and levels of the soluble protein antigen p57 increase (Turaga et al. 1987a,b). Evidence suggests that humoral immunity to p57 leads to immune complex formation and subsequent hypersensitive reactions in the glomeruli. Young and Chapman (1978) revealed immune complex-mediated glomerulonephritis in rainbow trout experimentally infected with Rs by immunohistological investigation. Specific antisera showed the presence and distribution pattern of piscine Ig or complexes. Improving on assumptions by Young and Chapman (1978), Razquin et al. (in press) demonstrated the presence of immune complexes in tissue and sera of experimentally infected coho salmon which are composed of p57 and salmon Ig, induced by perhaps type III hypersensitivity.

Virulence Factors

A number of putative virulence factors of Rs have been identified as reviewed by Toranzo and Barja (1993). Bruno and Munro (1986c) describe the presence of catalase and DNase, postulated to be involved in phagocytic avoidance and interfering with host DNA expression after intracellular invasion, as well as proteolytic and hemolytic activity. Recently Evenden et al. (1990)

cloned a hemolysin from Rs and found the sequence to be homologous in four other strains. Griffiths et al. (1991) and Rockey et al. (1991a) identified a serine protease. The larger of two proteolytic bands had activity against p57 (Rockey et al. 1991a). Although the function of the protease has not been determined, putative roles may include nutrative, by degrading host proteins into amino acids the bacterium can utilize as proposed by Ellis (1991) with another fish pathogen, *Aeromonas salmonicida*, upregulation of the fibrinolytic and blood coagulation cascades, liquification of tissue, inactivation of Ig, regulation of inflammatory reactions, or disruption of macrophage chemotaxis, as observed with some mammalian extracellular serine proteases (Bond and Butler 1987; Finlay and Falkow 1989). However, the Rs serine protease is known to cleave the soluble form of the p57 protein in ECP, which has been associated with suppression of the B cell response in coho (Turaga et al. 1987; Rockey et al. 1991a). Perhaps the Rs serine protease plays a role in regulating the host immune response. For example, in mammalian systems cellular proteinases are known to function in creating immunologically recognizable molecules (Whitaker and Seyer 1979).

Extracellular toxins may also exist *in vivo* (Bruno and Munro 1986a; Bell et al. 1990) and *in vitro* (Shieh 1988) in contrast to Bandin et al. (1991), who were unable to demonstrate toxicity of the ECP, but have detected the presence of a dermatoxin (Bandin et al. 1992). Reports of weak exotoxin production have led researchers to examine the putative virulence antigens associated with the cell envelope. The major cell surface and soluble ECP component, the 57 kDa antigen, p57, has been characterized and seems to be an important initial target for assessing the immune response by virtue of its surface exposed location allowing significant interaction with the salmonid immune system. The properties associated with this molecule include autoagglutination (Bruno 1988), salmonid spermagglutination (Daly and Stevenson 1989), hydrophobicity (Bruno 1988;

Daly and Stevenson 1990), and hemagglutination of mammalian erythrocytes. The short fimbrae-like structure of p57 proposed by Dubreuil et al. (1990a) may help mediate *in vitro* agglutination. A mutant strain of Rs, MT239 was originally described by Bruno (1988) as less virulent and lacking p57 (Bruno 1990; Daly and Stevenson 1990), perhaps due to routine subculturing, as well as nonagglutinating (Daly and Stevenson 1990). However Senson and Stevenson (1994) report the MT239 isolate does produce p57 yet the protein does not remain associated with the cell surface. These researchers also found MT239 to lack a capsule and a carbohydrate moiety associated with p57 from the autoagglutinating Rs strain JD24. Also, Wiens and Kaattari (1991) attribute leukoagglutinating capabilities to p57. These factors may contribute to serum resistance, intracellular survival, and invasion of ova and host endothelial cells (Toranzo and Barja 1993). Turaga et al. (1987b) reports production of antibodies *in vitro* from coho lymphocytes was suppressed when a soluble ECP fraction from Rs culture was added. Perhaps ECP, containing p57, is an important virulence factor due to its ability to suppress the humoral response.

The p57 molecule is known to be unstable and susceptible to proteolytic degradation (Dubreuil et al. 1990a; Griffiths and Lynch 1991; Rockey et al. 1991b). Griffiths and Lynch (1991) found more lower molecular weight bands after freeze/thaw, in older culture supernatants, and at 25 and 30^{o}C after a 10 h incubation, corresponding to a decrease in the 57 kDa protein. Because bands at 57 and 33-37 kDa had autolytic activity, the authors propose p57 may be autolytic or co-migrate with a protease. Rockey et al. (1991b) identified a high molecular weight serine protease (100 kDa) with activity against p57 at 17 and 37^{o}C. Digestion of the partially purified molecule yields a spectrum of breakdown products similar in molecular mass and antigenicity to those in ECP. This suggests the immunologically related constituents of ECP are generated by

degradation of p57. Recently, Chien et al. (1992) cloned and sequenced p57 from crudely purified soluble ECP by immunoaffinity chromotagraphy and PAGE. A potential cleavage site was found between residues 26-27, the end of a prokaryote secretory signal sequence. The authors feel p57 is synthesized as a precursor and processed into mature protein. A highly hydrophilic region (85-90) was predicted to be an antigenic determinant. Also, there were two imperfect direct repeats in two different regions. One repeat had a sequence slightly similar to trypsin-like and V8 *Staphylococcus*-type serine proteases. An indirect repeat at the C-terminus of the p57 gene had three stop codons. Virulence factors are important candidates for the role of protective immunogens, as both cellular and extracellular antigens of Rs have received attention as potential constituents of BKD vaccines. A better understanding of the host immune response to these and other putative cellular and extracellular virulence factors and antigens of Rs are needed to improve vaccine efficacy.

Host Immune Response

Limited information is available concerning the immunology of the host response. There is subtantial evidence that a specific, low level agglutinating antibody response experimentally and in natural infections is generated against Rs (Weber and Zwicker 1979; Sanders et al. 1978; Paterson et al. 1981a,b; Paterson et al. 1985; Bruno 1987; Sakai et al. 1989; Sami et al. 1992). Juvenile coho salmon had agglutinating titers lower than those reported by Evelyn (1971) and Paterson et al. (1981a,b) after responding to injections to whole cells emulsified in Freund's complete adjuvant (FCA) (Sanders et al. 1978). Paterson et al. (1981a) detected residual titers in controls (1:56) and low titers (1:122) at 94 weeks post-immunization in under-yearling Atlantic salmon parr injected with

heat-killed, sonicated whole cells by immersion and titers < 1:20 after IP injecting whole cells in saline without adjuvant. Low cutaneous and intestinal mucus titers (1:2-1:20) from immersion vaccinated fish were identical to controls, as were intestinal mucus titers from fish vaccinated with whole cells in FCA. Cutaneous mucus from fish injected with cells and FCA were not as low. Bruno (1987) also observed low agglutinating specific antibody titers from farmed rainbow trout between approximately 1:2 and 1:32, regardless of past history, contact with Rs or during a clinical outbreak. Variable low titers from farmed Atlantic salmon with and without a history of BKD ranged between 1:2 and 1:128. Wild Atlantic salmon samples negative for Rs over a four year period by IFAT and Gram stain also had low variable titers. Although agglutinating antibody levels do not appear to correlate with evidence of infection, Bruno (1987) postulates the immune system may have been active in removing the bacterium since no agglutinating antibody was detected in heavily infected smolts at one farm, but increased two months prior to the disappearence of infection. Analysis of serum agglutinating antibody titers in rainbow trout by Sakai et al. (1989) after artificial IP injection of formalin-killed whole cells in FCA and without adjuvant showed no difference in low titers (1:8) at 5 weeks post-injection. Unfortunaltely, studies were not extended to access antibody levels during the anemnestic response.

In addition, initial work by Evelyn (1971) and Paterson et al. (1981a) demonstrated the ability of immature Sockeye salmon and Atlantic salmon respectivley, to mount an anemnestic response characterized by elevated specific serum agglutinating antibody. Paterson and co-workers noted augmented antibody levels (1:320-1:10,240) three months after a booster at 13 months post primary vaccination IP with heat-killed whole BKD cells, compared with titers of 0 to 1:1,280 observed a year and a half post primary injection without boosting. Also, just 12 weeks after initial injection titers were elevated (0-

1:2,560). Further, these studies demonstrate that the primary antibody response to Rs is durable yet slow to develop. Paterson et al. (1981a) indicate an elevated agglutinating antibody response with a mean of 1:322 beginning 5 weeks post IP injection of Rs whole cells with FCA in under-yearling Atlantic salmon until a year and a half later (mean=1:4,322). Post-yearling Atlantic salmon also demonstrated high durable titers to formalin or heat-killed cells in FCA for approximately a year after initial injection.

Other work concerning the host humoral response has focused on identifying the specific Rs bacterial antigens recognized by fish using immunoblotting. Turaga et al. (1987a) detected four soluble antigenic bands of 70, 60, 33-37, and 26 kDa with molecular weights similar to purified ECP, after probing infected coho serum with polyclonal rabbit anti-soluble antigen. Although two cross-reactive antigens were found in control serum, the molecular weights were different. Antibodies to the 57-60 kDa antigen appear first, 10 days after infection, increase in density over time, and are the most prominant, while the 70 and 33-37 kDa antigens appear after 20 days. The 26 kDa antigen was not recognized. In addition, Bartholomew et al. (1991) detected salmon and mammalian antibodies to formalin-killed Rs antigens with a monoclonal antibody against salmonid Ig. Most of the response was to a 57-58 kDa complex on whole cells and in soluble ECP, as well as to four other proteins of lower, yet unidentified molecular weight. Chinook and coho salmon infected naturally also reacted with p57, but not with the other antigens. It has been postulated by S. L. Kaattari (in Bartholomew et al. 1991) that this may be due to low antibody titer in naturally infected fish because of immune complex formation.

Fiedler and Draxl (1986) demonstrated the presence of a 70 kDa antigenic protein attached to the cell wall after probing blots with rabbit anti-trypsinized cell serum. Reaction with nontrypsinized cell anti-serum was greater, indicating other

proteins attached to the cell wall were antigenic. The antigenicity of cell surface components remained after trypsinization as shown by agglutination with rabbit anti-trypsinized serum. Purified polysaccharide was found to be antigenic by a precipitin test using rabbit anti-whole cell serum. Further, trypsinized and nontrypsinized cell walls were agglutinated by anti-trypsinized serum. The authors speculate that because of the low levels of peptidoglycan in the cell wall and high substitution of N-acetylmuramyl residues, a predominant portion of the Rs cell surface may be comprised of polysaccharide. Therefore, surface polysaccharide may play an important role in pathogenesis and the immune response. Sorum and Robertsen (1994) demonstrated that trypsinized Rs cells produced antibodies to the galatose-rich surface polysaccharide (GPS) in rabbits. It is not known whether GPS is immunogenic in the natural salmon host.

Studies by Rose and Levine (1992) addressed another aspect of the early host immune defense, namely interactions between the bacterium and host complement. Rs was shown to activate the alternate complement pathway until C3 and the binding of C3b. C3b bound to Rs appears to be accessible on the surface of the cell to complement receptors unlike the phagocytosis resistant Strep A mutant cell surface, which interferes with C3b binding. The fact that Rs was found to be internalized by rainbow trout head kdney macrophages via a complement dependent phagocytic process seemed likely since in mammals, phagocytosis by complement receptors does not result in toxic superoxide release in macrophages and may allow intracellular survival. This bacterium has been shown to resist phagocytic killing by surviving (Young and Chapman 1978) and possibly multiplying within phagocytic cells (Bandin et al. 1993). The survival of virulent and avirulent strains waned after 3-4 days and correlated with a reduction in the superoxide anion response of macrophages, suggesting this type of killing mechanism is not operating with Rs.

Vaccination Against Bacterial Kidney Disease

The difficulty associated with developing immunoprophylactic preparations for protection against BKD persists. A summary of the major attempts at immunization against Rs are reviewed by Evenden et al. (1993). Several researchers failed to demonstrate protection after challenge with IP delivered heat or formalin-killed whole cells with or without FCA adjuvant (Baudin-Laurencin et al. 1977; Sakai et al. 1989a), with ECP, fractured cells, cell wall fractions with or without *A. salmoninarum*, *Vibrio angillarum*, *V. ordalii* or FCA by Evelyn and co-workers (in Evenden et al. 1993), or killed whole cells with or without muranyl dipeptide, FCA, *E. coli* LPS and *V. angillarum* extract or soluble ECP with or without *V. angillarum* conjugated by glutaraldehyde, cyanium Cl, tannic acid or ethyl carbodimide by Kaattari and co-workers (in Evenden et al. 1993). Turaga and Kaattari (1989) administered two antigens with the immunomodulating antigen *V. angillarum* extract, FCA, formalin-killed *V. angillarum* or heat-killed *mycobacterium chelonii,* and a third antigen, polysaccharide extracted from the cell wall. Agglutinating titers for all antisera except anti-polysaccharide sera, which was not tested, were minimal (64-128). Little prophylactic effectiveness based on % survival was noted using this forms of the immunogen. Soluble ECP exacerbated the disease, as mean time to death was more rapid than controls. The authors hypothesize this may have been the result of induced immune complexes, the presence of immunosuppressive components, or both.

Some success with experimental BKD vaccines have been reported, but without the correlation of serum antibody levels. McCarthy et al. (1984) performed a vaccination trial with various modified antigens, yet reported the

most beneficial protection with formalin-killed pH-lysed cells delivered and challenged IP. However, Fryer and Lannan (1993) caution that measurement of Rs in the animals by the insensitive Gram stain may not detect subclinical infection. Further, because the antibody response was not measured, the correlation of humoral immunity for this particular antigen is not known. Shieh (1989) noted all 10 fish from each of four groups injected four times in four weeks with a different purified soluble ECP fraction, survived challenge intermuscularly (IM) one month later with $5x10^5$ Rs cells. Each purified fraction of ECP contained a different concentration of protein in the vaccine dose injected every week, including a crude supernatant at 100 mg protein, an acetone precipitate at 60 mg protein, an ammonium sulfate precipitate (20 mg/protein) and a DEAE-cellulose fraction at 7mg. Also, two different challenge doses were used as well. Challenge with $2x10^6$ cells protected 8-9 of 10 fish, while the highest challenge dose of $1x10^7$ cells protected only 6-8 of 10 fish and was the least effective. Survival rates dropped as challenge dose increased. Unfortunately, Shieh did not include media infected fish as controls to rule out any nonspecific protective response from toxins or other media related components (Wiens 1992).

Studies by Paterson et al. (1981a,b) measured the antibody response to vaccine preparations reported to be protective. Atlantic salmon were immunized IP with heat-killed, formalin-killed, or heat-killed sonicated cells in adjuvant. High agglutinating titers appeared at 5 weeks and continued to endure for 1.5 years post primary injection. Only kidney lesions, a sign of overt disease, were reduced while the prevalence of asymptomatic infection in vaccinated and control groups were similar. Further, elevated mucosal antibodies appearing until 9 weeks post vaccination were not protective. This work provides a correlation between specific anti-Rs serum agglutinating antibodies and the limited protection provided against the bacterium. Also, despite the fact that specific

antibody against soluble ECP is not protective, coho salmon challenged with a mixture of live Rs cells and high titer anti-serum to ECP took 25% longer to die versus the controls (Kaattari et al. 1988). Evenden et al. (1993) suggests specific humoral immunity responses do have some effect on survival, as do nonspecific responses to adjuvant alone. Also, other researchers suggest humoral immunity may not be indicative of protection (Bruno 1987; Sakai et al. 1989a; Hsu et al. 1991).

Recent work by Piganelli et al. (unpublished, b) in our laboratory indicated IP vaccination and challenge of coho salmon with a heat exposed form of the Rs whole cell missing the 57 kDa putative virulence factor and immunosuppressive surface protein ($p57^-$) in FIA, significantly increased mean time to death compared to saline and FIA controls until 5.5 weeks post challenge with live Rs cells. In a second experiment, the route of exposure was more important with natural bath challenge. Orally fed $p57^-$ vaccinated fish had a significant decrease in soluble p57 in the kidney versus the controls. However, IP $p57^-$ injected fish had higher antibody titers throughout but were not protected when bath challenged. They also had a higher concentration of p57 at 12 weeks.

Masking of Bacterial Cell Surface Antigens

Gram-negative bacteria

There are a number of reports of occlusion by different components of gram-negative bacterial species, which include blocking of antigens by S protein layers, concealment of cell wall and O antigens by capsule polysaccharide, and shielding by the LPS O antigen and outer core. Dooley and Trust (1985) report masking by the S layer in the fish pathogen *Aeromonas hydrophila* by

immunoflurescence studies with a mAb specific for the LPS core oligosaccharide-polysaccharide linkage. Sulfo-NHS-biotin cell surface labeling shows occlusion of outer membrane (OM) antigens by the 52 kd major surface array protein or S layer in *A. hydrophila.* The restricted access of this compound as shown by immunoblotting, demonstrates that these OM proteins are shielded from immune recognition (Dooley and Trust 1988).

Further, polysaccharide capsules have long been known to exhibit masking properties (Orskov et al. 1963; Stirm et al. 1971; Wu and Park 1971; Kopecko and Formal 1984; Kadurugamuwa et al. 1985a, b; Cross et al. 1986; Williams 1987; Williams et al. 1986, 1988a). The exopolysaccharide capsule of many pathogens mask OM protein antigens (Williams 1988b). Other authors have observed that the capsule masks the presence of Ig at the cell surface (Williams et al. 1988) but does not prevent access of anti-O antibodies in *Klebsiella* or other *E. coli* strains (Cross et al. 1986).

There are many ways in which a physical barrier of a bacterium may be disrupted thereby unmasking surface antigens. It is well documented that heat treatment of gram-negative bacterial cells destroys the integrity of the OM allowing better penetration of antibodies for reaction with core epitopes in O-polysaccharide$^-$ mutants (Lieve 1974; Tsuchido et al. 1985). Further, removal of the *E. coli* K antigen by heating or enzymatically unmasks O antigens allowing agglutination by anti-O serum (Orskov et al. 1963; Stirm et al. 1971). In addition, heating is known to increase antibody binding to whole bacteria (Nelson 1991), perhaps by unmasking other protein or polysaccharide antigens allowing greater antibody accessibility. Also, chemical degradation is known to partially remove O antigens on rough mutants unmasking rough bacterial surface sites (Luderizt et al. 1966).

Another mechanism involves altering cell surface properties of the capsule when serum antibodies bind to *Klebsiella* which increases surface hydrophobicity. This appears to determine whether antibodies are exposed on the surface, not whether they penetrate the capsule (Williams et al. 1988). Chedid et al. (1968) proposed an enzymatic process in serum which attacked gram-negative cell wall components, unmasking rough antigenic structures. Also, heat-inactivated sheep serum seems to alter antigen expression of LPS and allow mAbs to bind to the core (Nelson 1991). In addition, nutritional conditions are known to effect the size of the polysaccharide capsule and the size of pores in the matrix causing LPS core determinants to become better exposed (Nelson 1991).

A greater number of protein antigenic sites become exposed at the cell surface and accessible to antibodies after growth in the presence of sub-inhibitory concentrations of antibiotics (Kadurugamuwa et al. 1985b) including cephalosporins (Kadurugamuwa et al. 1985a; Williams 1987) and ciprofloxacin (Williams 1987) which alter the *Klebsiella* exopolysaccharide capsule.

Also, capsule polysaccharide is known to block core LPS binding sites (Luk et al. 1991) and thought to have a role in masking these and other LPS binding sites (Nelson 1990, 1991). Luk et al. (1991) detected steric effects by bulky O-polysaccharide chains on antibody reactivity to *Salmonella typhimurium*. Epitopes in the core glycolipid are usually not accessible to the host for immune recognition, except in O-antigenic polysaccharide$^-$ mutants that allow core oligosaccarhide exposure on their surface.

Components of LPS are also known to occlude antibody binding sites. The O antigen of LPS shields antibodies to OM proteins (Graham and Stocker 1977; Mutharia and Hancock 1983; Jessop and Lambert 1985; Van der ley 1986; Williams et al. 1988b), and more specifically the core LPS (Gigliotti and Shenep

1985; Nelson 1990). Pollack et al. (1989) note epitope concealment of core-lipid A by overlying O chains and core sugars and also postulate steric interference by other surface structures as do other authors (Ng et al. 1976; Gigliotti and Shenep 1985).

Gram-positive bacteria

Brubaker (1985) reviews mechanisms used by some gram-positive bacterium to promote nonspecific binding of immunoglobulins or other proteins to the cell surface which may serve to prevent specific antibody-mediated effects and also to mask portions of the microbial surface. Treponemes occlude antigens by attaching host proteins to their cell surface (Alerete and Baseman 1979). King and Wilkinson (1981) hypothesize the capsule of *Staphylococcus aureus* does not cover surface antigens outright so that antibodies cannot bind. Instead antibodies are allowed to pass through the capsular layer and bind to cell wall antigens, and are then covered over by the capsule. Thus cell wall antibodies are not exposed at the true surface, allowing resistance to phagocytosis. Johne et al. (1989) emphasizes the potential of polysaccharide capsule from a gram-positive bacteria to mask important structures on the bacterial surface, thus influencing pathogenicity. Specifically, they identified masking of protein A and cell wall components of *Staphylococcus aureus* by capsular polysaccharide. Loss of polysaccharide under certain culturing conditions increased expression of the cell wall component. Further, masking was dependent on the concentration of the surface determinants as well as exopolysaccharide abundance.

3. ENHANCED ANTIBODY RESPONSE TO *Renibacterium salmoninarum* AFTER REMOVAL OF THE BACTERIAL CELL SURFACE-ASSOCIATED 57 kDa PROTEIN

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Abstract

A study was conducted to determine the effect of endogenous protease activation by heat treatment on the immunogenicity of *Renibacterium salmoninarum* cells in chinook salmon (*Oncorhynchus tshawytscha*). Salmon were repeatedly immunized with either *R. salmoninarum* cells with p57 ($p57^+$) or predominently depleted of p57 ($p57^-$). The resultant antisera were examined by whole cell ELISA and immunoblot procedures using $p57^+$, $p57^-$, proteinase-K-, and periodate-treated whole cells. These analysis revealed that the removal of p57 by the endogenous serine protease significantly enhanced the immunogenicity of the cell, resulting in a 20-fold increase in detectable antibody titers. The bulk of this antibody activity appeared to be blocked by the presence of the p57 molecule. Furthermore, proteinase-K and periodate treatment of *R. salmoninarum* cells revealed the increased antibody activity almost exclusively reacted with carbohydrate moieties on the $p57^-$ cell.

Introduction

Renibacterium salmoninarum, the causative agent of bacterial kidney disease (BKD) of salmonid fish, is a slow-growing, gram-positive, obligate intracellular diplobacillus (Young and Chapman 1978; Fryer and Sanders 1981; Evenden et al. 1993). Despite advances in the understanding of pathogenicity, immune interactions and the development of new disease management techniques, BKD continues to be a problem during the culture of salmonids. The ability of the organism to be transmitted both horizontally (Mitchum and Sherman 1981; Bell et al. 1984) and vertically (Evelyn et al. 1984), survive and potentially

replicate within phagocytic cells, together with its fastidious requirements, and slow growth have made it one of the most difficult fish pathogens to control (Evenden et al. 1993; Fryer and Lannan 1993).

Currently, there is no commercially available vaccine that demonstrates protection against BKD. Numerous attempts to vaccinate intraperitoneally (IP) with native heat-killed and formalin-killed whole cells (Baudin-Laurencin et al. 1977; Sakai et al. 1981), soluble extracellular proteins (ECP) or crude cell fractions (Evelyn et al. 1984; 1988) have failed to protect salmonids reliably (Paterson et al. 1981a,b; McCarthy et al. 1984; Shieh 1989). While native whole cells and soluble ECP antigens do not appear to be protective, it is possible other cell-associated antigens may serve as protective antigens in vaccines against BKD.

Despite previous studies aimed at elucidating immune interactions of *R. salmoninarum* with the host, specificity of the salmonid immune responses to the bacterium remain uncharacterized. Only limited information is available concerning the specific immune response of salmonids to antigens of *R. salmoninarum*. Weber and Zwicker (1979) detected agglutinins in Atlantic salmon after a natural outbreak. Elevated humoral antibody titers have been observed after vaccination with adjuvant and in natural infections (Evelyn 1971; Paterson et al. 1981, Bruno 1987, Munro and Bruno 1988; Sakai et al. 1989). Turaga et al. (1987a) demonstrated by immunoblotting that coho salmon recognize ECP, containing p57. Chinook salmon recognize p57 and three other proteins of unidentified molecular weight (MW) (Bartholomew et al. 1991).

The p57 molecule is well characterized and a focal point of extensive studies concerning diagnosis, pathogenesis and prophylaxis of BKD. This hydrophobic protein possesses hemagglutinating (Daly and Stevenson 1987) and leukoagglutinating (Wiens and Kaattari 1991) capabilities. Also, p57, and other

extracellular components (ECP) are known to suppress plaque-forming cells of salmonid leukocytes *in vitro* (Turaga et al. 1987a). Rockey et al. (1991a) in our laboratory identified an endogenous serine protease that degrades the soluble p57 component of ECP and particulate p57 associated with the cell surface (Piganelli et al. unpublished, a) at an elevated temperature of 37^0C. In addition, we demonstrated that heat induced proteolytic activity caused ECP to lose the ability to suppress the *in vitro* antibody response. We reasoned that removal of the immunosuppressive, putative virulence factor p57 may unmask antibody binding sites and enhance the antibody response to other cell surface antigens.

Therefore, we have compared the salmonid humoral immune response to formalin-killed *R. salmoninarum* vaccine preparations which either possessed cell-associated p57 ($p57^+$), or lacked a majority of cell-associated p57 ($p57^-$). Analysis of specific serum antibody titers indicated that removal of p57 from whole cells by 37°C treatment was critical for enhancing immunogenicity and antigenicity. Further, analysis of $p57^-$ serum reactivity against heat treated $p57^-$ cells digested with periodate provided indirect evidence that carbohydrate antigens may also be important immunogens. In addition, immunoblot analysis revealed that p57 and a couple of its breakdown products were the exposed immunogenic determinants on $p57^+$ and heat treated $p57^-$ cells.

Materials and Methods

Animals

Spring chinook salmon (*Oncorhynchus tshawytscha*) eggs that tested negative for BKD were obtained from the Dworshak National Fish Hatchery in Ahsahka, ID. Three tanks each of 35 fish weighing approximately 20-50g upon

initial injection were maintained in 12°C UV, filter sterilized, pathogen free lake water at the Northwest Biological Science Center of the National Biological Servey in Seattle, Washington. Fish were fed Oregon Moist Pellets (OMP)(Bioproducts, Warrington, OR). To produce standard antisera, coho salmon (*Oncorhynchus kisutch*), weighing 200-500 g upon initial immunization were maintained at the Salmon Disease Laboratory (SDL) at Oregon State University (OSU), Corvallis, Oregon.

Bacterial Strains

Renibacterium salmoninarum type strain ATCC 33209 and isolate D-6 (obtained from C. Banner, Oregon Department of Fish and Wildlife, OSU, Corvallis, OR) were cultured for 8-14 days to an optical density (OD) of 1.0 (525 nm) in kidney disease medium (KDM II) at 17°C (Evelyn 1977), excluding calf serum. After centrifugation (6,000 x g, 30 min, 4°C) bacterial pellets were washed (16,000 x g, 2 min) three times with 1 ml cold 10 mM phosphate buffered saline (PBS) (0.85% NaCl, 10 mM $NaPO_4$, [pH 7.2]), resuspended to a wet weight concentration of 200 mg/ml and stored at -70°C. Soluble ECP was harvested from the supernatant as previously described (Turaga et al. 1987). Isolate ATCC 33209 was used for the hyperimmunization of coho salmon. Pooled coho sera was used as a standard in the whole cell ELISA (WCE). Strain D-6 was used to immunize chinook salmon at Seattle.

Preparation of Whole Cells

The $p57^-$ cells were prepared by incubating $p57^+$ cells at 37°C for 10 h. Microfuged cell pellets were washed twice in cold PBS. Both cell preparations were then formaldehyde-killed with a 0.3% solution (EM science, Cherry Hill, NJ)

in PBS overnight at 4^{0}C. After washing cells twice, pellets were resuspended to 200 mg/ml in PBS and stored at -70^{0}C.

Production of Monoclonal Antibodies (mAbs)

Monoclonal antibodies (mAbs) 4D3, 3H1, and 1A1 (Wiens and Kaattari 1989, 1991) were used as positive control reagents to identify p57 and its breakdown products in immunoblots. Ascitic fluid was purified using the caprylic acid procedure of Russo et al. (1983). After precipitation with 50% ammonium sulfate, the fluid was dialyzed extensively against PBS, diluted 1:1 in glycerol and stored at -20^{0}C.

Densitometry

The total protein stained western blot was scanned with a helium neon laser beam at an optical density (OD) of 226% with a Personal Densitometer SI, model PDSI-PC, and Image Quant data recorder (Molecular Dynamics, Sunnyvale, CA).

Antigen Preparation and Administration for Immunization

Chinook were hyperimmunized with either p57$^+$ or p57$^-$ whole cell antigens (100 µg/fish) emulsified 1:1 in Freund's complete adjuvant (Sigma; FCA) and PBS for 4 min at 100 units on a Virtis "23" mixer (Virtis, Gardiner, NY). A preparation of FCA in PBS alone served as a negative control. Each fish was injected with a total volume of 50 µl intraperitoneally using a 1 cc syringe fitted with a 26 1/2 gauge needle (Becton Dickinson, Rutherford, NJ). A boost in Freund's incomplete adjuvant (FIA) followed 15 weeks after primary immunization

at half the concentration in the same total volume. During the repeat experiment, each chinook was injected with 100 µg/100µl. Boosts followed 9.5, and 21.5 weeks post primary injection. Hyperimmunized standard antisera was generated by injecting coho with $p57^+$ whole cells (1.0 mg/fish) in FCA in a volume of 100 µl. Boosts in FIA followed 8.5, 10, and 17 weeks at 500 µg/fish in 100 µl.

Collection of antiserum

Individual serum samples were collected from selected fish in the $p57^+$, $p57^-$ and control groups at 0, 12, 32.5, and 43 weeks post immunization. Blood was taken from the caudal vein of animals anaesthetized in 10% benzocaine (Sigma). Chinook used to reproduce the experiment were individually tagged and bled at 0, 9.5, 21.5, and 32.5 weeks. The $p57^+$ hyperimmunized coho to be used as the standard were bled 0, 8.5, 11.5, 17, 19.5 and 26 weeks post primary injection. Blood was allowed to clot at RT for several hours. Samples were centrifuged (Beckman model TJ-6) at 500 x g for 5 min at 4°C. Anti-$p57^+$ serum at 17, 19.5 and 26 weeks from four hyperimmunized coho were pooled. Serum was aliquoted and stored at -70°C.

Protein and Carbohydrate Digestion of Whole Cells

Aliquots of 80 µl of a $p57^+$ or $p57^-$ cell stock (10 mg/ml) were treated with 20 µl of 0.2 M meta-periodate (Sigma) in dd-H_2O (which was warmed to get the compound into solution prior to use), or 20 µl PBS as a control. Cells were incubated at 4°C for 24 h as described by Liang et al. (1992).

The same cell concentration was used with proteinase-K treatment (1 mg/ml) (Sigma) in 0.1 M tris HCl, pH 8.0. Cells were incubated in the dark at room temperature (RT) for 24 h (Mack et al. 1992). Reagents were removed by washing the cells twice in PBS, and resuspending back into the original buffer.

Determination of Antibody Activity by Whole Cell ELISA (WCE)

The concentration of antibody activity to $p57^+$, $p57^-$ and cells treated with proteinase-K or periodate was obtained by the use of an enzyme-linked immunosorbent assay (ELISA) as previously described (Arkoosh and Kaattari 1990), yet slightly modified and optimized for *R. salmoninarum* whole cell antigens. To determine equivalent antigen coating concentrations assuring 50% saturation of the wells, the peroxidase saturation technique of Munoz et al. (1986) was applied with minor modifications. Briefly, whole cells were coated overnight at 17°C, followed by the addition of horseradish peroxidase (HRPO) (Sigma) for 1 h at RT. Plates were washed 10 times with TTBS with a Titertek Automatic Microplate Washer (Flow Laboratories, McLean, VA) and blocked at 200 ml/well with 1% bovine serum albumin (Fraction V, Sigma, St. Louis, MO; BSA) and 0.1% Tween 20 in tris-buffered saline (50 mM tris, 1 mM EDTA, 8.7% NaCl, pH 8.0; TTBS) for 1 h at RT. Plates were read kinetically for 10 min at 405 nm with a Titertek Multiskan Automatic Reader (Flow Laboratories) (Figure 3.1).

The antigens were coated on an E.I.A./R.I.A. ELISA plate (Costar, Cambridge, MA) using formalin-fixed $p57^+$ (50 µg/ml) or $p57^-$ cells (70 µg/ml) overnight at 17°C. Plates were blocked with 1% BSA-TTBS for 1 h at RT and washed five times in TTBS. Each plate contained 5 dilutions of a standard coho $p57^+$ hyperimmune sera titration in 1% BSA-TTBS used for normalization of the data. One unit of antibody activity is equivalent to the volume of standard sera

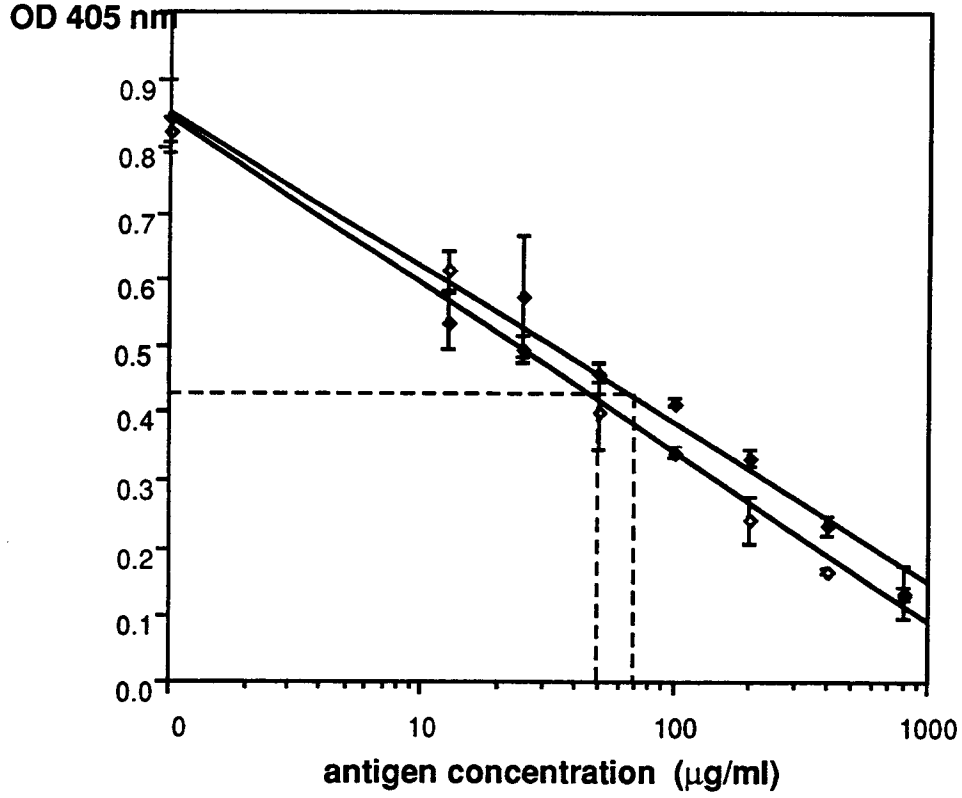

Figure 3.1. Determination of the whole cell antigen concentration needed to occupy 50% of the surface of an ELISA plate well. Plates were coated with different concentrations of each antigen and antigen adsorption in the well was analyzed using horseradish peroxidase to determine the percent non-occupied surface sites. Logarithmic curve-fit interpolation was calculated and surface-saturating antigen concentration was calculated as that corresponding to the intersection of the 50% saturation line. Results corresponding to $p57^+$ cell antigens (◆) and $p57^-$ cell antigens (◆) are plotted together.

(1 µl) required to produce 50% of the maximum OD (Figure 3.2). Plates were washed 10 times after each remaining step. The presence of IgM in the serum samples was detected using a primary antibody, a mouse anti-trout IgM mAb (mAb I-14) developed by Deluca et al. (1983), at 1:1,500 in 1% BSA-TTBS. After washing, streptavidin-conjugated HRPO (Sigma) at 1:200 in 1% BSA-TTBS was added for 45 min. After washing, substrate (ABTS) was added. Optical densities were read kinetically for 10 min.

Preparation of Whole Cells for SDS-PAGE and Immunoblotting

Whole cells (20 mg/ml) were mixed 1:1 in sample application buffer (SAB) (120 mM tris, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 3 mM bromophenol blue) or 5 µl (100 mg/ml) of cells were mixed with 120 µl dd-H_2O and 125 µl of SAB and loaded into a 5 mm well for immunoblotting. After boiling for 3 min, samples were electrophoresed in 10% SDS-polyacrylamide gels (SDS-PAGE) with a Mini-protean II electrophoresis apparatus (Bio-Rad, Richmond, CA) at 200 V for 1 h. Gels were equilibrated in transfer buffer (192 mM glycine, 20 mM tris, pH 8.3, 20 % methanol), and proteins electrophoretically transferred to immobilon-P (Millipore, Bedford, MA) at 100 V for 1 h with cooling.

Immunoblotting was performed after blocking membranes with 1%BSA-TTBS for 1 h at room temperature (RT) or overnight at 4°C. Blots were washed three times for 10 min in TTBS and rinsed in dd-H_2O. Individual salmon serum samples (70 units/70 µl) and positive control mAbs 4D3, 3H1 and 1A1 (3 µg/ml) in 1% BSA-TTBS were each added to a well in a Miniblotter apparatus (Immunetics, Cambridge, MA) and incubated for 1.5-2 h at RT with gentle shaking. After rinsing wells three times with TTBS, blots were removed and rinsed with TTBS three times for 10 min. After washing, blots were incubated for

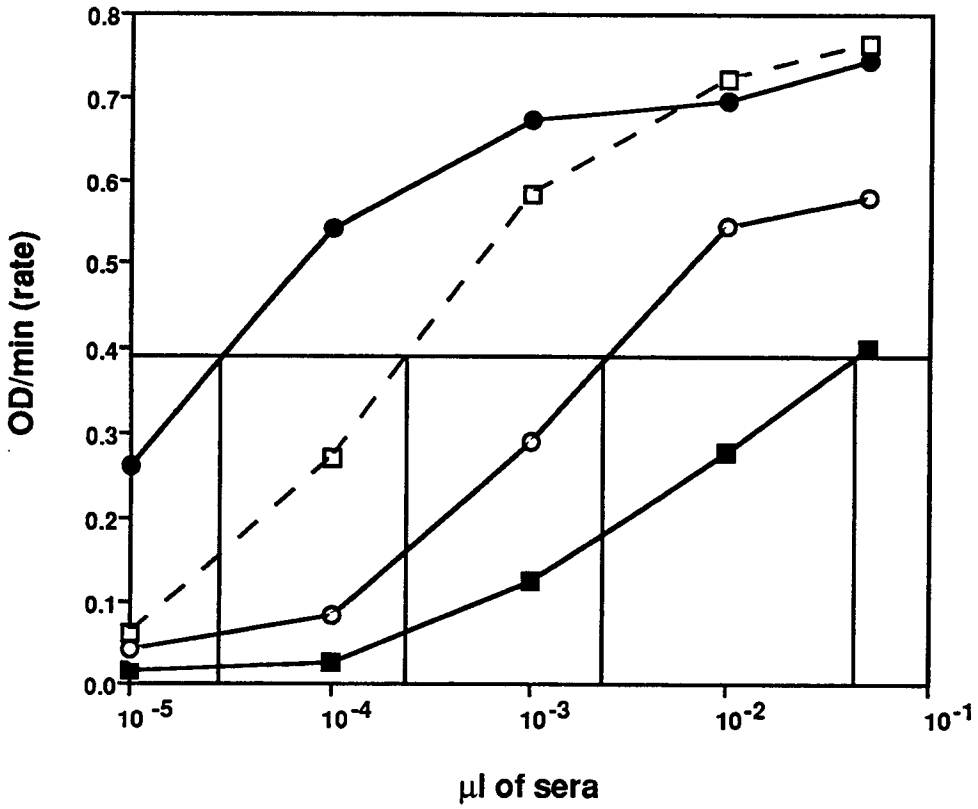

Figure 3.2. Quantitation of hyperimmune serum antibody activity from three samples and the pooled p57+ hyperimmune standard antisera. Data was expressed in OD/min for kinetic-based analysis over a 10 min period. The standard µl value was obtained by interpolation of a µl of serum giving 50% maximum optical density (OD) and assigned a value of 1 unit of antibody activity (U/µl). The amount of antibody activity was calculated using the equation cited by Arkoosh and Kaattari (1990). In this example the standard (□) was assigned a value of 10,000 U/µl; sample 1 (●), 93,000 U/µl; sample 2 (o), 1,000 U/µl; and sample 3(■), 80 U/µl.

1 h in mouse anti-trout-Ig (5 µg/ml) in 1% BSA-TTBS and washed, followed byperoxidase-conjugated goat anti-mouse-Ig (Hyclone, Logan, UT) diluted 1:5000 in 1% BSA-TTBS for 45 min at RT. Molecular weight markers and a lane of protein bands were stained for total protein with colloidal gold solution (Bio-Rad) using a procedure provided by the manufacturer. Blots were washed extensively and developed for 15 s using chemiluminescence (Amersham, Arlington Heights, IL).

Statistical Analysis

Antibody titers were log transformed and analyzed using a student t-test program on the Statgraphics software package to determine significance of each group of mean sera sample values tested in an ELISA against both $p57^+$ and $p57^-$ cells. The reported values in units of antibody activity per µl of serum (U/µl) were back transformed. P-values exceeding 0.05 were not considered significant.

Results

Quantitative and Qualitative Analysis of $p57^+$ and $p57^-$ *R. salmoninarum* Cells

To visualize the effects of 10 h heat treatment on surface proteins, a western blot of $p57^+$ and $p57^-$ cells was stained for total protein (Figure 3.3). A majority of the p57 molecule was reduced, while an increase in p36 as well as a number of additional lower molecular weight breakdown products was observed. Figure 3.4 illustrates the protein profiles of each cell type before and after heat treatment. The densitometric profile indicated p57 comprised approximately 70%

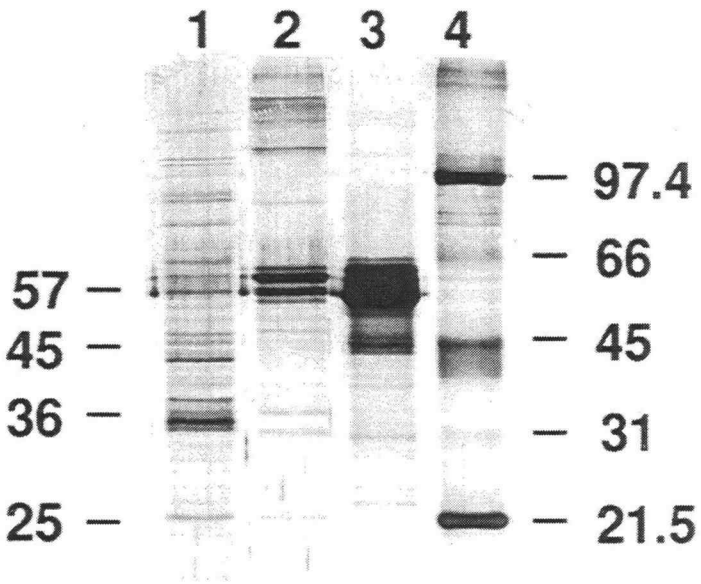

Figure 3.3. Analysis of p57 reduction on the cell surface of *Renibacterium salmoninarum* by 10% SDS-PAGE and total protein staining. Total protein stain of 50 µg/ml of *R. salmoninarum* ECP, native cells ($p57^+$), and cells that have been heat treated at 37°C for 10 h ($p57^-$). Lanes: 1, Formalin-killed $p57^-$ cells; 2, Formalin-killed $p57^+$ cells; 3, ECP; 4, Molecular weight standards are indicated in kilodaltons.

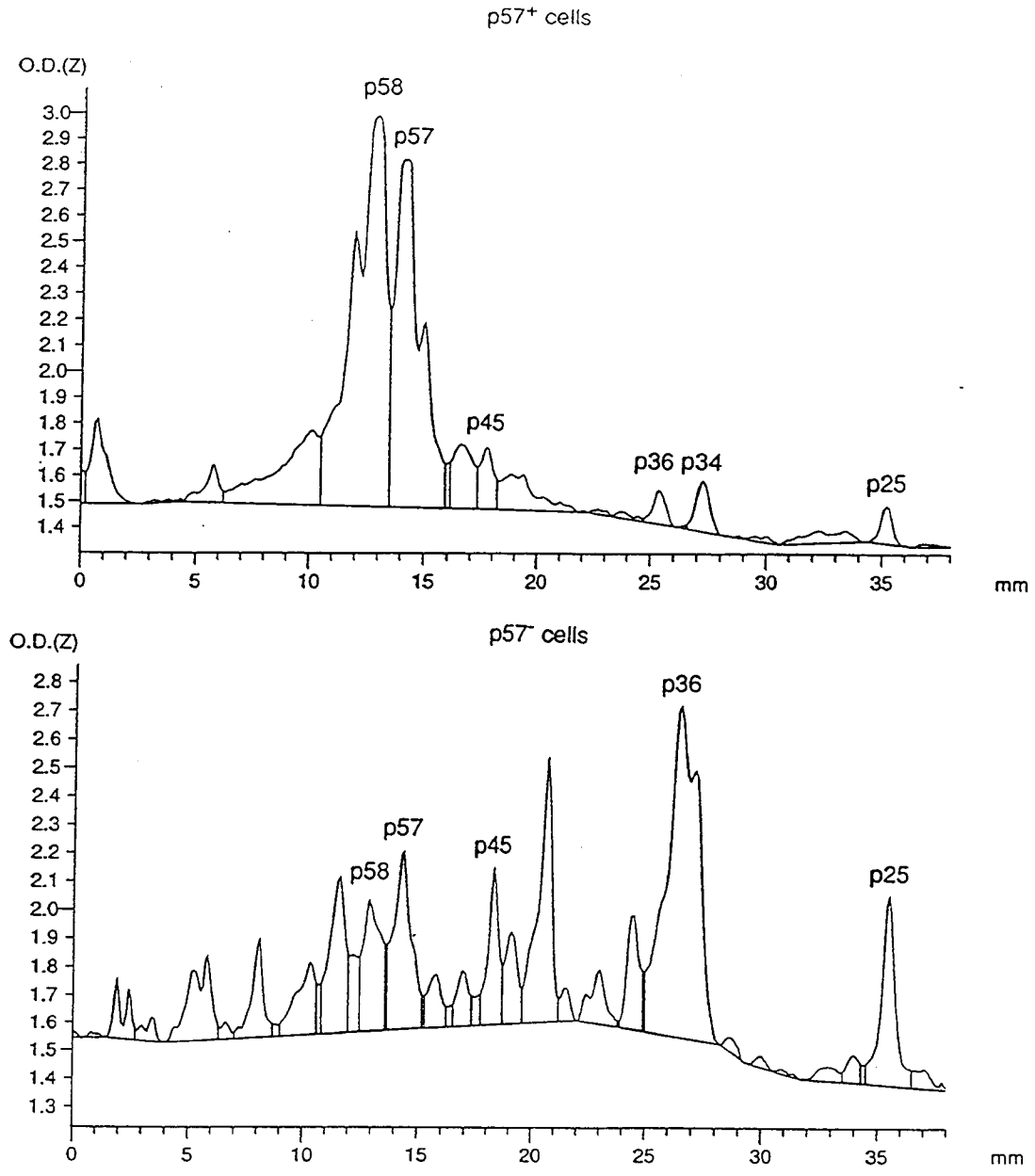

Figure 3.4. Densitometer scan of a total protein stained blot of *Renibacterium salmoninarum* p57+ and p57- cells as detected by colloidal gold staining. Peaks are labeled with proteolytic breakdown products corresponding to their molecular weight in kilodaltons. The x-axis signifies signal strength represented by intensity units (Z). The y-axis represents the distance scanned in millimeters (mm).

of all protein on p57+ cells, yet only 14% of the p57 molecule remained after heat treatment.

Determination of Antibody Activity to p57+ and p57- *R. salmoninarum* Whole Cells by ELISA (WCE)

A whole cell ELISA (WCE) was optimized to compare differences in antibody levels in serum samples from a population of fish hyperimmunized with p57+ or p57- cells. The range of antibody activity present in individual chinook salmon at 12 weeks post primary injection was <45 U/µl to 8,571 U/µl (Table 3.1). The mean titer against p57- cells from fish immunized with p57- cells was approximately 5-fold higher than the mean titer from fish immunized with p57+ cells. Sera from adjuvant injected fish (controls) had less than detectable titers. At 32.5 weeks post primary injection, chinook immunized with heat treated p57- cells had significantly higher antibody titers ($p \leq 0.01$) with an average of 25,704 U/µl compared to an average of 1,288 U/µl from fish immunized with native p57+ cells when reacted with p57- cells (Table 3.2). The mean titers of p57+ and p57- immune sera were comparable when assessed against p57+ cells. All control fish demonstrated less than detectable titers.

Detection of Primary Immunogenic Surface Proteins on p57+ and p57- *R. salmoninarum* Whole Cells by Immunoblotting

Western blots of p57+ (Figure 3.5, panel A) or p57- cells (Figure 3.5, panel B) of *R. salmoninarum* were probed with 32.5 week sera from 10 individual chinook immunized with p57+ cells and 5 chinook immunized with p57- heat treated cells to identify any differences in the primary immunogenic proteins that are recognized on non-heat treated and heat treated cells. The only particulate

Table 3.1. Quantitation of serum antibody from chinook salmon immunized with p57+ or p57- *Renibacterium salmoninarum* whole cells at 12 weeks post primary injection. Hyperimmune sera raised against p57+ cells was used as a relative standard

	Distribution of antibody activity as measured by ELISA (units/µl)[a]					
Fish No.	p57+ cells bound to ELISA plate wells			p57- cells bound to ELISA plate wells		
	p57-[b] sera	p57+[c] sera	control[d] sera	p57-[b] sera	p57+[c] sera	control[d] sera
1	55	58	<45	857	90	<70
2	55	88	<45	75	300	<70
3	55	58	<45	987	90	<70
4	<45	144	<45	750	300	<70
5	<45	88	<45	8,571	225	<70
Mean sera log titers[e]	51	83		832	174	

[a] Amount of fish antibody titer present corresponds to that found in a µl of serum giving 50% of the maximum OD reading and assigned a value of 1 unit of antibody activity as described in the Materials and Methods for 10 min at 405 nm.

[b] Corresponds to fish 1b-5b.

[c] Corresponds to fish 1c-5c.

[d] Corresponds to fish 1d-5d injected with adjuvant in PBS. Data corresponds to less than detectable titers.

[e] Mean of log transformed data was backtransformed.

* Statistically significant at $p<0.05$ when comparing each sera type against one kind of ELISA.

Table 3.2. Quantitation of serum antibody from chinook salmon immunized with p57+ or p57- *Renibacterium salmoninarum* whole cells at 32.5 weeks post primary injection[a]. Hyperimmune sera raised against p57+ cells was used as a relative standard

Fish No.	Distribution of antibody activity as measured by ELISA (units/µl)[b]					
	p57+ cells bound to ELISA plate wells			p57- cells bound to ELISA plate wells		
	p57-[c] sera	p57+[d] sera	control[e] sera	p57-[c] sera	p57+[d] sera	control[e] sera
1	136	93,000	<45	70,707	122,580	<70
2	95	1,000	<45	1,077	1,520	<70
3	12,667	80	<45	77,778	95	<70
4	475	3,500	<45	8,750	10,556	<70
5	1,357	1,000	<45	73,684	1,056	<70
6	88	987	<45	1,280	1,650	<70
7	6,563	113	<45	145,450	138	<70
8	636	75	<45	8,889	206	<70
9	NE[f]	5,807	<45	NE[f]	917	<70
10	65,625	487	<45	800,000	1,375	<70
Mean sera log titers[g]	1,096	955		*25,704	1,288	

[a] Fish were boosted at 190 days post primary injection as described in the Material and Methods.

[b] Amount of fish antibody titer present corresponds to that found in a µl of serum giving 50% of the maximum OD reading and assigned a value of 1 unit of antibody activity as described in the Materials and Methods for 10 min at 405 nm.

[c] Corresponds to fish 1c-10c.

[d] Corresponds to fish 1d-10d.

[e] Corresponds to fish 1e-10e injected with adjuvant in PBS. Data corresponds to less than detectable titers.

[f] Not enough serum available to perform the assay.

[g] Mean of log transformed data was backtransformed.

* Statistically significant at $p \leq 0.01$ when comparing each sera type against one kind of ELISA.

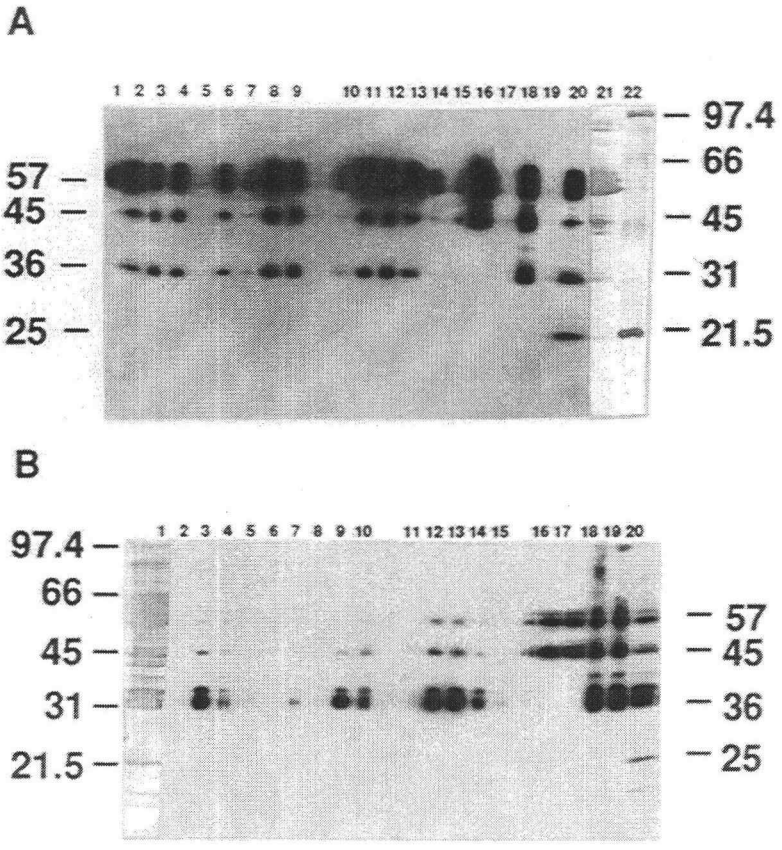

Figure 3.5. Immunoblot analysis with p57$^+$ and p57$^-$ *Renibacterium salmoninarum* hyperimmune chinook sera. *Renbacterium salmoninarum* p57$^+$ whole cells (panel A) or p57$^-$ whole cells (panel B) were probed with serum against p57$^+$ cells and p57$^-$ cells from individual fish, and three different p57 mAb controls. Lanes: 1-9, p57$^+$ sera; 10-14, p57$^-$ sera; (panel A), 2-10, p57$^+$ sera; 11-15, p57$^-$ sera; (panel B), 16, mAb 4D3; 18, mAb 3H1; 20, mAb 1A1; 21 (panel A), total protein stain of p57$^+$ cells; 1 (panel B), total protein stain of p57$^-$ cells. Molecular weight standards are indicated in kilodaltons.

cell-associated proteins recognized by either p57+ or p57- hyperimmunized chinook salmon antibodies were p57 and its breakdown products (ECP). Most of the antibody response to p57+ cells was directed against p57, while serum antibodies reacted predominantly with p36 on p57- cells. Recognition patterns of mAbs from groups I (4D3), II (3H1), and III (1A1) to the bacterial associated breakdown fragments were consistent with those described by Wiens and Kaattari (1991).

Immunogenic Composition of *R. salmoninarum* p57+ and p57- Cells After Protein and Carbohydrate Disruption

To determine the extent of antibody recognition to different immunogenic components on *R. salmoninarum* with serum from chinook immunized with p57+ or p57- cells, each cell type had surface protein degraded by proteinase-K or surface carbohydrate degraded by periodate (Table 3.3). Comparable coating concentrations of each digested cell type (using the peroxidase saturation technique), revealed only an average of 4% of p57- serum activity remained after carbohydrate disruption. In addition, most (80-85%) of the antibody response in p57- serum remained after protein digestion. Further, periodate treatment of p57+ cells slightly enhanced reactivity of p57- serum to p57+ cells. However, titers to untreated p57+ cells were approximately 180-fold lower than to p57- cells (1,404 U/µl versus 250,000 U/µl), indicating this apparent increase was relatively marginal.

Discussion

The nature of protective antigens of *R. salmoninarum* and their immunogenicity in salmonids remain largely uncharacterized. This study utilized

Table 3.3. Enzymatic and chemical digestion of $p57^+$ and $p57^-$ *Renibacterium salmoninarum* whole cells

Whole cell type	Average serum antibody activity (%) detected after digestion with:[a]		Average serum antibody as measured by ELISA (units/µl)[b]
	Proteinase-K	Periodate	Untreated cells
$p57^+$ cells	80	430	1,404
$p57^-$ cells	85	4	250,000

[a] Values represent the average percentage of antibody activity (units/µl) remaining from two chinook bled 32.5 weeks post primary injection after digestion of whole cell antigen with proteinase-K for 24 h in 0.1 M tris HCl buffer (pH 8.0) and periodate for 24 h at 4°C, as described in the materials and methods.

[b] Amount of fish antibody produced was determined as described in Table 3.1, footnote b.

a novel method for removing a majority of the immunosuppressive, putative virulence factor, p57, from the surface of whole cells by heating at 37°C (Piganelli et al. unpublished, a) which enhances the activity of a *R. salmoninarum* endogenous serine protease. This removal was associated with increased antigenicity and immunogenicity. Whole cell ELISA analysis of hyperimmune chinook antiserum to $p57^-$ *R. salmoninarum* revealed significantly elevated (20-fold) specific serum antibody titers.

In carp, vaccines prepared from heat-killed *Aeromonas hydrophila* cells induced a higher agglutinating antibody titer over a period of 32 weeks than did formalin-killed cells (Lamers and Van Muiswinkel 1986). The authors speculate that heating may release more antigenic material without altering antigen structure and hence proper antigen processing by macrophages. Our heat treated $p57^-$ whole cell preparation would appear to be a promising candidate for an experimental vaccine.

In an attempt to identify the nature of the antigens remaining on the $p57^-$ cell which induce the high titered response, immunoblot analysis was performed with $p57^+$ and $p57^-$ sera samples using $p57^+$ and $p57^-$ cells. Only three or four denatured immunogenic proteins were detected on either cell type by the salmon antisera, namely the p57 molecule and its proteolytic breakdown products. Few antigenic fungal protein bands were detected by Wethered et al. (1988) in immunoblots probed with infected human sera. Their result coupled with significant IgM levels, led these authors to suggest the antibody response may be against carbohydrate antigens which are not detectable on an immunoblot. While every fish tested responded to $p57^+$ and $p57^-$ cell-associated ECP, there was individual variation in intensity of recognition to these antigens despite incubating blots with serum of equivalent antibody activity (1 U/µl). Also, the greater recognition of p57 on $p57^+$ cells shifted to p36 on $p57^-$ cells. This

correlated with the decrease in total protein and band density of p57 and the increase in p36 after 37°C treatment as analyzed by total protein staining and densitometry. It did not appear that band density corresponds to the amount of antibody activity as other non-reactive bands had signals similar in intensity to some of the breakdown products.

Additionally, disruption of $p57^-$ cells by periodate caused $p57^-$ serum titers to diminish. Although cell-associated proteins did elicit an antibody response, periodate sensitive moieties, probably carbohydrate components, may be responsible for a majority of the elevated serum antibody titers to $p57^-$ heat treated cells. These results suggest the importance of *R. salmoninarum* carbohydrates as immunogens. It is unknown why reactivity to $p57^+$ cells increased slightly after periodate exposure. Perhaps chemical cleavage exposes carbohydrate antigens via conformational changes causing displacement of protein conjugated to carbohydrate, yet remaining protein is still able to sterically hinder a portion of the carbohydrate antibody binding sites. Also, if $p57^+$ cells only induce anti-protein antibodies, periodate treatment may increase reactivity with these proteins. Heat treatment may change the physicochemical properties of the cell surface as well, such that more carbohydrate is uncovered.

We propose several hypothetical models by which this organism may minimize antibody effectiveness and may explain the heightened antibody response and improved antigenicity of $p57^-$*R. salmoninarum*. In one model, p57 may occlude the antibody interaction with other immunogenic surface antigens. In this case, proteolytic modification by an endogenous serine protease after heating may increase surface exposure of immunogenic carbohydrate epitopes or other protein antigens normally masked by p57. There are a number of reports of occlusion by different components of gram-negative bacterial species, which include blocking of antigens by S layer proteins, concealment of cell wall

and O antigens by capsule polysaccharide (Orskov et al. 1963; Stirm et al. 1971; Wu and Park 1971; King and Wilkison 1981; Kopecko and Formal 1984; Kadurugamuwa et al. 1985a, b; Cross et al. 1986; Williams 1987; Williams et al. 1986, 1988a; Johne et al. 1989), and shielding by the LPS O antigen (Ng et al. 1976; Graham and Stocker 1977; Mutharia and Hancock 1983; Jessop and Lambert 1985; Van der ley 1986; Williams et al. 1988b; Pollack et al. 1989) and outer core (Gigliotti and Shenep 1985; Nelson 1990).

Dooley and Trust (1985) report masking of the LPS core oligosaccharide-polysaccharide linkage by the S layer protein in the fish pathogen *Aeromonas hydrophila*. Sulfo-NHS-biotin cell surface labeling shows occlusion of outer membrane (OM) antigens by the 52 kd major surface array protein or S layer in *A. hydrophila*. The restricted access of this compound as shown by immunoblotting, demonstrated that the OM proteins from selected strains of *A. hydrophila* are shielded from immune recognition (Dooley and Trust 1988). *Treponema pallidum* is known to absorb a surface coat of host proteins which may play a role in masking key immunogens (Alderete and Baseman 1979).

There are many ways in which a physical barrier of a bacterium may be disrupted thereby unmasking surface antigens. Chemical degradation is known to partially remove O antigens on rough mutants unmasking rough bacterial surface sites (Luderizt et al. 1966). Also, it is well documented that 55^0C heat treatment of gram-negative bacterial cells destroys the integrity of the OM allowing better penetration of antibodies for reaction with core epitopes in O-polysaccharide$^-$ mutants (Lieve 1974; Tsuchido et al. 1985). Further, removal of the *E. coli* K antigen by heating or enzymatically unmasks O antigens allowing agglutination by anti-O serum (Orskov et al. 1963; Stirm et al. 1971). In addition, heating is known to increase antibody binding to whole bacteria (Nelson 1991). Aydintug et al. (1989) demonstrate that monoclonal antibodies specific to lipid A

of *E. coli* had greater reactivity with boiled than with live cells. Further, antibody reactivity in this model was dependent on the physical state of the bacterium or LPS, the assay used , and the specificity of the antibody. These findings support our proposed model for increased antibody activity after removal of the *R. salmoninarum* p57 antigen by heating, which may unmask other protein or polysaccharide antigens allowing greater antibody accessibility.

Finally, we cannot rule out that the conformation of heat treated cells immobilized to a solid matrix may differ from untreated cells in suspension. This may have made determinants more accessible to $p57^-$ antibodies by alteration during binding, a premise suggested by Nelson et al. (1990). However, we feel this kind of effect probably contributed minimally if at all to the overall antibody activity to $p57^-$ versus $p57^+$ cells. In fact, in specific cases the ELISA can offer greater sensitivity.

Another effective abrogating mechanism of specific humoral immunity are processes that prevent antibody synthesis. In this study, perhaps the immunosuppressive protein, p57, was inactivated after cleavage by the heat activated serine protease. Bacterial components from a number of mammalian and fish pathogens have been demonstrated to alter immune responsiveness of the animal host. Extracellular products of oral bacteria have been known to inhibit lymphocyte mitogenesis (Higerd et al. 1978). Hastings and Ellis (1988) speculate that the poor immunogenicity of the formalin-activated ECP protease of *A. salmonicida* in trout may be due to suppression of the antibody-producing system by the protease. The soluble ECP of *R. salmoninarum* has been shown to inhibit antibody production in coho salmon B-cells *in vitro* (Turaga et al. 1987b). In addition, heating purified extracellular and cell-associated ECP, which resulted in the degradation of p57 and other immunoreactive ECP components, caused a reduction of its immunosuppressive function (Rockey et al. 1991).

Perhaps in this study, we are seeing the same effect as indicated by the increase in antibody production after p57 degradation by a proteolytic enzyme.

In conclusion, we have identified an enhanced antibody response to a novel form of a potentially protective whole cell antigen. We reasoned that perhaps eliminating virulence factors from experimental vaccine material may enhance immunogenicity and benefit vaccine design. This was done by physically modifying the whole cell antigen by heat without destroying its antigenicity which significantly increased the magnitude of the anamnestic antibody response as well as antigenicity. Our results would be more consistant with the possibility that p57 may be occluding crutial polysaccharide antigens. Future studies however, will be required to determine if passive immunization with heat-treated $p57^-$ sera antibodies may protect salmon. Campbell et al. (1994) report greater serum killing and lower mortalities in fish injected with $p57^+$ Rs challenge survivor serum after 8 weeks exposure versus controls in rainbow trout.

4. IDENTIFICATION OF A IMMUNOLOGICALLY CROSS-REACTIVE 60 KILODALTON *Renibacterium salmoninarum* PROTEIN WHICH IS DISTINCT FROM p57: IMPLICATIONS FOR IMMUNODIAGNOSTIC ASSAYS

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Abstract

Renibacterium salmoninarum produces a major surface 57 kDa protein (p57) targeted for use in antibody-based diagnostic assays. Recently, reports suggest that p57 has immunologically related comigrating homologs in other species of gram-positive bacteria. Because of the implications of cross-reactivity to the utility of these assays, we used immunoblotting to evaluate both monoclonal antibodies (mAbs) directed at p57 and polyclonal anti-*R. salmoninarum* sera as probes for lysates of *R. salmoninarum* and other selected bacteria. Immunoblots were also probed with polyclonal antisera against the 60 kDa heat shock protein (HSP 60) from a phylogenetically distinct species. Anti-*R. salmoninarum* polyclonal serum recognized a 57 kDa protein in three other gram-positive bacteria, but commercially available antisera which had been affinity-purified with *R. salmoninarum* whole cells did not. We also confirmed that anti-p57 mAbs do not detect similarly-sized proteins in other bacterial species. Finally, we demonstrated that antigenically conserved 60 kDa heat shock proteins which comigrate with p57 may be responsible for cross-reactivity previously demonstrated with anti-*R. salmoninarum* polyclonal sera. These results provide a possible explanation for cross-reactivity seen when anti-*R. salmoninarum* polyclonal sera are used in immunoassays, and confirm the need to examine the specificity of both polyclonal and monoclonal antibody reagents to avoid false-positive reactions.

Introduction

Many immunodiagnostic techniques for the salmonid kidney disease bacterium, *Renibacterium salmoninarum,* have been employed to detect this slow growing, fastidious pathogen (Chen et al. 1974; Bullock and Stuckey 1975; Bullock et al. 1980; Kimura and Yoshimizu 1981; Cipriano et al. 1985; Dixon 1987; Pascho and Mulcahy 1987; Sakai et al. 1987; Turaga et al. 1987; Gudmonsdottir et al. 1993). Cross-reactivity is a common problem encountered in immunoassays which utilize polyclonal antisera directed against *R. salmoninarum* (Bullock et al. 1980; Evelyn et al. 1981; Austin et al. 1985; Dixon 1987; Turaga et al. 1987; Yoshimizu et al. 1987; Pascho et al. 1991; Barbash 1992; Foott et al. 1992; Gudmonsdottir et al. 1993). While cross-reactive monoclonal antibodies (mAbs) against *R. salmoninarum* have been produced (Arakawa et al. 1987), several mAbs directed at a major cell surface protein (p57) are specific as they do not cross-react with other tested fish pathogens (Wiens and Kaattari 1989). Subsequetly, Wiens and Kaattari (1991) developed six additional anti-p57 mAbs and mapped the general location of their epitopes based on the differential recognition of proteolytic breakdown products of p57 present in culture supernatant. Group I mAbs (4D3, 4C11, and 4H8) recognize epitopes proximal to the amino-terminus of p57, group II mAbs (3H1 and 2G5) bind to epitopes in the central region, while group III mAbs (1A1, 4D10, and 2G9) recognize epitopes associated with the carboxy-terminus. The presence of unique epitopes on p57 combined with the high levels of expression has proven useful in the development of diagnostic assays to detect the presence of p57 and breakdown products in fish sera and tissues (Hsu et al. 1991; Pascho and Mulcahy 1987; Rockey et al. 1991). Antibodies from group I and II are used in

ELISA-based diagnostic assays and can detect *R. salmoninarum* in symptomatic and asymptomatic infected salmonids (Hsu et al. 1991; Rockey et al. 1991).

Recently, it has been reported that a 57 kDa cross-reactive antigen from selected gram-positive organisms is recognized by polyclonal antisera from rabbits immunized with *R. salmoninarum* whole cells (Bandin et al. 1993; Toranzo et al. 1993). These authors have suggested that a common p57 protein is produced by *R. salmoninarum, Corynebacterium aquaticum,* and *Carnobacterium piscicola.* Because of the implications of this possibility in the use of antibody-based immunoassays for the detection of *R. salmoninarum* in fish populations, we evaluated these results using anti-*R. salmoninarum* polyclonal sera and seven anti-p57 mAbs as probes for immunoblots of several bacterial species. We also used antisera against a phylogenetically conserved 60 kDa common antigen (HSP 60) to probe the same group of bacterial species to examine the possibility that this molecule is a cross-reactive protein which may comigrate with *R. salmoninarum* p57. The results indicated that anti-p57 mAbs and affinity-purified polyclonal antisera do not cross-react with 57-60 kDa proteins in lysates of other tested bacterial species. In addition, anti-HSP 60 sera recognized a 60 kDa protein produced by *R. salmoninarum* and a number of other gram-positive bacteria. Therefore, we propose that the HSP 60 molecule, a protein which is structurally, functionally and antigenically conserved across all phylogenetic groups, is a likely candidate for the cross-reactivity reported in previous papers (Bandin et al. 1993; Toranzo et al. 1993).

Materials and Methods

Bacterial Isolates and Culture Conditions

Renibacterium salmoninarum isolate D-6 was grown in kidney disease medium (KDM II) for 14 d at 17°C (Evelyn 1977) excluding calf serum. After centrifugation (6,000 x g, 30 min, 4°C) cell pellets were washed and microfuged (16,000 x g, 2 min) three times with 1 ml cold 10 mM phosphate buffered saline (PBS)(0.85% NaCl, 10 mM $NaPO_4$, [pH 7.2]), resuspended to a concentration of 200 mg/ml and stored at -70°C. Soluble ECP was harvested from the supernatant as previously described (Turaga et al. 1987). Strain MT239, a non-agglutinating, less virulent, p57 deficient *R. salmoninarum* isolate (Bruno 1988) was grown in biphasic medium at 17°C (Daly and Stevenson 1985). *Chlamydia psittaci* elementary bodies were cultured and purified as described by Caldwell et al. (1981). Other bacterial strains and their culture conditions are shown in Table 4.1.

Production of Antibodies

One milliliter of formalin-killed *R. salmoninarum* (1 mg/ml) was mixed with an equal volume of Freund's incomplete adjuvant (FIA) and injected subcutaneously and intramuscularly into a New Zealand white rabbit. After a boost 75 d post-primary immunization, the animal was euthanized and bled 60 d post-secondary immunization. Serum was collected, aliquoted, and stored at -70°C. Production and characterization of the mAbs 4D3, 4C11, 4H8, 3H1, 2G5,

TABLE 4.1. Strain designation and culture conditions of the tested bacterial species

Bacteria	Strain designation	Medium[a]	Growth temperature (°C)	Growth time (hours)
Aeromonas hydrophila	OSU culture collection	BHI	30	24
Arthrobacter globiformis	ATCC 8010	TSB/yeast	25	36
Bacillus cereus	OSU culture collection	BHI	37	24
Bacillus subtilis	ATCC 6633	BHI	37	24
Carnobacterium piscicola	ATCC 35586	BHI	25	48
Corynebacterium aquaticum	ATCC 14465	BHI	25	48
Pseudomonas aeruginosa	ATCC 10145	BHI	37	24
Pseudomonas fluorescens	OSU culture collection	BHI	30	24
Renibacterium salmoninarum	D-6	KDM II	17	14 days
Renibacterium salmoninarum	MT 239	CA	17	14 days
Vibrio anguillarum	LS1-74	TSB	25	24

[a] Media: BHI, brain heart infusion broth (Difco, Detriot, MI); TSB, trypticase soy broth supplemented with 2 g/L yeast (pH 7.0) (Difco); CA, charcoal agar (Sigma, St. Louis, MO)(Daly and Stevenson 1985); KDM II, kidney disease medium (Evelyn 1977).

1A1 and 4D10 used in these experiments have been described previously (Wiens and Kaattari 1989, 1991). Ascitic fluid was purified using the caprylic acid procedure of Russo et al. (1983). After precipitation with 50% saturated ammonium sulfate, pellets were dissolved and dialyzed extensively against PBS. Monoclonal antibodies were diluted 1:1 in glycerol and stored at -20°C.

Polyclonal antisera to the 60 kDa chlamydial heat shock protein (HSP 60) were produced in Hartley strain guinea pigs. Approximately 100 mg of affinity-purified HSP 60 in 2 ml of sterile saline was mixed with the Ribi trivalent adjuvant (Ribi Immunochem Research, Hamilton, MT). Guinea pigs were immunized and sera were collected using procedures supplied by the manufacturer.

Electrophoresis and Immunoblotting

Bacterial lysates were prepared using the method of Bandin et al. (1993) for the two different blotting procedures. Washed bacteria were mixed 1:1 in sample buffer and boiled for 3 min. Immunoblots probed with rabbit anti-*R. salmoninarum* polyclonal serum, commercially available affinity purified-peroxidase labeled goat anti-*R. salmoninarum* sera (Kirkegaard and Perry Laboratories, Gaithersburg, MD), or anti-p57 mAbs were prepared using a mini-gel format. Samples were electrophoresed through 10% polyacrylamide gels in a Mini-Protean II electrophoresis apparatus (Bio-Rad Laboratories, Richmond, CA) at 200 V for 1 h. After electrophoresis, gels were equilibrated in transfer buffer (192 mM glycine; 20 mM tris, pH 8.3; 20% methanol), and proteins were electrophoretically transferred to Immobilon-P (Millipore, Bedford, MA) at 100 V for 1 h with cooling. Whole cell lysates were stained for total protein with colloidal gold solution (Bio-Rad) using a procedure provided by the manufacturer.

To corroborate cross-reactivity seen with anti-*R. salmoninarum* polyclonal sera, membranes were blocked with 3% gelatin in tris-buffered saline (TBS; 50 mM tris, 1 mM EDTA, 0.87% NaCl, pH 8.0) for 1 h. After washing 30 min, immunoblots were incubated for 1 h in rabbit anti-*R. salmoninarum* polyclonal serum diluted 1:1,000 in 1% gelatin-TBS. Blots were washed three times in TBS with 0.1% Tween-20 (TTBS) and incubated for 1 h in a 1:1,500 dilution of biotinylated-goat anti-rabbit-Ig (Zymed, San Francisco, CA). Blots were washed three times in TTBS and incubated for 1h in streptavidin-conjugated horseradish peroxidase (Vector, Burlingame, CA) at a dilution of 1:3,000. After extensive washes, immunoreactive proteins were visualized using chemiluminescence (Amersham, Arlington Heights, IL). A commercial peroxidase-conjugated anti-*R. salmoninarum* polyclonal sera was also examined for cross-reactivity. For this procedure, blots were blocked in 1% bovine serum albumin (Fraction V, Sigma; BSA) in TTBS overnight at 4^{o}C. After washing in TTBS, blots were probed with this antisera for 2 h at room temperature. Blots were washed and developed using chemiluminescence.

To test whether anti-p57 mAbs were cross-reactive with antigens in other bacterial species, blots were blocked in 1% BSA-TTBS overnight. After three, 10 min washes in TTBS, blots were incubated with each mAb (1 μg/ml) separately for 1 h and washed. A 45 min incubation in peroxidase-conjugated, goat anti-mouse-Ig (Hyclone, Logan, UT) at a dilution of 1:1,500 was followed by extensive washing and detection of bands with chemiluminescence.

To examine cross-reactivity of anti-HSP 60 sera, bacterial lysates were electrophoresed through 12.5% polyacrylamide gels and proteins were transferred to nitrocellulose in 25 mM phosphate buffer as described (Rockey and Rosquist 1994). Following transfer, blots were blocked in 2% BSA in PBS (150 mM NaCl, 10 mM PO_4, pH 7.4) plus 0.1% Tween-20 (TPBS). Antisera from

two immunized guinea pigs were pooled and diluted 1:1,000 in BSA-TPBS prior to incubation on the blots. Blots were incubated 2 h in primary antisera, and washed three times with TPBS. Immunoblots were incubated for 1 h in ^{125}I-labeled staphylococcal protein A (approximately 124 nCi/ml in BSA-TPBS; New England Nuclear, Boston, MA). Blots were washed three times in TPBS, dried, and exposed overnight to autoradiography film.

Results

Immunoblot Analysis with Anti-*R. salmoninarum* Polyclonal Serum

Rabbit polyclonal serum directed against *R. salmoninarum* was used as a probe for immunoblots of selected gram-positive bacterial species. This antiserum reacted with p57 and its breakdown products (Turaga et al. 1987; Figure 4.1), as well as other uncharacterized antigens. There were also reactive bands with a molecular weight similar to p57 in each of the other tested strains, consistent with data described by Bandin et al. (1993) and Toranzo et al. (1993). Affinity-purified anti-*R. salmoninarum* sera did not cross-react with any of the other tested gram-positive or gram-negative bacterial lysates (not shown).

Specificity of Anti-p57 mAbs to *R. salmoninarum*

Whole cell lysates of seven gram-positive and four gram-negative bacteria were probed by immunoblotting to verify the specificities of a panel of anti-p57 mAbs. The mAbs only recognized *R. salmoninarum* p57 and breakdown products (Figure 4.2, panel A-C, lane 5) and none of the proteins produced by *C. aquaticum, C. piscicola* and *A. globiformis.* Total protein profiles shown in Figure 2, panel D indicate that approximately equal amounts of total protein were

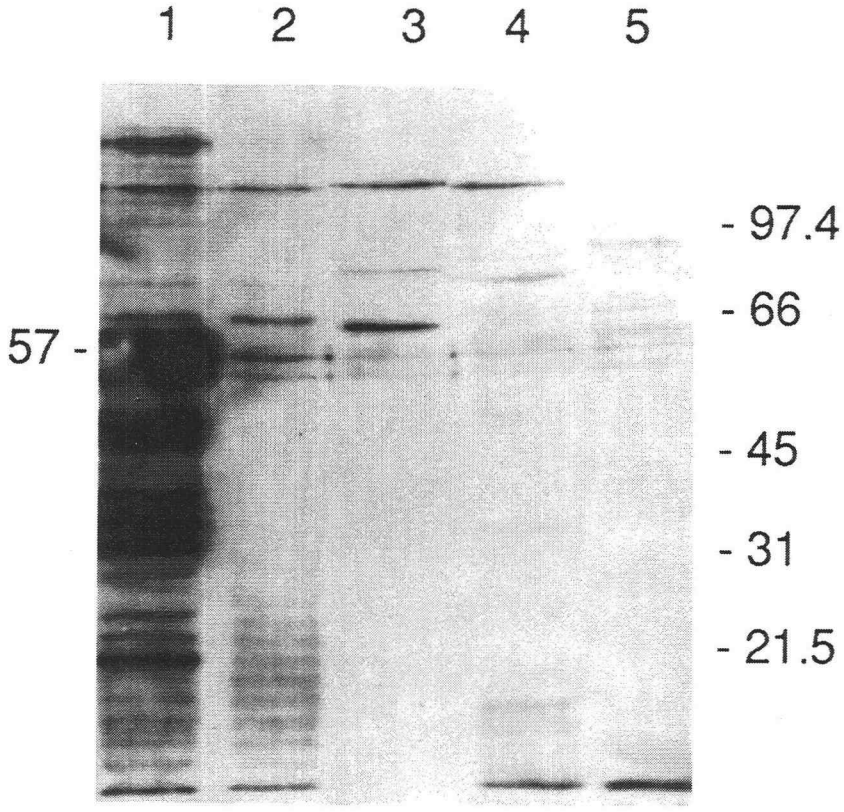

Figure 4.1. Immunoblot analysis with anti-*Renibacterium salmoninarum* polyclonal serum. Lysates of *R. salmoninarum* and other gram-positive bacteria were probed with serum against *R. salmoninarum*. Lanes: 1, D-6; 2, MT239; 3, *Corynebacterium aquaticum*; 4, *Carnobacterium piscicola*; 5, *Arthrobacter globiformis*. Molecular weight standards are indicated in kilodaltons.

Figure 4.2. Specificity of anti-p57 monoclonal antibodies by SDS-PAGE and immunoblotting. Total protein profile from lysates of *Renibacterium salmoninarum* and other gram-positive bacteria as detected by colloidal gold staining (panel D), and immunoblot analysis with mAb 4D3 (panel A), 3H1 (panel B) and 1A1 (panel C). Lanes: 1, *Arthrobacter globiformis*; 2, *Carnobacterium piscicola*; 3, *Corynebacterium aquaticum*; 4, MT239; 5, D-6. Molecular weight standards are indicated in kilodaltons.

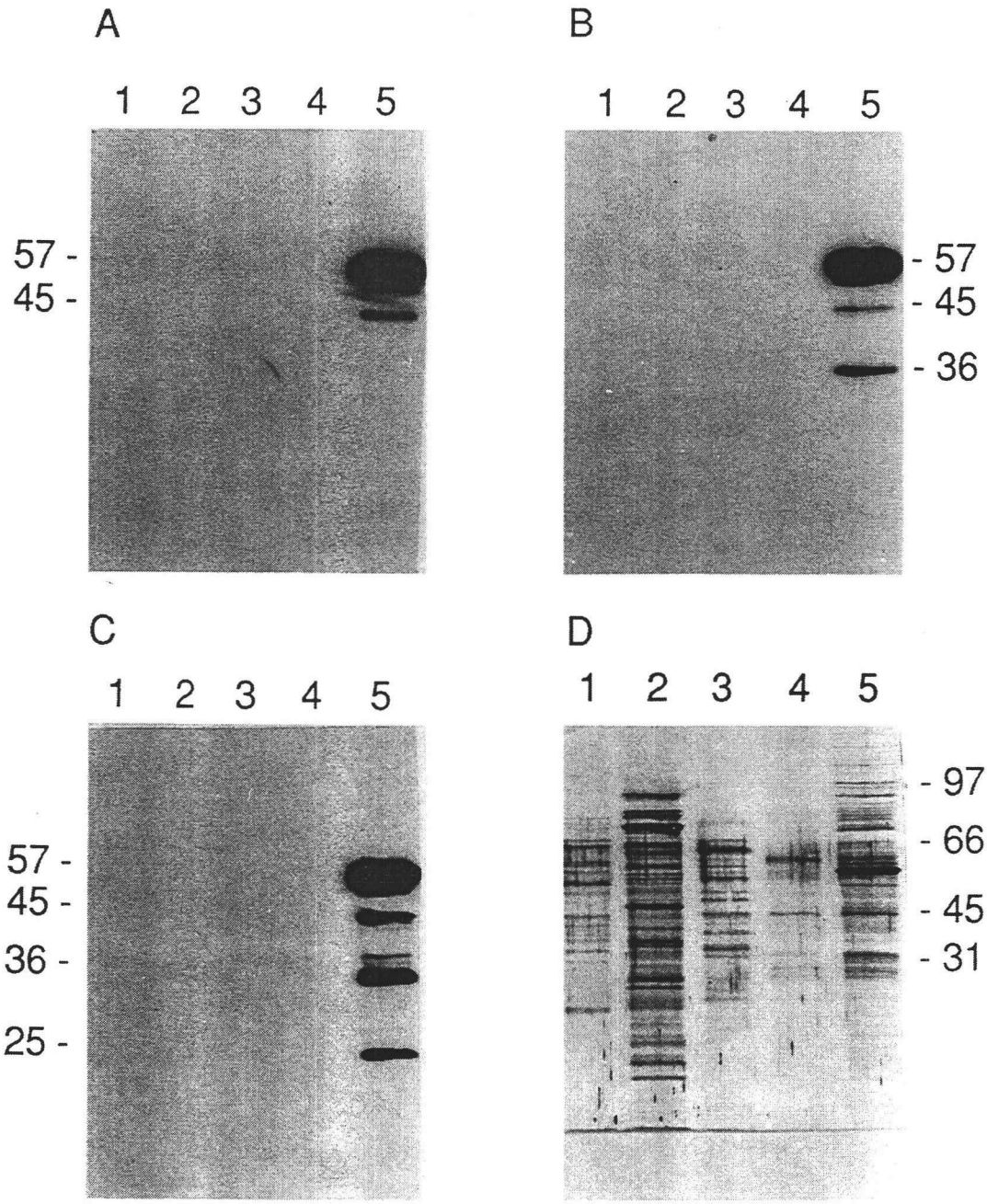
A
1 2 3 4 5
57 -
45 -
B
1 2 3 4 5
- 57
- 45
- 36
C
1 2 3 4 5
57 -
45 -
36 -
25 -
D
1 2 3 4 5
- 97
- 66
- 45
- 31

Figure 4.2.

present on the immunoblots. The immunoreactivty patterns of these mAbs against lysates of *R. salmoninarum* whole cells are consistent with those described by Wiens and Kaattari (1989, 1991) for groups I, II, and III mAbs. Monoclonal antibodies 4D3, 4C11, 4H8, 3H1, 2G5, 1A1, and 4D10 were also tested in immunoblots against all of the different bacteria listed in Table 4.1. Anti-p57 mAb 1A1 and 4D10 (group III) recognized a protein of approximately 30 kDa in *Vibrio anguillarum,* but there was no other evidence of cross-reactivity between the seven mAbs and any proteins in the bacterial lysates (Table 4.2). No reactivity of the anti-p57 mAbs with *R. salmoninarum* strain MT 239 was detected (Figure 4.1, panel A-D, lane 4), which has been previously shown to be deficient in p57 (Daly and Stevenson 1990).

Immunoblot Analysis with Anti-Chlamydial HSP 60 Sera

Immunoblot analysis using guinea pig anti-chlamydial HSP 60 sera detected a cross-reactive protein which migrated at a MW of approximately 60 kDa in lysates of *R. salmoninarum, C. aquaticum, C. piscicola* and *C. psittaci* (Figure 4.3, panel A). Proteins of approximately 60 kDa were detected in the other tested strains, with the exception of two *Bacillus* species (Table 4.2). No immunoreactive proteins were detected in *R. salmoninarum* ECP by anti-HSP 60 sera (Figure 4.3, panel A), nor was chlamydial HSP 60 recognized by anti-p57 mAb 4D3 (Figure 4.3, panel B; other mAbs not shown).

Discussion

Both public fish health management professionals and the private aquaculture industry rely on the specificity and sensitivity of methods for the

Table 4.2. Immunoblot analysis of bacterial lysates probed with seven anti-p57 monoclonal antibodies to *Renibacterium salmoninarum* or anti-chlamydial HSP 60 sera

Bacteria	p57 mAbs	HSP 60 Sera
Aeromonas hydrophila	-	+
Arthrobacter globiformis	-	+
Bacillus cereus	-	-
Bacillus subtilis	-	-
Carnobacterium piscicola	-	+
Corynebacterium aquaticum	-	+
Pseudomonas aeruginosa	-	+
Pseudomonas fluorescens	-	+
Renibacterium salmoninarum D-6	+	+
Renibacterium salmoninarum MT 239	-	+
Vibrio anguillarum	+[a]	+

[a] Group III anti-p57 mAbs 1A1 and 4D10 reacted with a 30 kDa protein.

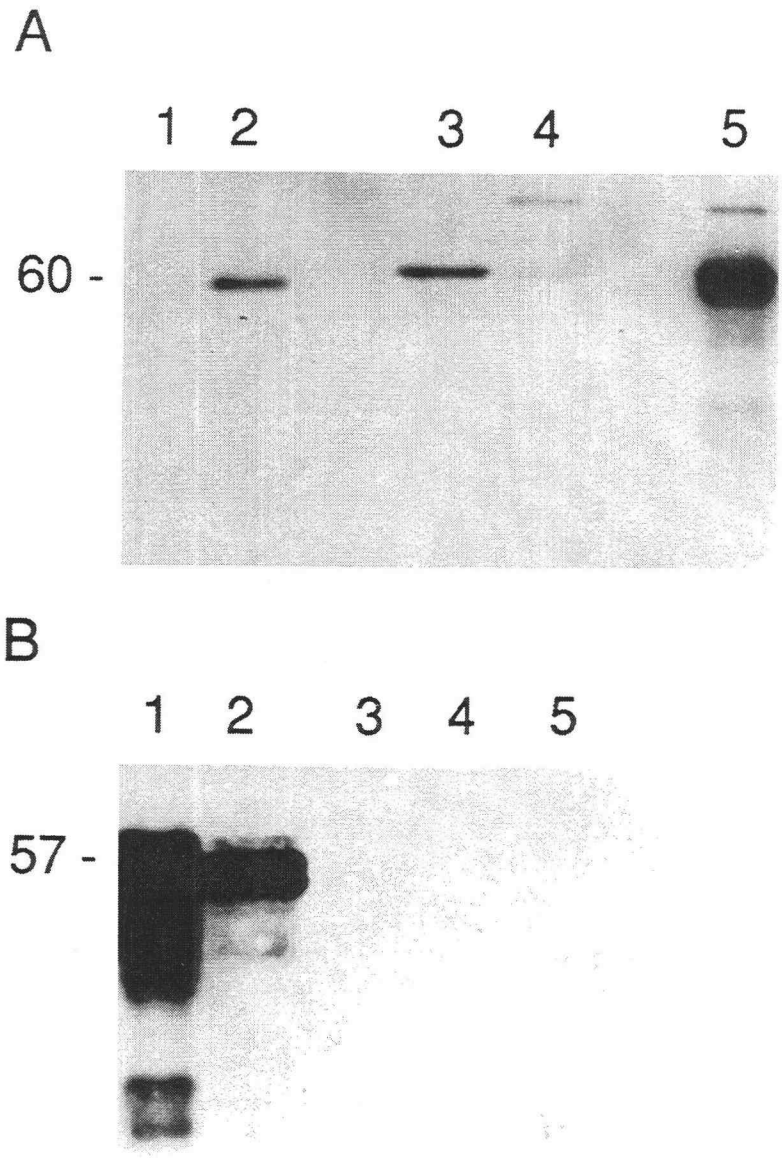

Figure 4.3. Immunoblot analysis with anti-chlamydial HSP 60 sera and anti-p57 monoclonal antibodies. Lysates of *Renibacterium salmoninarum*, other gram-positive bacteria and a gram-negative control bacteria were probed with anti-chlamydial HSP 60 sera (panel A), or mAb 4D3 (panel B). Lanes: 1, *R. salmoninarum* ECP; 2, D-6; 3, *Carnobacterium piscicola*; 4, *Corynebacterium aquaticum*; 5, *Chlamydia psittaci* strain GPIC elementary bodies. Molecular weight standards are indicated in kilodaltons.

detection of pathogenic microorganisms. Rapid and inexpensive diagnostic tools are important for the certification of pathogen-free stocks and for monitoring the overall health of wild and hatchery populations. The reduction of false-positive reactions using specific reagents will allow the aquaculture industry to make more accurate, cost-effective management decisions, regarding the treatment and prevention of bacterial kidney disease (BKD) in salmonids. Many immunologic assays for *R. salmoninarum* detect free or cell-associated p57 within tested fish tissues. These assays are based on the premise that p57 is a unique protein antigen which is not found in other bacterial species.

A recent set of publications has examined this premise and concluded that the p57 protein is present in at least two other bacterial species, *C. aquaticum* and *C. piscicola.* This conclusion was based on the reactivity of polyclonal anti-*R. salmoninarum* sera with comigrating proteins in immunoblots of these bacteria (Bandin et al. 1993; Toranzo et al. 1993). Because the concept of a cross-reactive protein is crucial to the specificity and sensitivity of individual detection assays, we repeated and expanded upon these experiments.

The data shown in Figure 4.1 demonstrated that our preparation of anti-*R. salmoninarum* polyclonal rabbit serum cross-reacts with proteins of a MW similar to p57 in lysates of several other tested bacterial species. This result was consistent with those of Bandin et al. (1993) and Toranzo et al. (1993). Based on these data, the previous reports conclude that p57 and the cross-reactive 57-60 kDa protein found in the other species are related. However, it is possible that p57 is not related to the described cross-reactive proteins, and that the cross-reactivity is a function of recognition by polyclonal antisera of two unrelated proteins with a similar molecular weight.

The cross-reactivity observed with anti-*R. salmoninarum* polyclonal serum contrasted to results seen with monoclonal anti-p57 antibodies, which never

detected a 57-60 kDa comigrating protein in any tested species except *R. salmoninarum*. These results demonstrate that anti-p57 mAbs do not recognize epitopes present on these putative cross-reactive proteins and support the conclusion that p57 is not present in the other tested strains. These results are also consistent with other reports which demonstrate the specificity of group I or II anti-p57 mAbs in ELISA or immunoblot experiments (Wiens and Kaattari 1989; Reddington 1993). The lack of cross-reactivity observed with these antibodies is important because mAbs from groups I and II are used in published and commercially available ELISAs for the detection of *R. salmoninarum* in fish tissues. (Hsu et al. 1991; Rockey et al. 1991). Group III mAbs 1A1 and 4D10, however, did recognize a 30 kDa protein produced by *V. anguillarum* and also emphasize the importance of determining the specificity of monoclonal antibody reagents.

Other investigators have been aware of cross-reactivity with anti-*R. salmoninarum* polyclonal sera, and used immunoadsorbtion techniques to remove problematic non-specific antibodies from their sera prior to experimentation. After immunoadsorbtion of sera with *Rothia dentocariosa* and *Bacillus sphaericus,* Dixon (1987) reports no cross-reactivity with the heterologous bacteria tested by ELISA. Commercially available Kirkegaard and Perry goat anti-*R. salmoninarum* polyclonal reagents are affinity purified to *R. salmoninarum* whole cells and lack cross-reactivity to a battery of tested bacteria (Henry-Ford and Chen 1994).

To investigate the relationship between p57 of *R. salmoninarum* and the 57-60 kDa proteins present in other species, we examined the possibility that comigrating proteins were distinct molecular species with no structural or antigenic relationship, except for a similar migration rate in SDS-PAGE. One well-characterized prokaryotic protein which is both antigenically conserved and

migrates with a MW of 57 to 60 kDa is the HSP 60 chaperonin, a protein present in all organisms. Chaperonins are proteins which assist in the folding of polypeptides into structurally correct molecules (Young and Elliott 1989). Many reports have demonstrated that the HSP 60 protein from a large spectrum of bacterial species is structurally, functionally and antigenically related, and that antisera directed against purified HSP 60 from one species will commonly recognize HSP 60 from a variety of other species (Yuan et al. 1992).

We used antisera directed against the chlamydial HSP 60 molecule to investigate the cross-reactivity seen with polyclonal antiserum directed against *R. salmoninarum*. These experiments demonstrated that anti-chlamydial HSP 60 recognized a protein of about 60 kDa in eight out of ten tested species, including several previously shown to be recognized by anti-*R. salmoninarum* polyclonal sera in fluorescent antibody tests and other assays. Although the anti-chlamydial HSP 60 did not react with the two *Bacillus spp.* this does not indicate that the protein is absent within these organisms. All bacteria possess HSP 60, and the HSP 60 protein and corresponding gene have been examined in at least two *Bacillus spp.* (Schmidt et al. 1992; Schoen and Schumann 1993). Instead, this may indicate that the antigenic relatedness among these proteins is not absolute, and that anti-chlamydial sera did not contain antibodies which were directed at sequences within the *Bacillus spp.* HSP 60 protein. Finally, while each mAb reacted strongly with p57 and its breakdown products in ECP, there was no recognition of ECP components by anti-HSP 60 antisera. This result was expected because it is uncommon for HSP 60 to be liberated from viable bacteria.

Collectively, these experiments identify cross-reactive bands consistent in molecular weight with those reported by Bandin et al. (1993) which are recognized by anti-HSP 60 sera, but not by mAbs directed at p57. We conclude

that it is likely the cross-reactivity described by Bandin et al. (1993) was a result of fortuitous comigration of a common antigen, possibly HSP 60, and the p57 protein. Although we have only tested HSP 60 in our described system, other highly conserved eubacterial proteins (for example HSP 70 proteins and elongation factors) may also cause cross-reactivity problems in diagnostic assays when antisera to whole bacterial cells are used as the primary detecting reagent. Additionally, normal bacterial flora may stimulate antibody responses to other cross-reactive moieties such as lipopolysaccharide or bacterial cell wall constituents. These possibilities may explain reported cross-reactivities which involve carbohydrate or unidentified epitopes (Bullock 1980, Evelyn et al. 1981, Feidler and Draxl 1986, Arakawa et al. 1987, Turaga et al. 1987, Pascho et al. 1991, Barbash 1992, Gudmundsdottir et al. 1993, Henry-Ford and Chen 1994).

In conclusion, we have presented a model which may explain previously observed cross-reactivities with anti-*R. salmoninarum* polyclonal antisera which, we feel, have been inappropriately attributed to the presence of *R. salmoninarum* p57-like molecules in other bacterial species. A common bacterial antigen, the 60 kDa heat shock protein, comigrates with p57 and is a probable source of observed cross-reactivity. All bacteria possess these homologous proteins which have common functions in a variety of species, and antisera directed against whole cells is likely to contain antibodies directed toward these molecules. Our results suggest two methods to minimize cross-reactivity problems: (1) to affinity purify polyclonal antisera and assay these sera for reactivity to common antigens or; (2) to use well-characterized mAbs in the development of such assays.

SUMMARY AND CONCLUSIONS

1. Two *Renibacterium salmoninarum* candidate vaccine antigens were formalin-killed and repeatedly injected into chinook salmon to study the humoral response to this bacterial pathogen. Native whole cells, including p57 and its major extracellular breakdown products ($p57^+$ cells) were prepared. In addition, an endogenous serine protease was activated at 37°C for 10 h. This heat treatment removed a major portion of the immunosuppressive, putative virulence factor p57 from the cell surface ($p57^-$ cells).

2. Densitometry performed on a total protein stained western blot, indicated p57 comprised approximately 70% of all protein on $p57^+$ cells, while only 14% of the p57 molecule remained after heat treatment.

3. A whole cell enzyme-linked immunosorbent assay (WCE) using $p57^+$ and $p57^-$ cells was optimized to compare differences in antibody activity in serum samples from fish hyperimmunized with $p57^+$ and $p57^-$ cells.

4. Whole cell ELISA (WCE) studies indicated a significantly enhanced antibody response to $p57^-$ cells at 32.5 weeks post primary injection with $p57^-$ serum versus $p57^+$ serum.

5. Both WCE and densitometry analysis suggest that a reduction in approximately 85% of the p57 molecule results in a 20-fold increase in antibody activity.

6. 10% SDS-PAGE analysis of $p57^+$ and $p57^-$ cells against $p57^+$ and $p57^-$ serum demonstrated that p57 and its breakdown products were the only immunogenic cell surface-associated proteins.

7. Exposure of $p57^+$ and $p57^-$ cells to enzymatic digestion with proteinase-K and carbohydrate disruption with periodate revealed $p57^-$ serum retained only 4% of the original antibody activity. There was virtually no change in antibody response after proteinase-K treatment. While both carbohydrate

and protein seem to be recognized by this salmonid species, most of the humoral response to $p57^-$ cells appears to be to carbohydrate.

8. Studies with anti-*Renibacterium salmoninarum* polyclonal rabbit sera claimed p57 may be found in other gram-positive bacteria and cause false-positive reactions in immunodiagnostic assays. In contrast, anti-p57 monoclonal antibodies did not react with other proteins of similar molecular weight in selected gram-positive or gram-negative bacteria, indicating these epitopes of p57 were not present in the tested bacterial species.

9. Immunoblot analysis with anti-60 kDa heat shock protein sera indicated the existence of a comigrating 60 kDa protein common to almost every bacteria tested. Perhaps this 60 kDa protein, distinct from p57, is a source of cross-reactivity in immunodiagnostic assays.

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