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Selected secreted and cellular virulence factors of *Aeromonas salmonicida* were examined. A protocol was developed for the separation of two secreted proteases (P1 and P2 protease), and a trout erythrocyte specific hemolysin (T-lysin) from supernatants of cultures of the bacterium. Distinctions between the proteases were demonstrated using molecular weight determinations, substrate specificities, sensitivity to chemical protease inhibitor sensitivities, and polyacrylamide gel electrophoresis using gels containing protease substrates (G-PAGE). P1, but not P2, protease was detected in G-PAGE analyses of protease from lesions of coho salmon (*Oncorhynchus kisutch*) infected by injection. Other proteases of apparent host origin were also detected in these assays. Analysis of the T-lysin demonstrated that although the bacterium produced high titers of the enzyme *in vitro*, no hemolytic activity was detected

in vivo nor in cultures grown in salmonid sera. Subsequent experiments demonstrated that salmonid sera possess an inhibitor of hemolysis capable of protecting erythrocytes from enzymatic or chemical lysis. The inhibitor was partially purified using molecular sieve chromatography and preparative isoelectric focusing. Analysis of P1 protease, P2 protease, and T-lysin production was continued by examining their production in the presence of salmonid sera and in the presence of high concentrations of selected salts added to brain heart infusion broth (BHI). The spectrum of proteases produced in serum was similar to the spectrum produced in BHI. However, a larger phenylmethylsufonyl fluoride sensitive fraction was detected in supernatants from bacterial cells grown in serum. Analysis of supernatants from the cultures grown in high salts indicated that P1 protease and T-lysin production were inhibited by these salts but P2 protease production was not. Growth in high concentrations of magnesium salts also affected the cellular morphology of the bacterium and this effect was associated with the presence of an outer membrane protein layer, the A layer.

Four monoclonal antibodies (Mabs) were produced with specificity towards *A. salmonicida* lipopolysaccharide (LPS). These Mabs were used to identify two distinct epitopes on LPS and to show that the presence of each epitope varied among different strains. The antibodies were also used to demonstrate the difference in the host response of rabbits and rainbow trout (*Oncorhynchus mykiss*) to *A. salmonicida*.

Virulence Factors of *Aeromonas salmonicida* and Their Interaction with the Salmonid Host

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Chapter 1

Introduction

Aeromonas salmonicida, causative agent of furunculosis disease of salmonids, is a pathogen of salmon and trout and is endemic to North America, Europe, and Asia. Non-salmonids are also infected by the bacterium. Furunculosis is characterized by liquefactive lesions in the musculature, systemic bacterial colonization, and other clinical signs typical of septicemic infections by gram-negative pathogens of fish. Identified bacterial components associated with virulence include both cell-associated and secreted factors. The major cell-associated virulence factor is the A layer, an outer membrane layer consisting of a repeated array of a single protein which covers the surface of the cell. The A layer is responsible for bacterial aggregation, tissue adhesiveness, and survival in serum. Secreted factors include proteases and cytolysins but the function of these enzymes in the disease process is not clear.

Although an efficaceous vaccine is the practical goal of furunculosis research, certain subjects important to vaccine development must first be addressed. A major problem is that the general understanding of the interaction between the piscine

host and pathogenic agent is inadequate. Experiments clarifying the identity and function of virulence factors, and analyses of bacterial antigenic structure need to be undertaken before vaccine design can be rationally examined. These approaches have produced successful vaccines against diseases of other species where classical bacterin vaccines have not been practical or effective. A recent example of this is streptococcal sore throat in humans (Fischetti et al. 1989). Investigations delineating the interaction between potential virulence components with the salmonid host are needed to adequately understand the pathogenic mechanisms used by the bacterium, and to address possible ways to defeat those mechanisms and thereby protect the fish.

The work described in this thesis focuses on the interaction of selected *A. salmonicida* virulence factors and the salmonid host. Our goals were to: 1. Investigate the nature and identity of selected potential virulence factors or antigens produced by *A. salmonicida*, and; 2. Examine the interaction of these factors with the fish host. The first three chapters following the literature review are analyses of enzymes secreted by the bacterium and the last two chapters are investigations of cell-associated factors. In each case, the bacterial component is examined *in vitro* and then selected aspects of the described factor are examined in relation to the host. This thesis does not address vaccine production or other direct means of preventing furunculosis in fish but the information presented will be useful to other

scientists working towards the prevention and detection of the disease. Additionally, methods used and results obtained during the analyses of the host-pathogen interaction may assist investigators addressing other diseases of fish.

Chapter 2

Literature Review

The Etiology of Furunculosis in Fish and the Classification of the Causative Agent

A detailed review of the early research describing furunculosis was presented by McCraw (1952). The initial report of furunculosis of salmonids was in 1894 (Emmereich and Weibel 1894). These authors described the bacterium as a gram-negative nonmotile coccobacillus which produces a water soluble brown pigment, and named the organism *Bacterium salmonicida*. Shortly thereafter the disease was observed in cultured brown trout (*Salmo truttae*) and brook trout (*Salvelinus fontinalis*) in the United States and was named *Bacterium truttae* (Marsh 1902). The first isolation of *A. salmonicida* in Oregon was in 1939 (Shaw and Seghetti 1940). To date, *A. salmonicida* has been detected throughout the northern hemisphere and Australia in hatchery and wild salmonids, cyprinids, and other fish (Herman 1968; Bucke 1989; Trust et al. 1980; Fryer et al. 1988).

The first placement of the species within the genus *Aeromonas* (family Vibrionaceae) was by Griffin et al. (1953), but accurate taxonomic classification has been difficult. Authors placed the species in association with *Serratia sp.* (Stevenson

1959) or proposed a new genus, *Necromonas*, to emphasize differences between *A. salmonicida* and the motile aeromonads (Smith 1963). These changes in taxonomy have not been supported and currently the Bergey's Manual of Systematic Bacteriology retains the generic name *Aeromonas* (Popoff 1984).

The determination of the characteristics within the species has also been difficult. Initially, the definitive characteristics of *A. salmonicida* included production of a brown water soluble pigment, lack of motility, indole reaction (negative), and the inability to utilize sucrose. Currently however, other bacteria pathogenic to fish with less similar traits have been added to the species and the classical *A. salmonicida* placed in the subspecies *A. salmonicida salmonicida*. One of the additional organisms is *A. salmonicida masoucida*, identified by Kimura (1969) as a non-pigmented, indole-positive subspecies of *A. salmonicida*. A second non-pigmented group was initially classified as *Haemophilus piscium* (Snieszko et al. 1950), but was combined with other non-pigmented isolates into the subspecies *A. salmonicida achromogenes* (Schubert 1969). This addition was accepted on the basis of GC content, susceptibility to bacteriophage that infect aeromonads, and the inability to satisfy the taxonomic requirements of the genus *Haemophilus* (McCarthy 1975; Killian 1976; Paterson et al. 1980). Isolates of *A. salmonicida* which cannot be placed into any of the described subspecies have been discovered in a variety of freshwater and marine fish species (Evelyn 1971; Hastein et al. 1978; Kitao et al. 1985). These are

called 'atypical' *A. salmonicida*. Recently, the subspecies *A. salmonicida nova*, was introduced to accomodate these strains (McCarthy and Roberts 1980; Belland and Trust 1988). These authors proposed that *A. salmonicida achromogenes* and *A. salmonicida masoucida* be combined into a single subspecies with the former epithet. Under this system, isolates from salmonids that do no conform to the characteristics of *A. salmonicida salmonicida* are placed in the subspecies *A. salmonicida achromogenes* and those isolates from non-salmonids that do not conform to the characteristics of *A. salmonicida salmonicida* are placed in the subspecies *A. salmonicida nova*. This may not be an adequate scheme for the description of the species and future modifications may be warranted. For the remainder of this thesis, the subspecies will be indicated only if isolates other than *A. salmonicida salmonicida* are discussed.

The Pathology of Furunculosis and Virulence Factors Produced by *Aeromonas salmonicida*.

Transmission and general pathology of *A. salmonicida* infection. Furunculosis occurs after contact with waterborne bacteria or cohabitation with infected fish (Spence et al. 1965; Bullock et al. 1976; Cipriano 1982b). There is no evidence for vertical transmission of the organism (Wood 1974; Bullock and Stuckey 1987). The bacteria can survive in natural waters for

extended periods of time (Allen-Austin et al. 1984; Wedemeyer and Nelson 1974; Sakai 1986a) which facilitates transfer of the organism without fish to fish contact. Transmission of this and other diseases of cultured salmonids is often mediated by bacteria released from migratory carrier fish upstream of a hatchery intake (Wood 1974).

The gills and abraded skin are portals of entry for bacteria into fish (Bowers and Alexander 1982; Tatner et al. 1984). After entry, the bacteria are transferred systemically in the circulation. Tatner et al. (1984) detected *A. salmonicida* in the spleen, heart, liver, and kidney of rainbow trout (*Oncorhynchus mykiss*) one day after bath exposure. As the disease progresses, the organism is recoverable from all organs (Ferguson and McCarthy 1978; Morikawa et al. 1981).

Once the bacterium successfully invades the host, the disease can manifest itself in several forms. McCraw (1952) divided infections into four types: acute, sub-acute, chronic, and latent. Acute infection often leads to rapid death of a large number of fish. Frequently no clinical signs occur prior to death. Sub-acute or chronic infections result in the spectrum of clinical signs and the outbreaks are of longer duration. Latently infected fish show no clinical signs nor serious mortality, but may shed bacteria which infect other fish in the population. Each of these types has been described (Ferguson and McCarthy 1978; Horne 1928; Bullock and Stuckey 1975).

The external pathology caused by *A. salmonicida* in salmonids is described by McCarthy and Roberts (1980). Clinical signs include exophthalmia, lethargy, darkening of the skin, and a characteristic necrosis of the musculature which leads to ulcerative lesions. Lesions are observed as blue to purplish regions which break through the integument in later stages of infection. This characteristic sign of infection led to the naming of the disease furunculosis, because of its superficial similarity to the human furuncle, or boil.

Aeromonas salmonicida achromogenes infections characteristically produce a less distinct 'furuncle' and may not become systemic (McCarthy 1975). This infection is common in cyprinids and the disease is known as carp erythrodermatitis (Bootsma et al. 1977; Pol et al. 1980). In salmonids *A. salmonicida achromogenes* and many atypical strains cause ulcer disease (Snieszko et al. 1950)

Another type of external pathology associated with *A. salmonicida* infection is fin erosion. This was observed in hatchery-reared Atlantic salmon (*Salmo salar*) at low water temperatures (Schneider and Nicholson 1980). It is likely, however, that the association of *A. salmonicida* with this condition is opportunistic and not causative (P. Reno, personal communication).

Varied internal damage from *A. salmonicida* infections has been reported. Ferguson and McCarthy (1978) designated splenomegaly and cardiac injury as the most common disorders in

natural infections of brown trout. These authors observed that necrosis in the spleen is centered in the sheathed capillaries and that hemorrhage, edema, circulatory, and renal damage are also common during infection. Many of these signs are not specific to *A. salmonicida* and are associated with diseases caused by other gram-negative septicemic bacterial pathogens of salmonids (Bullock et al. 1971; Mellergaard and Larsen 1981).

The role of extracellular products in pathology. Munro et al. (1979) demonstrated that extracellular product (ECP) of *A. salmonicida* has proteolytic, hemolytic, and leukocytolytic activity. These components were concentrated from spent culture supernatants with ammonium sulfate. Ellis et al. (1981) reported that injection of ECP causes many of the same signs as those associated with injection of the bacteria. These include lesion development, splenomegaly, tissue destruction, and ventricular epicarditis. After the identification of ECP as a major factor in the establishment of disease during infection, investigators focused on the components of ECP which facilitate the disease process.

Purification of proteases. Dahle (1971) reported the first efforts to purify *A. salmonicida* secreted factors. Dahle described a single proteolytic component with molecular weight 43,600 that has a pH optimum of 9.0. Cobalt chloride and ferric sulfate both enhance the yield of protease from culture. Dahle found that the activity is sensitive to heat in the absence of a casein substrate, but more resistant to heat in the presence of substrate.

Shieh and MacLean (1975) isolated an extracellular protease with a molecular weight 11,000 from *A. salmonicida*. The enzyme has a pH optimum of 10.5 against a casein substrate in glycine buffer. Activity increases at temperatures up to 60 C, after which it decreases rapidly. The enzymatic activity is sensitive to phenylmethylsulfonyl fluoride (PMSF), insensitive to ethylenediaminetetraacetic acid (EDTA), and insensitive to sulfhydril binding compounds. This pattern of sensitivity to inhibitors is characteristic of a serine protease (Powers and Harper 1986a).

The work of Shieh and MacLean (1975) was the first analysis of the serine protease produced by *A. salmonicida*. Other investigators have expanded and modified the description of this enzyme (Tajima et al. 1984; Hastings and Ellis 1985; Fyfe et al. 1987a). These authors agree that the serine protease of *A. salmonicida* has a molecular weight of 70,000, that activity can be determined using a casein substrate, and that protease causes significant pathology during infection. The described molecular weight is different than that reported by Shieh and MacLean (1975), but otherwise the descriptions are similar.

Sheeran and Smith (1981) identified another extracellular proteolytic activity in culture supernatants. This enzyme digests gelatin but not casein, is insensitive to PMSF, and is EDTA sensitive. The sensitivity profile is characteristic of metalloproteases (Powers and Harper 1986b). The described activity elutes from a diethyl aminoethyl cellulose (DEAE) ion

exchange column at a higher NaCl concentration than the serine protease. Based on the different elution profiles, Sheeran and Smith (1981) developed a nomenclature for these different activities, labeling the protease that elutes first (the serine protease) P1 and the protease that elutes second (the metalloprotease) P2. The existence and significance of the P2 protease was disputed by Hastings and Ellis (1985) who used preparative isoelectric focusing to demonstrate that only a single isoelectric peak of gelatinase activity is present in culture supernatants of *A. salmonicida* and that this peak also contains all caseinolytic activity.

Mellergaard (1983) described a fibrinolytic activity from *A. salmonicida* culture supernatants. This enzyme has a pH optimum of 9, a temperature optimum of 48 C, and a molecular weight of 87,500. The fibrinolytic activity is partially sensitive to both PMSF and EDTA.

The function of protease in the pathogenic process. *Aeromonas salmonicida* protease was first proposed as a virulence factor by Sakai (1977). Sakai (1985a) later produced mutants deficient in P1 protease. These mutants were avirulent, implicating the enzyme as a major virulence determinant. Fyfe et al. (1986) subsequently demonstrated that injection of purified P1 protease was sufficient for tissue destruction and some pathology associated with infection. Sakai (1985b) also determined that supplemented P1 protease allows these mutants to grow in the presence of casein as a sole amino acid source. This established

that the organism requires P1 protease to survive in an environment deficient in peptides or amino acids. Sakai used casein as substrate for his enzyme assays and therefore did not investigate the presence or absence of P2 protease.

Although the evidence supporting the role of P1 protease as a major virulence determinant is strong, this enzyme is not the only secreted factor that causes pathology *in vivo*. Fyfe et al. (1986, 1988) supported the conclusion that P1 protease is a major factor in virulence, but demonstrated that other factors also cause clinical signs. Tajima et al. (1983a, 1983b) determined that the protease and a cytolytic glycoprotein in combination cause the disease state associated with furunculosis. Other authors dispute the importance of P1 protease in the infective process. The mutant produced by Sheeran et al. (1983) is deficient in P1 protease and is still pathogenic to salmonids (Drinan and Smith 1985, Drinan et al. 1989). Finally, Tajima et al. (1987) isolated a P1 protease-deficient *A. salmonicida* that is still capable of infecting goldfish.

In addition to being a likely direct cause of pathology, P1 protease activates other potential virulence factors secreted by the organism. Titball et al. (1985) and Titball and Munn (1981, 1983) showed that P1 protease is specifically responsible for the activation of a hemolysin secreted by *A. salmonicida* in static culture, and for facilitating complete lysis of trout red blood cells (RBC) partially lysed by a second hemolysin secreted by the organism in shaken cultures.

Discovery and purification of cytolytic factors. The reported lytic components in the ECP of *A. salmonicida* involve three types of cells: leukocytes (LC's), RBC's, and tissue culture cells. Leukocytolytic factors were proposed by Klontz et al. (1966) because of the absence of leukocytes at the site of a lesion during infection. Fuller et al. (1977) partially purified a leukocytolytic factor from *A. salmonicida*. This factor has activity *in vivo* and *in vitro* and has a molecular weight between 100,000 and 300,000. Cipriano et al. (1981) reported that a fraction of ECP which elutes from a DEAE column at 0.6 M NaCl (pH 8.0) has cytolytic activity against both LC's and tissue culture cells. Extracellular product inhibits certain cells of the immune system without causing cytolysis. Pourreau et al. (1987) showed that supernatants from 96 h cultures contain leukoeffectors which suppress the *in vitro* mitogenic response of cells from the head kidney of carp, but supernatants from 20 h cultures do not. The component that inhibits the response is sensitive to heat (70 C, 30 min).

Aeromonas salmonicida also produces hemolytic enzymes. Nomura and Saito (1982) demonstrated that a hemolytic factor produced by the organism is toxic to RBC's of fish and sheep and that RBC's from different species of fish have different susceptibilities to the enzyme. These authors also observed that addition of different compounds to a basal medium regulates enzyme production. In a later report these authors further purified and characterized the hemolysin, which they label salmolysin (Nomura et al. 1988). Salmolysin is stable in a pH

range of 3-11, is sensitive to 60 C heating, and its undenatured molecular weight is 200,000. The molecular weight of denatured hemolysin is 54,000 (Fyfe et al. 1987) which suggests that the native enzyme is a tetramer.

Titball and Munn (1981) demonstrated two independent hemolytic factors in culture supernatants. One of these has activity against trout cells (T-lysin), and the other has activity against a variety of cells, but limited activity against trout cells (H-lysin). The T-lysin is enriched in cultures that are shaken while H-lysin is enriched in static cultures. The T-lysin is less sensitive to heat than H-lysin, has greater serum sensitivity, and is able to pass a 0.45μ cellulose acetate filter. H-lysin is bound by such a filter. There are distinctions between the enzyme described by Nomura et al. (1982, 1988) and the T-lysin of Titball and Munn (1981) which cause difficulty in determining whether the hemolysins are the same. Salmolysin completely lyses trout RBC while the T-lysin requires a serine protease for complete hemolysis. The enzymes also can use erythrocytes from a different range of species as targets.

Fyfe et al. (1988) examined the role of T-lysin in the pathology of furunculosis by injecting purified P1 protease or purified protease plus hemolysin into Atlantic salmon. This work established that the combination of protease and hemolysin is responsible for many of the clinical signs associated with the injection of total ECP. This report, however, does not demonstrate

the purity of the T-lysin. Therefore, other activities such as P2 protease may have contaminated their preparations.

The interaction of *A. salmonicida* secreted factors with host components also may contribute to the pathological process. Sakai (1984) demonstrated that complement from rainbow trout serum is activated by ECP and *A. salmonicida* protease activity is decreased after incubation with the serum. Prolonged incubation of ECP with serum reduces the *in vivo* toxicity of ECP, indicating that serum factors are important in host defense against the pathogen. Sakai also proposed that ECP protease may cause depletion of the host complement system, allowing for proliferation of the bacterium. Ellis and Grisley (1985) and Ellis (1987) examined the interaction of *A. salmonicida* P1 protease with trout serum antiproteases. They found that factors in the alpha-macroglobulin fraction of serum inactivate P1 protease *in vitro*. Well characterized alpha-macroglobulin antiproteases with a major role in the regulation of host proteases are found in this fraction of mammalian serum (Barratt and Starkey 1973). Therefore, although inactivation of *A. salmonicida* proteases can be accomplished by serum protease inhibitors, saturation of these antiproteases by *A. salmonicida* proteases may reduce the homeostatic effect of the inhibitors and facilitate tissue destruction by unregulated host enzymes.

Other identified components of ECP. Two additional components of ECP which have been identified and characterized have not been associated with virulence. The first of these is the

brown pigment produced by many strains. This was first thought to be a melanin pigment, but was subsequently shown to be a polymer of a different tyrosine derivative (Donlon et al. 1983). An enzyme with phospholipid: cholesterol acetyl transferase activity has also been identified but not associated with pathogenicity (Buckley et al. 1982).

Cell-associated virulence factors. The first description of a cell-associated virulence factor is the A layer (Udey and Fryer 1978). The A layer is an additional outer membrane protein layer present in virulent strains of *A. salmonicida* and is responsible for autoaggregation of the organism and adhesion of the bacterium to tissue culture cells. These traits are dependent on the presence of divalent cations in the medium. Electron microscopy of A layer positive (A+) cells demonstrated that the A layer consists of a repeated tetragonal array on the surface of the bacterium (Stewart et al. 1986). These tetramers are organized into two different general patterns covering the surface of the cell. Kay et al. (1981) reported that the A layer consists of a hydrophobic 49 kdal protein which they called A protein. These authors proposed that the A layer functions as a molecular barrier because it protects other membrane components from radiolabelling. Evenberg et al. (1982a,1982b) made similar observations with an *A. salmonicida* strain pathogenic to carp, but indicated that the A protein has a molecular weight of 54,000. Kay et al. (1984) subsequently compared properties of the A protein of *A. salmonicida* isolates from a variety of sources. Although

differences were observed between isolates of the typical and atypical types of the bacterium, the molecular weight, amino acid composition, isoelectric point, immunological relationships, and other characteristics of the A protein were shown to be similar throughout the species. The A layer increases the hydrophobicity of *A. salmonicida* cells (Van Alstine et al. 1986) and causes a change in the net electrical charge of the cell (Sakai 1986b). Ishiguro et al. (1981) determined that upon culture of A+ cells at 30 C, the ability to produce A layer is lost from the population. This discovery allowed these authors to accurately compare isogenic strains of bacteria having or lacking the A layer. These analyses demonstrated that the A layer shields the bacterium from some bacteriophage receptors and blocks yeast and sheep erythrocyte agglutination receptors available in A layer negative (A-) cells. Conversely, trout and salmon erythrocytes are agglutinated by A+ cells, but not by A- cells (Sakai and Kimura 1985).

Differential media have been formulated for determining if a strain of *A. salmonicida* is A+ or A-. Ishiguro et al. (1985) demonstrated that if the dye Congo Red is incorporated into brain heart infusion agar (BHIA), the colonies of A+ bacteria are red and colonies of A- bacteria are white. Wilson and Horne (1986) used Coomassie Blue, a non-specific protein stain, incorporated into BHIA to distinguish between A+ and A- colonies. During growth on this medium, colonies of A+ bacteria bind the dye and are blue. Colonies of A- bacteria do not bind the dye and are white.

Although the A layer functions in both autoaggregation and virulence, autoaggregating virulent A- isolates have been reported. Johnson et al. (1985) showed that in a survey of 10 clinical isolates, three autoaggregating strains did not have an A layer. The mechanism of aggregation in these strains is not described.

Biological role of A layer. Udey and Fryer (1978) and Ishiguro et al. (1981) demonstrated the importance of A layer in virulence. The A layer is important to the survival of the bacterium in the presence of immune and non-immune serum and also provides resistance to complement-mediated lysis (Munn et al. 1982). The A protein binds to porphyrins such as the iron-binding prosthetic group of hemoglobin (Kay et al. 1985). This provides evidence for the hypothesis that the A layer is involved with other proteins (Chart and Trust 1983) for the sequestering of iron from the host. The A layer also facilitates hemagglutination of trout erythrocytes (Sakai 1985c), possibly placing the bacterium close to its source of iron.

The ability of A+ bacteria to adhere to tissue cells is another possible role of the A protein in virulence (Udey and Fryer 1978). Sakai (1986b) showed that the A layer causes a charge shift in viable whole bacteria; A+ cells are electronegative and A- cells are electropositive. Sakai (1987) subsequently demonstrated that properly treated tissue culture cells migrate towards the cathode, and this migration is eliminated in the presence of A+ bacteria. This showed that the charge shift facilitated by the A layer is

responsible for the adhesive properties of the bacteria. Outside the host, the A layer facilitates the survival of the organism in river water (Sakai 1986a). River water components such as sand and various organics are also important in bacterial survival in water.

Lipopolysaccharide as a virulence factor. Another cell-associated factor which has a possible role in virulence is lipopolysaccharide (LPS). *Aeromonas salmonicida* LPS consists of two major components, a high molecular weight chain component and a low molecular weight core polysaccharide, and is homogeneous in both structure and antigenicity among most isolates (Chart et al. 1984; Evenberg et al. 1985). Lipopolysaccharide also functions in protection against lysis by non-immune serum, when compared to mutants deficient in both A layer and LPS (Munn et al. 1982). Contrary to its effect in mammals, LPS does not induce endotoxin mediated anaphylaxis in fish. Fish tolerate, with no ill effects, up to 30 times the endotoxin levels required to kill mice (Paterson and Fryer 1974b). Wedemeyer et al. (1968) supported these results with evidence that fish have no histamine response to endotoxin, nor do tested enzyme systems appear to react to the presence of endotoxin.

Aeromonas salmonicida and the Immune Response in Fish

Vaccination against furunculosis. This review will provide a summary of the development of potential vaccines designed to protect salmonids against *A. salmonicida* infection. Detailed presentations of the history of reported vaccinations against furunculosis are presented by Munro (1984) and Hastings (1988).

Attempts to vaccinate fish using bacterins of *A. salmonicida* began with a report by Duff (1942) who demonstrated that cutthroat trout are protected by long term oral administration of chloroform killed *A. salmonicida*. Unfortunately, this result was not consistently repeated. Certain authors described successful oral vaccination attempts (Klontz et al. 1970) but most authors indicate that orally administered bacterins do not provide protection (Snieszko and Friddle 1949; Udey and Fryer 1978; McCarthy et al. 1983). This inconsistency continued after the development of successful injection, oral, and immersion bacterins against vibriosis (Rohovec 1974; Croy and Amend 1977; Gould et al. 1979) or enteric redmouth disease (Anderson and Nelson 1974). Successful vaccinations against furunculosis have been described using A+ bacterins, passive antisera, or A- live cells (McCarthy et al. 1983; Olivier et al. 1985a; Cipriano and Starliper 1982) but in general, protection is low or absent with any

delivery method (Michel 1985; Udey and Fryer 1978; Hastings 1988). The inconsistency of vaccination results caused confusion in the analysis of potential protective cellular antigens.

Successful vaccinations using ECP or ECP fractions as antigen have also been reported. Cipriano (1982) demonstrated that a chromatographic fraction (fraction 4) which elutes from a DEAE column at 1.2 M NaCl protects fish when injected in the presence or the absence of Freund's incomplete adjuvant. The protection is conferred by fraction 4 from either virulent or avirulent isolates. Shieh (1985) reported successful vaccination using protease from an avirulent isolate of *A. salmonicida*. Sakai (1985d) demonstrated protection using protease inactivated by normal rainbow trout serum. Ellis et al. (1988) also demonstrated that protease is a potential protective antigen using passive immunization.

The immunology of the host response to *A. salmonicida*. Although some results were promising, in the final analysis, decades of vaccination trials designed to protect fish against furunculosis have produced a single commercial vaccine of debatable efficacy (Ellis 1988) for use by the fish culture industry. Analyses of the immune response of fishes to *A. salmonicida* were required to define why these vaccines were not effective. This was facilitated by the growing understanding of the humoral and cellular immune capabilities of fish.

The antibody response in fish to *A. salmonicida* was first investigated by Smith (1940) who demonstrated that carp and

rainbow trout produce agglutinins after parenteral injection of bacteria. Development of antibodies in brook trout and brown trout was demonstrated by Krantz et al. (1964a, 1964b) who also showed that mineral oil adjuvants facilitate antibody production and protection against challenge. Spence et al. (1965) protected coho salmon (*Oncorhynchus kisutch*) with passive immunization using hyperimmune rainbow trout serum. A detailed kinetic, qualitative and quantitative analysis of the antibody response to *A. salmonicida* LPS was presented by Paterson and Fryer (1974a, 1974b). These authors determined that fish as small as 1.2 g develop high antibody titers against the pathogen and that vaccination results in moderate protection against challenge by the bacteria. This collection of research demonstrates that salmonids and other fish are capable of producing high titers of specific antisera against *A. salmonicida.* These antisera, however, are never fully protective against challenge, and subsequent authors have demonstrated that increased agglutinin titers do not correlate with increased survival of challenged fish (Michel 1985; Cipriano and Heartwell 1986).

The specific antibody response of salmonids and rabbits has been compared to determine if there are differences between these groups. Olivier et al. (1985a) examined the ability of rabbit or coho salmon antisera to passively protect juvenile coho salmon. In all cases, the rabbit antisera protects better than the antisera from salmon. Hastings and Ellis (1988) compared the antibody response of rainbow trout and rabbits to ECP, and although the

quantitative responses are comparable, they found that rabbits develop antibodies against several antigens not recognized by the trout. These studies indicated that even though fish can develop specific antibody titers against *A. salmonicida*, protective antigens are not recognized. Therefore, either alternate immunization methods or different antigenic preparations which facilitate recognition of these antigens need to be developed, or other components of immunity such as cell-mediated or non-specific protection need to be enhanced for successful vaccination.

Specific salmonid cell mediated immune mechanisms analogous to those present in mammalian systems have not been thoroughly investigated (Trust 1986). There are no reports of activating this mechanism for protection against *A. salmonicida*.

Non-specific resistance mechanisms. Non-specific humoral and cellular immune mechanisms have been investigated to determine if these systems can be augmented for increased resistance to *A. salmonicida* infection. Incubating salmonid serum with ECP decreases its toxicity upon subsequent injection into fish (Munro et al. 1979; Sakai 1984; Cipriano 1982). Sakai (1984) correlated the decrease with activation of serum complement. Cipriano et al. (1981) reported that serum from fish species considered more resistant to *A. salmonicida* (rainbow trout) is more active in the inhibition of ECP toxicity than serum from a less resistant species (brook trout). This was also demonstrated by Ellis and Stapleton (1987), who reported that ECP proteolytic activity is more sensitive to rainbow trout serum than to Atlantic

salmon or brown trout serum. In fact, the proteolytic activity of low concentrations of ECP in brown trout serum is higher than the ECP alone. The interaction of sera from different species with ECP has also been demonstrated *in vivo*. Cipriano (1983) injected serum from rainbow trout into brook trout and this protected the recipient fish from subsequent waterborne challenge. The inhibitory nature of serum probably results from mechanisms involved in homeostasis and not actual host defense mechanisms (Grisley et al. 1984) and it may not be possible to augment these mechanisms to protect fish from infection (Munro 1984).

Cipriano and Heartwell (1986) identified non-specific mucus precipitins which precipitate solublized cellular antigens from a collection of gram-negative bacteria. The concentrations of these precipitins are higher in fish stocks which are more resistant to furunculosis. Additionally, fish stocks selectively bred for resistance to infection by *A. salmonicida* have higher mucus precipitin activity than outbred stocks.

Non-specific cellular mechanisms facilitating the avoidance of furunculosis infection have been decribed by several authors. Olivier et al. (1985b) and Cipriano and Pyle (1985) reported that injection of adjuvant alone yields protection equivalent to the injection of adjuvant plus *A. salmonicida* cells. This protection apparently involves the macrophage. Adams et al. (1988) also showed that non-specific immunity is important in protection against *A. salmonicida*, but these authors claimed that adjuvant plus antigen facilitates better protection than adjuvant alone.

Lipopolysaccharide from gram-negative bacteria is mitogenic to salmonid lymphocytes (Yui and Kaattari 1987) and this non-specific stimulation may be responsible for some protection afforded by bacterin vaccines. *Aeromonas salmonicida* cells contain large amounts of LPS, and protection derived from injection of bacterins plus adjuvant may potentiate only a non-specific response.

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Chapter 3

Separation and *in vivo* analysis of two extracellular proteases and the T-hemolysin from *Aeromonas salmonicida*

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Running title: Proteases and hemolysin from *A. salmonicida*

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Abstract

A procedure using DEAE cellulose and hydroxyapatite was developed for the separation of *Aeromonas salmonicida* P1 protease, P2 protease, and trout cell specific hemolysin (T-lysin) from supernatants of broth cultures. The different proteases were demonstrated using protease inhibitors, substrate specificities and polyacrylamide gel electrophoresis with protease substrates in the gels. Isolated P1 protease and T-lysin were shown to act independently in the complete lysis of rainbow trout erythrocytes *in vitro*. The T-lysin acted on the outer membrane and P1 protease acted on the nuclear membrane. Analysis of cell free exudate from lesions of trout infected with *A. salmonicida* by injection demonstrated that more than one protease was also present *in vivo*. P1 protease was present in both lesions and in culture supernatants, but P2 protease was detected only in culture supernatants. No T-lysin activity was detected in cell free exudate from lesions caused by *A. salmonicida* infection.

Introduction

Aeromonas salmonicida, the causative agent of fish furunculosis inflicts serious losses in hatchery-reared salmon and trout in many parts of the world (McCarthy and Roberts 1980). Although efficacious vaccines have been developed for other, similar, gram negative fish pathogens, commercial furunculosis vaccines have only recently been made available and documentation of efficacy is absent in the literature (Michel 1985). Therefore, virulence factors of this organism have been the object of much research. Previous work has included investigations of cell-associated and secreted components of the bacterium. An outer membrane protein matrix, the A layer, has been directly associated with virulence and the role of lipopolysaccharide in bacterial survival has also been addressed (Udey and Fryer 1978; Munn et al. 1982). Non-cellular factors secreted by *A. salmonicida* have been shown to be directly responsible for many of the clinical signs associated with the disease (Ellis et al. 1981). Secreted factors which have been investigated include proteases, hemolysins, and a leukocidin. Several authors have reported purification of these secreted proteins, as well as their individual and interactive characteristics (Fuller et al. 1978; Hastings and Ellis 1985; Fyfe, et al. 1987; Sheeran and Smith 1981; Titball and Munn 1981, 1983, 1985).

Investigation of *A. salmonicida* proteases began with Dahle (1971) who reported characteristics of proteases of *A. salmonicida* and *A. hydrophila*. Subsequent research described a variety of proteolytic factors. The major secreted protease is a serine protease of molecular weight 70,000 with activity against casein and gelatin (Fyfe et al. 1986a; Tajima et al. 1983). Sheeran and Smith reported two activities in culture supernatants of *A. salmonicida* one of which (P1) was the major serine protease; the other was an ethylene diamine tetraacetic acid (EDTA) sensitive protease (P2). P1 protease was active against casein and gelatin, while P2 protease had activity against gelatin but not casein. Other authors have reported a low molecular weight protease and a fibrinolytic protease with molecular weight 87,500 (Shieh and MacLean 1975; Mellergaard 1983). The presence of alternate proteases was debated by Hastings and Ellis (1985) based on results associating all gelatinolytic activity with a single isoelectric point in preparative isoelectric focusing. This suggested that a single component accounted for the activity.

In order to facilitate analysis of secreted virulence factors of *A. salmonicida,* we have developed a single technique for the separation of P1 protease, P2 protease and T-lysin from culture supernatants. Using this technique, we demonstrated that multiple proteases are present and have separated these activities. The relationship between the T-lysin and P1 protease in the complete lysis of trout RBC was also investigated *in vitro.* In addition, proteolytic and hemolytic activities were investigated

in vivo. It was shown that more than one protease were present in lesions caused by injection of the microorganism, but *in vivo* hemolytic activity was not detected.

Materials and Methods

Bacterial strains. Experiments were conducted with a recent isolate of *A. salmonicida* (RC1) from diseased spring chinook salmon (*Oncorhynchus tshawytscha*) at the Round Butte Salmon Hatchery, Madras, OR, USA. Comparisons for strain variation were conducted with strain SS70 (Udey and Fryer 1978). Cultures were stored on brain heart infusion (BHI) agar (Difco) at 4 C and grown for harvest in 500 ml BHI broth cultures at 17 C for 48 h with shaking.

Separation of virulence factors. Forty-eight hour cultures of *A. salmonicida* were pelleted at 2500 x *g* for 15 min and the supernatant removed and recentrifuged. The resulting supernatant was transferred to a flask on ice and powdered NH_4SO_4 (Mallinckrodt, GenAR grade) was added over a 10 min period with constant stirring to 45% saturation (32 g/100 ml supernatant). Fifty µl of 1 N NaOH was added for every 10 g NH_4SO_4. The solution was stirred on ice for 20 min and then centrifuged for 20 min at 10,000 x *g*. The pellet was dissolved in water, filter sterilized, and extensively dialysed against 20 mM Tris pH 7.9 at 4 C. The supernatant from the previous

centrifugation was concentrated with a second NH_4SO_4 precipitation by increasing the NH_4SO_4 concentration to 65% saturation (45.8 g/100 ml supernatant) and repeating the pelleting and dialysis. After dialysis, ion exchange chromatography using DEAE cellulose (Whatman DE 23) was performed and fractions were eluted using step-gradients of 0.1, 0.15, 0.2, and 0.35 M NaCl in 20 mM Tris pH 7.9. Fractions containing proteolytic enzymes or hemolytic activity against trout red blood cells (T-lysin) were collected and dialysed into a 1 mM potassium phosphate buffer, pH 7.0. These samples were chromatographed on a hydroxylapetite column (HAP, Bio-Rad Laboratories) and step-eluted at 0.01, 0.1, and 0.5 M phosphate. The T-lysin was further purified on Sepharose 6 B (Pharmacia) if necessary to remove contaminating proteolytic activity as described by Titball and Munn (1981). All manipulations were performed at 4-8 C.

Protease assays. Proteolytic activity was qualitatively assayed using a 10% gelatin overlay on 1% skim milk agar in petri dishes. Seven µl of sample was injected through both layers with a micropipettor. Plates were incubated 3 h at 18 C before reading. Preparations with both caseinolytic and gelatinolytic activity cleared a zone in the skim milk and liquified the gelatin. Preparations with only gelatinolytic activity liquified the gelatin and left the skim milk turbid.

Quantitative protease assays were conducted using a modification of the Lowry method of protein quantification

adapted for detection of digested proteins (McDonald and Chen 1965). The assays utilized gelatin as the substrate at a final concentration of 0.2% in phosphate buffered saline (PBS, 0.85% NaCl, 10 mM PO_4, pH 7.0). Twenty µl of enzyme was added to 400 µl of the gelatin solution and incubated for 40 min at room temperature before addition of 750 µl of 30% trichloroacetic acid (TCA). After a 15 min incubation on ice, precipitable material was pelleted in a microcentrifuge and 1 ml of the supernatant transferred to another tube. This was adjusted to neutral pH and subjected to a Lowry assay. Reactions were allowed to develop for 1 h. Optical density (OD) of each sample was determined at 525 nm on a Spectronic 20 spectrophotometer (Bausch and Lomb). One unit of enzyme was defined by an increase of 0.01 OD in this assay.

Protease inhibition. Differential inhibition of the proteases was demonstrated by two methods. The first was to add a 0.1 M suspension of phenylmethylsulfonyl fluoride (PMSF Sigma) in 50% isopropanol to equal volumes of the protease preparations. The other was inhibition with EDTA at a final concentration of 50 mM EDTA, pH 8.0. These techniques selectively inhibit two different classes of proteases, serine proteases and metalloproteases, respectively (Powers and Harper 1986a, 1986b). In both cases enzyme was incubated with inhibitor for 5 min before the quantitative protease assay. Negative controls were made by adding enzyme and inhibitor to the reactions after addition of TCA.

Gel electrophoresis. Purity of the various preparations was determined by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Schleif and Wensink (1981). Gels were silver stained using the Bio-Rad Laboratories silver stain kit (Cat. #161-0443, Bio-Rad Laboratories).

Analysis of proteases by substrate gel electrophoresis followed the protocol described by Heussen and Dowdle (1980) with modifications. Gelatin or iso-casein (Difco) was added to 12% resolving gels at 0.01% from 10% stocks made in water (G-PAGE or C-PAGE respectively). No substrate was added to the stacking gels. Samples were mixed with equal volumes of tracking dye (5% SDS, 2 % sucrose, 8 ug/ml phenol red) and were not boiled before electrophoresis. Electrophoresis was conducted on a Bio-Rad mini-Protean II apparatus according to the manufacturer's instructions. After electrophoresis, gels were incubated in 2.5% Triton-X 100 (Sigma) in water for 30 min to remove SDS and then in 0.1 M glycine-HCl pH 8.3 for 2 h. Gels were stained with coomassie blue as described by Schleif and Wensink (1981). All electrophoresis and incubations were at room temperature. The effect of the protease inhibitors PMSF (10 mM) and EDTA (25 mM) was determined by adding the inhibitors to each incubation step in G-PAGE.

***In vivo* sample analysis.** Lesions were induced in coho salmon with *A. salmonicida* by injecting 10^3 colony forming units in 10 µl into the body musculature adjacent to the dorsal fin. The tissue surrounding the injection site was removed aseptically from

two fish on the fourth day post-injection, and pooled. A liquid fraction from this tissue was harvested by centrifugation (12,000 x *g*) and prepared for G-PAGE analysis. Negative controls were prepared with identical methods from mock injected fish.

Preparation of red blood cells (RBC). Four different species of salmonids were used in this study: chinook salmon and coho salmon (*O. kisutch*) and cutthroat (*O. clarki*) and rainbow (*O. mykiss*) trout. Blood was harvested from fish anesthetized with benzocaine by inserting a heparin-treated hypodermic needle into the caudal vein behind the anal fin. Blood was removed and kept in heparin (Sigma grade 1, 150 I.U./10 ml whole blood) for 20 min on ice then centrifuged at 800 x *g* for 10 min and the plasma discarded. The cells were washed once in PBS and then resuspended in two volumes PBS. The suspension was left on ice for 4-16 h for separation of cell types and the leukocyte layer removed with a transfer pipet.

Preparation of blood cell nuclei. Red blood cell outer membranes were lysed non-enzymatically in one of three ways. First, using a modification of the method described by Bayne et al. (1986), RBC were treated with 0.1% Nonidet P-40 (NP40, Sigma) at a ratio of 50 µl packed cells to 1 ml of the NP-40 solution. Cells were incubated for 5 min and then diluted 1/10 in sterile PBS. The second lysis technique osmotically lysed the cells by lowering the salt concentration on a blood cell suspension. Cells were exposed for 5 min to dilutions of PBS in deionized water, centrifuged, and resuspended in PBS. Cells were examined

microscopically to determine the PBS dilution that would lyse the outer membrane but leave the nucleus intact. Nuclei were also prepared by allowing RBC suspensions to incubate for extended periods of time in unrinsed Microtiter plates (Dynatech). This facilitated spontaneous lysis of the outer membranes of a high percentage of cells.

Hemolysin assays. One hundred µl of approximately 2.5 x 10^6 RBC/ml PBS was placed in each well of a PBS-rinsed Microtiter plate and 10 µl of each column fraction was added. Wells were examined for evidence of lysis with an inverted microscope at 250 x over a 90 min period. Units of T-lysin present in culture supernatants were determined by log 2 dilutions of supernatant in PBS. An equal volume (50µl) of RBC was added to each well and the plate was incubated at 18 C. Wells were examined for lysis for 20 min and the titer was reported as the reciprocal of the highest initial dilution that had 90% lysed cells.

Detailed examination and photography at 1000 x were accomplished with wet mounts of cells and nuclei preparations. Nuclear lysis was observed microscopically by adding 10 µl P1 protease (2 units/µl PBS) or bovine chymotrypsin (Sigma, 1 mg/ml PBS) to the edge of a wet mount containing nuclei preparations.

Results

Separation of protease and T-lysin. Figure 3.1 shows a schematic representation of the protocol used to isolate proteases and T-lysin from *A. salmonicida* culture supernatants. Two NH_4SO_4 concentrations, 0-45% and 45-65%, resulted in enrichment of the proteases and T-lysin, respectively, although all activities were found at both concentrations. During separations, the P1 protease eluted at two major steps from the DEAE column- 0.1 and 0.15 M NaCl. Fractions eluting at 0.15 M NaCl were the source of the P1 activity for continued separation. P1 activity also eluted with the P2 activity at 0.35 M, but this contamination was removed on the HAP column. P1 activity eluted from the HAP column at 0.1 M PO_4 and P2 activity eluted at 0.01 M PO_4. Hemolysin eluted from the DEAE column at 0.35 M NaCl and subsequently from the HAP column at 0.1 M PO_4. Any P1 protease contamination was removed using Sepharose 6 B (Titball and Munn 1981). This separation scheme resulted in P1 purified to near homogeneity as determined by silver stained SDS-PAGE gels, and P2 free of contaminating P1 and T-lysin (Figure 3.2). The T-lysin preparations resulted in three bands on SDS-PAGE gels (Figure 3.3) and were free of detectable contaminating proteolytic activity. No difference was observed in the column separations using *A. salmonicida* strain SS70.

Protease substrate specificity and sensitivity to inhibitors. The two layer substrate plates differentiated

Figure 3.1. Flow diagram of protocol used to isolate secreted virulence factors from *Aeromonas salmonicida* culture supernatants.

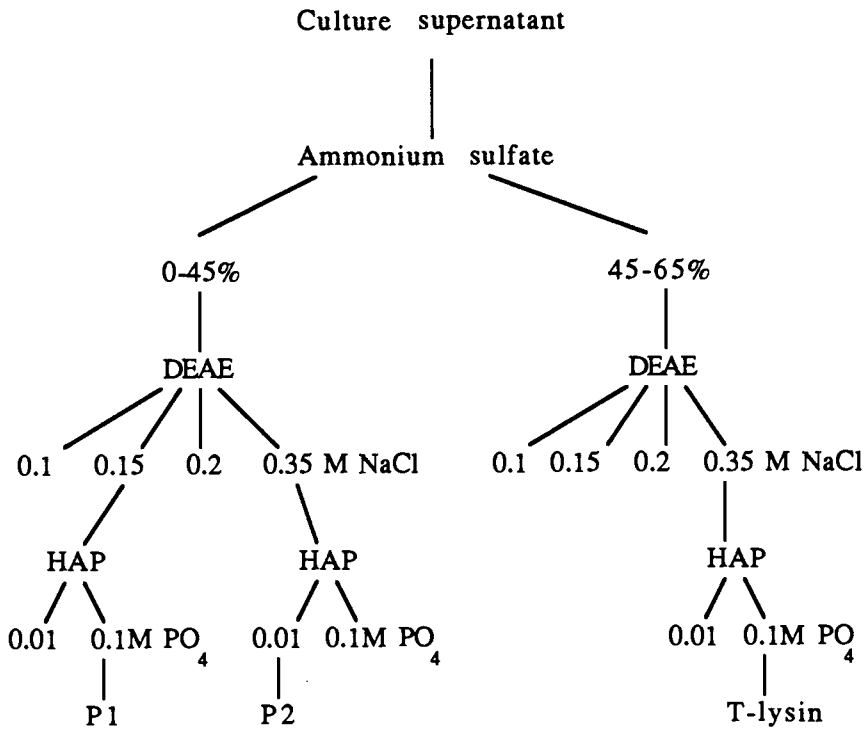
Culture supernatant
Ammonium sulfate
0-45%
45-65%
DEAE
DEAE
0.1
0.15
0.2
0.35 M NaCl
0.1
0.15
0.2
0.35 M NaCl
HAP
HAP
HAP
0.01
0.1M PO4
0.01
0.1M PO4
0.01
0.1M PO4
P1
P2
T-lysin

Figure 3.1

Figure 3.2. Results of SDS-PAGE of *Aeromonas salmonicida* P1 and P2 proteases. 1. Molecular weight standards, values in kilodaltons. Standards apply to lanes 2 and 3. 2. Material eluted from DEAE at 0.15 M NaCl. 3. P1 from hydroxylapetite (HAP). 4 and 5. P2 from HAP (4) electrophoresed adjacent to a P1 preparation (5).

Figure 3.3. Results of SDS-PAGE of T-lysin and P1 protease of *Aeromonas salmonicida*. 1. Molecular weight standards, values in kilodaltons. 2. T-lysin preparation. 3. P1 protease preparation.

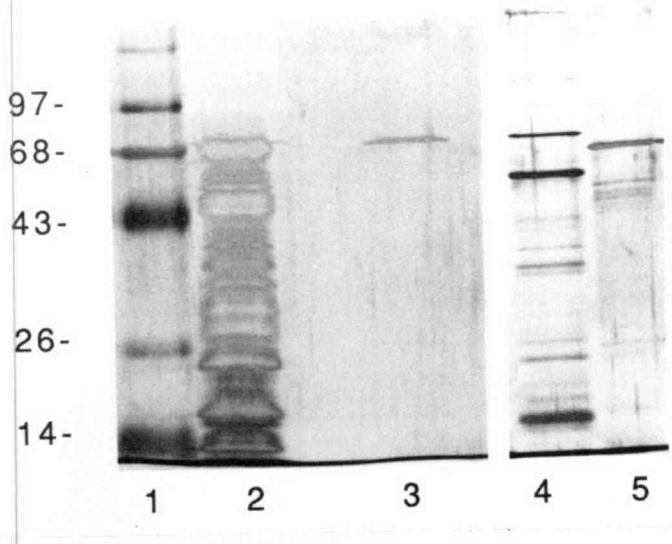

Figure 3.2

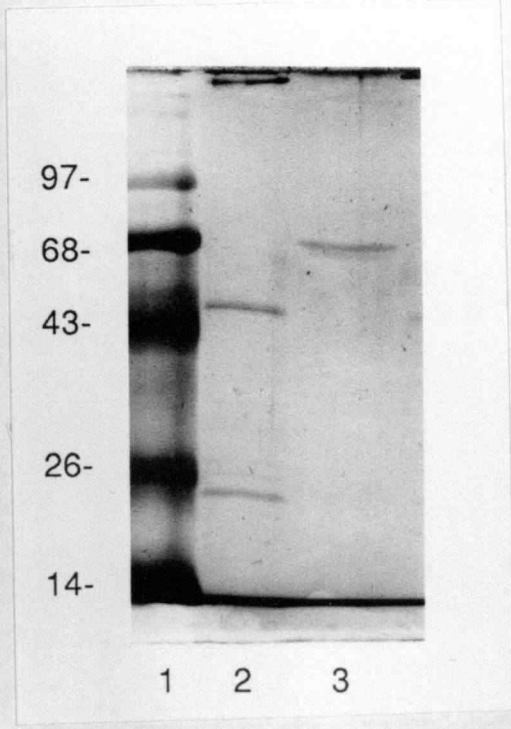

Figure 3.3

substrate specificities and demonstrated that purified preparations of P1 were both gelatinolytic and caseinolytic, while P2 was only gelatinolytic. Before separation on HAP, the protease assays of the peaks eluting at 0.15 M and 0.35 M NaCl from the DEAE columns were both caseinolytic and gelatinolytic in qualitative assays. However, distinctions were evident in the quantitative assays in the presence of inhibitor. The activity present in the 0.15 M peak was sensitive to PMSF while activity in the 0.35 M peak contained a PMSF resistant component (Figure 3.4). After separation on HAP, protease sensitivities were conducted using both PMSF and EDTA as inhibitors. Isolated P1 was shown to be completely sensitive to PMSF while P2 had limited sensitivity (Figure 3.5). The profile was reversed with EDTA; P2 was sensitive while P1 was insensitive.

Substrate gel electrophoresis. Samples of culture supernatant and the P1 and P2 proteases were subjected to substrate gel electrophoresis (Figure 3.6). Supernatants were shown to have more than one gelatinolytic component and a single caseinolytic component. Molecular weight standards are included for reference but are not indicative of actual protein molecular weight because of the incomplete denaturing electrophoretic conditions. P1 was shown, in these gels, to be a single factor while P2 preparations consisted principally of a lower molecular weight protein, with two higher molecular weight components having low activity in these conditions. Sensitivity to PMSF and EDTA was determined for the purified preparations.

Figure 3.4. Enzyme activities of samples from the 0.15 M and 0.35 M NaCl elutions from a DEAE column using the material from the 0-45% NH_4SO_4 concentration.

Figure 3.5. Enzyme activities of samples of hydroxylapetite-separated proteases in the presence of PMSF or EDTA. Dark bars; P1, light bars; P2.

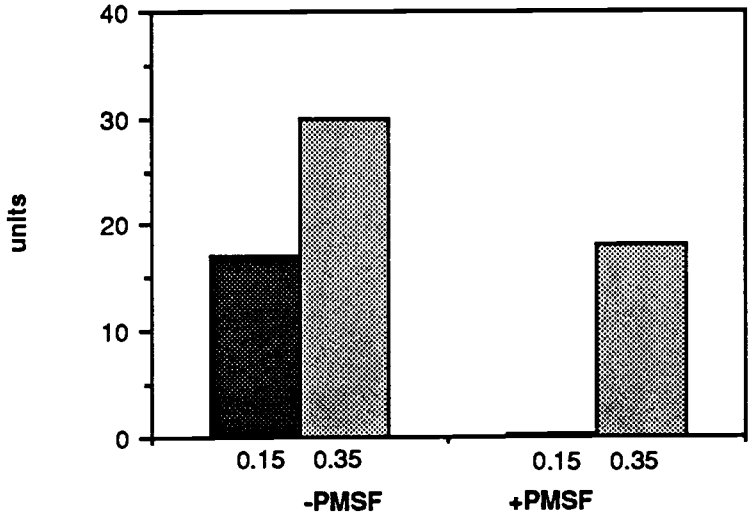

Figure 3.4

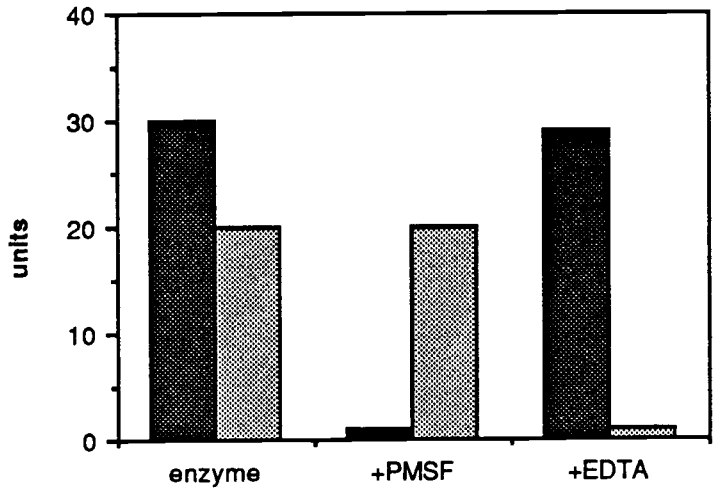

Figure 3.5

Figure 3.6. Substrate PAGE of *Aeromonas salmonicida* culture supernatant and purified proteases. 1. Gelatin-PAGE of culture supernatant. 2. Casein-PAGE of culture supernatant. 3 and 4. Gelatin-PAGE of isolated P1 protease (3) and P2 protease (4).

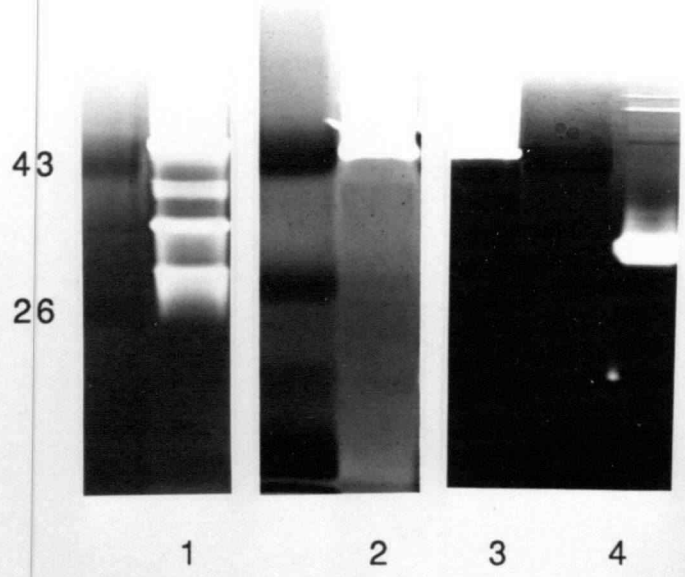

Figure 3.6

Gelatin-PAGE demonstrated that P1 was sensitive to PMSF and insensitive to EDTA, while P2 proteases were insensitive to PMSF and sensitive to EDTA (not shown).

The smearing visible at the top of our gels was also reported by Huessen and Dowdle (1980). In their work, this was eliminated by electrophoresis at 4 C. We could not remove the smearing with this treatment. The source of the smearing was shown to be digestion of substrate by the P1 enzyme during electrophoresis (not shown).

Electrophoretic analysis demonstrated that multiple proteolytic components were also present in samples taken from lesions of fish infected with *A. salmonicida*, but the only band that was present in G-PAGE of both culture supernatants and lesions was the P1 protease band (Figure 3.7). No lower molecular weight P2 protease band was seen in G-PAGE of lesions. All of the proteases detected in material from lesions were PMSF sensitive and EDTA resistant.

Hemolysin and P1 protease interaction. After separation of the proteases and T-lysin, we investigated the interaction of T-lysin and P1 protease in the complete lysis of trout RBC to define the role of each activity in the lytic process. This was facilitated by differential lysis of the RBC outer membrane using non-enzymatic means. The outer membrane of trout RBC was lysed with no apparent effect on the nuclear membrane when subjected to NP40, osmotic, or T-lysin mediated lysis (Figure 3.8). Blood cells from all species tested responded

Figure 3.7. Gelatin-PAGE of *Aeromonas salmonicida* proteases from culture supernatant (1) and of cell free exudate from infected fish (2) in the presence and absence of the protease inhibitors PMSF and EDTA.

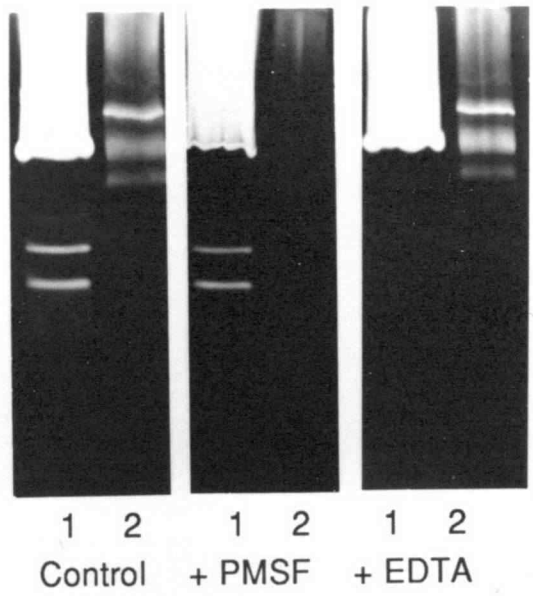

Figure 3.7

Figure 3.8. Rainbow trout red blood cell (RBC) preparations subjected to treatments for selective lysis of outer membranes. Normal RBC (1), treated with T-lysin (2), 35% PBS (3), and 0.1% NP40 (4).

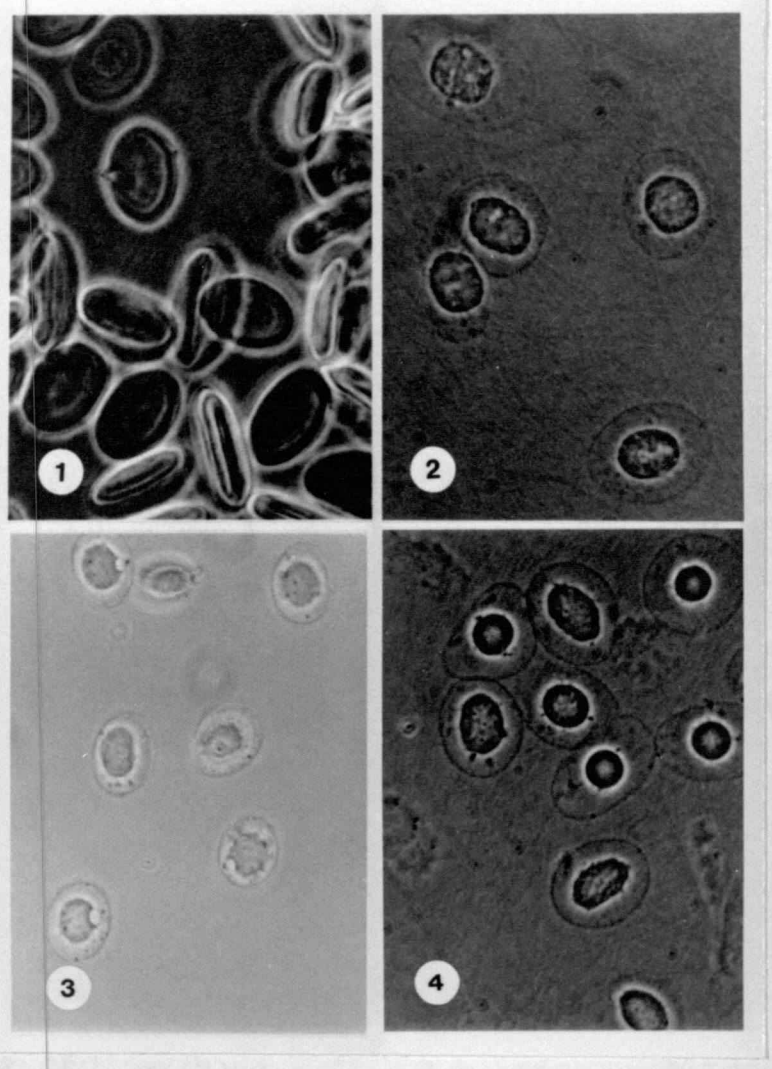

Figure 3.8

similarly to NP40 lysis. Osmotic lysis of rainbow trout RBC outer membranes required 30-40% PBS in water (0.25 or 0.34% NaCl). Cutthroat trout and coho salmon RBC had similar osmotic sensitivities, but the chinook salmon RBC completely lysed at these PBS concentrations. Chinook salmon RBC required 50 % PBS (0.43% NaCl) for selective outer membrane lysis. In each species tested, RBC with the outer membrane lysed and subsequently treated with P1 protease lost their nuclear integrity and contrast differences between the nucleus and empty cytoplasm disappeared. Similar results were obtained with RBC having spontaneously lysed outer membranes (not shown). These results indicated that T-lysin was not necessary for nuclear lysis. Protease-dependent nuclear lysis of NP40- or saline-treated RBC was visualized using microscopy. Lysis was indicated by nuclear expansion to fill the empty, but intact cytoplasmic membrane. The nuclei were apparently under positive internal pressure.

As reported elsewhere (Titball and Munn 1983), complete lysis of RBC did not specifically require the P1 protease of *A. salmonicida*. Bovine chymotrypsin also facilitated complete lysis of the nuclei. PMSF inhibited the lytic action of all nuclear lysis by P1 or chymotrypsin. PMSF had no effect on the lysis of outer membranes in the presence of P1, indicating no P1 contribution to outer membrane lysis. P2 preparations did not lyse RBC nuclei.

Hemolytic activity in infected tissues was compared to activity in culture supernatants. While T-lysin activity in

supernatants ranged from 128-1024 units, no hemolytic activity was observed in cell free exudates from lesions.

Discussion

There are several reports in the literature describing individual purifications and analyses of potential virulence factors from *A. salmonicida*. In this work a protocol for the separation of three previously identified factors from a single culture supernatant was developed. Using the described methods, two proteases, P1 and P2, and the T-lysin were partially purified and separated from one another for analysis. The P1 protease was purified to near homogeneity and contaminating activities were removed from both the P2 and T-lysin preparations.

The presence of P1 and P2 proteases in culture supernatants of *A. salmonicida* was demonstrated by different elution profiles from hydroxylapetite, substrate specificity, and sensitivity to inhibitors. These data support the results of Sheeran and Smith (1981) who documented two proteases in culture supernatants. However, by using G-PAGE and C-PAGE, we have also demonstrated that culture supernatants of *A. salmonicida* contained more than two gelatinases. All except P1 protease are EDTA sensitive and only P1 can digest both casein and gelatin. Investigators not detecting these different factors were

apparently limited by the sensitivity of their assays or by substrates used in detection.

Separation of these factors also facilitated analysis of the *in vitro* relationship between P1 protease and T-lysin in the complete lysis of trout RBC. Selective lysis of outer membranes using NP40 and decreased salinity was also used in these experiments. We determined that T-lysin lyses the outer membranes of trout RBC and P1 protease lyses the nuclear membrane. The functions are independent of one another. This relationship was first investigated by Titball and Munn (1983) and our results agree with the sequence of events they proposed. These results suggested that if enzymatic lysis of RBC is a component of pathology it is solely attributable to the T-lysin, because lysis of the outer membrane is sufficient to destroy the function of the cell.

Substrate gel electrophoresis and T-lysin activity assays were also used to investigate activities associated with these factors in coho salmon artificially infected with *A. salmonicida.* Although fish infected artificially had more than one gelatinolytic component, P1 protease was the only protease detected in lesions which was produced by the bacterium in culture supernatants. No low molecular weight P2 protease was detected in lesions. Gelatin-PAGE demonstrated that proteases found in lesions were PMSF sensitive, EDTA resistant. Non-P1 proteases in culture supernatants were EDTA sensitive, PMSF resistant. The non-P1 proteases detected in lesions were possibly host proteases

activated by P1 protease or otherwise liberated during the course of infection, but their source was not determined. Proteases other than P1 produced by *A. salmonicida* may have been present in lesions, but they were not within our limits of detection. This information suggested that P1 protease is the major proteolytic factor produced by *A. salmonicida* that is responsible for tissue destruction and lesion formation. Other investigators using alternate methods have supported the same conclusion (Sakai 1985a; Fyfe et al. 1986b). There is evidence, however, that *A. salmonicida* isolates lacking detectable caseinase activity (P1) can induce pathology and mortality in fish (Tajima et al. 1987). These infections do not involve lesion formation. Future research should address the potential role of non-P1 proteases in infection.

Attempts to detect hemolytic activity in cell free exudate from lesions were unsuccessful. This suggested that released T-lysin was either not present, or was incapable of RBC lysis *in vivo*. A recent report by Fyfe et al. (1988) indirectly associated T-lysin with specific tissue damage after injection with P1 protease into Atlantic salmon. They did not, however, associate the T-lysin with lysis of RBC. Their work supports our conclusion that the major target tissue for T-lysin *in vivo* is not the erythrocyte.

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Chapter 4

Salmonid Serum Inhibits the Hemolytic Activity of the Secreted Hemolysin of *Aeromonas salmonicida*

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Abstract

Rainbow trout serum protected trout red blood cells (RBC) from lysis by the hemolysins of *Aeromonas salmonicida* and *Aeromonas hydrophila*. Serum also protected RBC from non-enzymatic lysis by Nonidet P40. Sera from other salmonid species and cabezon were also protective, sera from catfish, largemouth bass and newt were less protective, and sera from higher vertebrates were not protective. Cell-free exudate collected from lesions of coho salmon artificially infected with *A. salmonicida* was also inhibitory to RBC lysis by hemolysin *in vitro*. The inhibitory factor was not sensitive to selected proteases, was not removed by centrifugation at 100,000 x g, and was sensitive to incubation at 60 C or higher for 30 min. Preparative isoelectric focusing and chromatography on Sepharose 6B resulted in partial purification of the anti-hemolytic factor.

Introduction

Analysis of the host-pathogen interaction in diseases of fish is an important area of research of investigators attempting to maintain and enhance the health of cultured fish. The relationship of *Aeromonas salmonicida* with its hosts has been one of the most investigated because of the widespread occurrence of this pathogen and its effects on hatchery and wild salmonids. Research has focused on the virulence factors of the bacterium and has a goal of a widely accepted efficaceous vaccine. Virulence mechanisms of the pathogen potentially include both cell associated and secreted factors. The A-layer protein is the major cell associated factor (Udey and Fryer, 1978; Munn et al. 1982), while secreted factors include proteases and cellular lytic activities (Hastings and Ellis, 1985, Titball et al. 1985). Fyfe, Coleman and Munro (1988) suggested that the trout cell specific hemolysin and the secreted serine protease are responsible for a majority of the clinical signs associated with the disease.

The fish host possesses a variety of barriers and protective mechanisms which the pathogen encounters during infection. Integument and slime, cellular and humoral immunity, and complement are primary in host defense (Ellis 1982; Ingram 1980; Cipriano and Heartwell, 1986) but other mechanisms also have potential defense roles. Fish and many other vertebrates

possess alpha-macroglobulin proteins in their serum which covalently bind and neutralize active proteases (Ellis and Grisley, 1985; Barratt and Starkey, 1973). These proteins have major roles in controlling host enzymes and may also serve an important function in defense against invasive agents which secrete proteolytic enzymes. Trout serum has also been shown to possess an antihemolytic activity of an undescribed nature (Munro et al. 1980; Titball and Munn, 1981; Ellis et al. 1981). In the present paper we partially characterize a component of piscine serum which protects salmonid erythrocytes against lysis by *A. salmonicida* hemolysin and other hemolytic agents.

Materials and Methods

Bacterial cultures and hemolytic agents. *Aeromonas salmonicida* strain RC1 and *Aeromonas hydrophila* strain 20354 cultures were maintained on brain heart infusion agar (BHIA). Cultures were grown for harvest in brain heart infusion broth (BHI) and filter-sterilized supernatants from 48 h cultures grown with vigorous shaking at 18 C were the source of hemolysin.

Cell-free exudate was collected from coho salmon (*Oncorhynchus kisutch*) infected by injection with 10^4 *A. salmonicida* cells in the dorsal musculature. Tissue surrounding the resulting lesion was excised four days after injection and the liquid fraction collected after centrifugation at 12,000 x *g*. This

material was examined for T-lysin activity and hemolytic inhibition activity.

The non-ionic detergent, Nonidet P40, (NP40, Sigma Chemical Co, St Louis, MO.) was used at a concentration of 0.01% in phosphate buffered saline (PBS, 0.85% NaCl, 10 mM PO_4, pH 7.2) as a non-enzymatic hemolytic agent (Rockey et al. 1988).

Plasma, sera, and erythrocytes. Coho salmon, rainbow trout (*Salmo gairdneri*), and cabezon (*Scorpaenichthys marmoratus*) were anesthetized with benzocaine and then bled from the caudal vein. Sera were obtained by centrifugation (1200 x *g*) of blood clotted at 4 C for 6 h. To obtain plasma and RBC, fish were bled into heparin at a final concentration of 150 I.U./ml blood. Red blood cells were either kept in plasma or washed in two volumes PBS and resuspended in an equal volume PBS. Plasma was collected after centrifugation of the blood. R.P. Hedrick (University of California, Davis CA) provided sera and heparinized RBC from largemouth bass (*Micropterus salmoides*) and channel catfish (*Ictalurus punctatus*). Plasma was obtained from newts (*Taricha granulosa*) by centrifugation of heparinized blood. Chicken serum was obtained after jugular bleeding. Sheep, cow, and horse sera were obtained from commercial sources. Plasma and sera were stored at -20 C and RBC were stored at 4 C. Erythrocytes from coho salmon, cabezon, largemouth bass, and channel catfish were used as substrate in hemolysin assays with *A. salmonicida* culture supernatant as the source of hemolysin. All sera were used in hemolytic inhibition assays to determine if

heterologous serum protected rainbow trout RBC from lysis by *A. salmonicida* culture supernatants.

Hemolysin assays and hemolytic inhibition assays. The hemolytic activity of *A. salmonicida* culture supernatants was determined by making twofold dilutions in PBS in 96-well plates which had been washed with PBS. An equal volume of RBC (10^6/ml in PBS) was added to each dilution and the assay was read after 5, 10, 20 and 30 min using an inverted microscope. The reported titer was the inverse of the highest initial dilution at which 90% of the cells were lysed after 20 min incubation

Hemolytic inhibition assays were conducted by making twofold dilutions of serum or plasma in PBS, followed by addition of an equal volume of culture supernatant which had a hemolytic titer of 256. After a 5 min incubation, RBC were added and the assays read at 5, 10, 20, and 30 min using an inverted microscope. The reported titer was the highest initial serum dilution where 90% of the RBC appeared normal after 20 min incubation.

Physical characterization of the hemolysis inhibition factor (HIF). Thermal sensitivity of the HIF was determined by heating serum at temperatures from 40 C to 90 C for 30 min. Samples were then cooled on ice and assayed for hemolytic inhibition.

To determine the sensitivity of the HIF to proteolytic degradation, serum was incubated at 4 C for 16 h with proteinase K (Sigma), or papain (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 0.1 mg/ml. Digestion of serum was confirmed

with discontinuous polyacrylamide gel electrophoresis followed by staining of gels with Coomassie blue (Schleif and Wensink, 1981). Phenylmethylsulfonyl fluoride (PMSF, Sigma) was then added to a concentration of 10 mM to inhibit the proteases and these preparations were used in a hemolytic inhibition assay. Controls included serum incubated with PMSF alone and proteinase K plus PMSF without serum.

Preparative isoelectric focusing of rainbow trout serum which had been treated with proteinase K and ultracentrifuged (60 min, 100,000 x *g*) was performed using a Bio-Rad Laboratories (Richmond CA) Rotofor isoelectric focusing device. Bio-lyte ampholytes (Bio-Rad) with a pH range of 3-10 were used for the separation. After completion of the focusing, fractions were collected, the pH of each fraction was measured, and samples from each fraction were used in a hemolytic inhibition assay.

Column chromatography was performed with 1 ml of ultracentrifuged serum on an 80 x 2.5 cm Sepharose 6B (Sigma) column at a flow rate of approximately 30 ml/h. Optical density of fractions was measured using a Beckman model 35 spectrophotometer and fractions were assayed for their ability to inhibit hemolytic activity.

Results

Protection of RBC by salmonid serum. Rainbow trout serum or plasma protected RBC from lysis by *A. salmonicida*

culture supernatants. Erythrocytes which had been washed once in PBS were readily lysed while RBC which had not been washed were not lysed (Figure 4.1). Inhibition of hemolysis by serum was not specific to supernatants of *A. salmonicida*. Although the hemolytic activity of *A. hydrophila* culture supernatants was lower than *A. salmonicida*, inhibition of hemolysis using *A. hydrophila* as a source of hemolysin was similar to that when *A. salmonicida* was used (Figure 4.2). Serum also inhibited the non-enzymatic lysis of RBC outer membranes by 0.01% NP40. Washed RBC were completely sensitive to NP40 at this concentration.

To determine if the HIF protected the RBC or inhibited the hemolysin, inhibition assays were conducted with serum mixed first with either the RBC or the culture supernatant, followed by addition of the other component of the assay. Serum incubated with RBC first followed by addition of culture supernatant resulted in higher inhibition titers than if the serum was first incubated with culture supernatant (Figure 4.2). This suggested that the inhibitor protects the RBC.

Cell-free exudate from lesions caused by *A. salmonicida* infection was used as the source of enzyme in hemolysin assays and was not hemolytic to washed RBC. Cell-free exudate was also used as inhibitor in hemolysis inhibition assays, and had hemolytic inhibition titers of 128.

Interaction of *A. salmonicida* hemolysin with sera and RBC of other species. To determine if hemolytic inhibition was unique to salmonid serum or was a general property of fish

Figure 4.1. Hemolysis by *Aeromonas salmonicida* culture supernatants of washed (□) or unwashed (■) rainbow trout erythrocytes

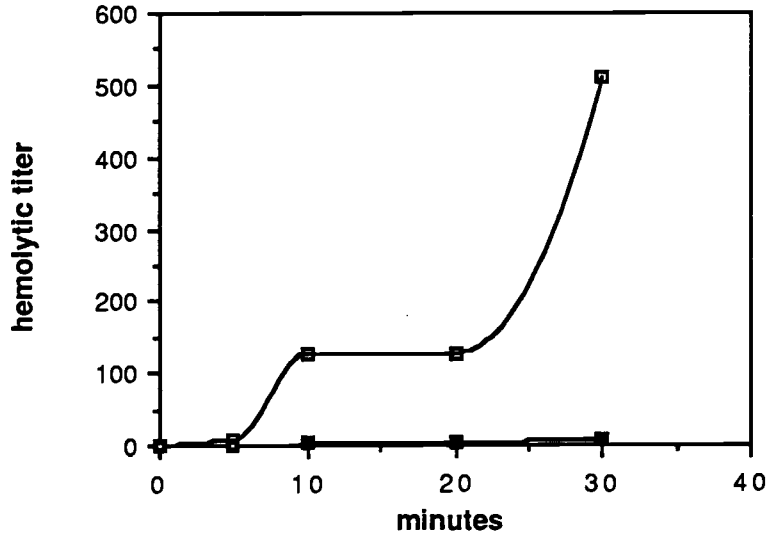


Figure 4.1

Figure 4.2. A. Hemolysis inhibition by rainbow trout serum in assays with *Aeromonas salmonicida* supernatants (a) added to serum before rainbow trout erythrocytes or (b) with supernatant added after the erythrocytes. B. Hemolysis and hemolysis inhibition assays using *A. salmonicida* (dark bars) or *A. hydrophila* (light bars) culture supernatants as source of hemolysin and rainbow trout serum as the source of hemolytic inhibitor.

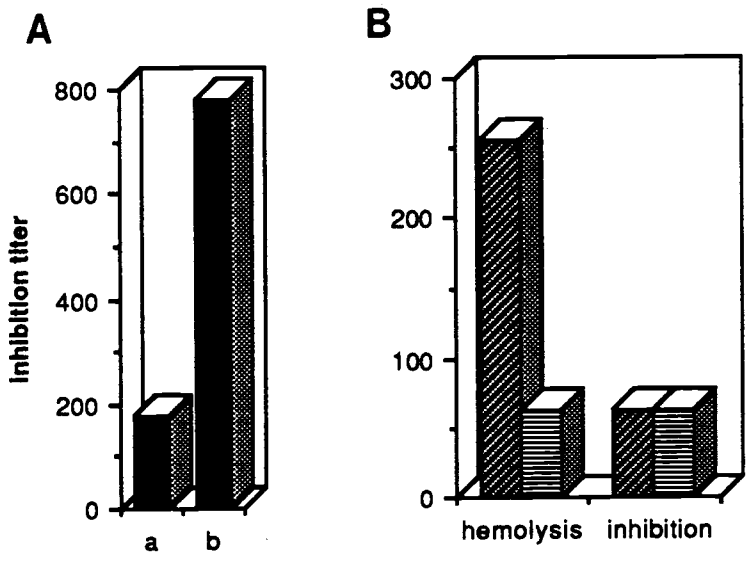

A
800
600
400
200
0
Inhibition titer
a
b
B
300
200
100
0
hemolysis
inhibition

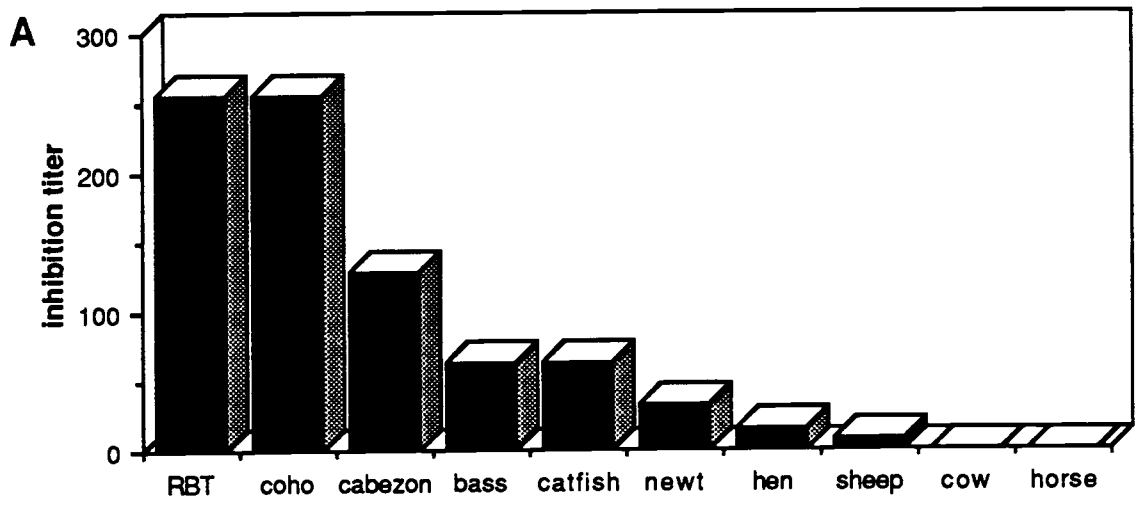
Figure 4.2

or other vertebrate serum, cross-protecting hemolytic inhibition assays were conducted. Of the fish species tested, rainbow trout and coho salmon sera were equally inhibitory to hemolysis of rainbow trout RBC, while cabezon, catfish and largemouth bass sera were inhibitory but to a lesser degree (Figure 4.3). Plasma and sera from non-piscine species had HIF activity which decreased with the species phylogenetic distance from salmonids. Assays using RBC from the selected fish species other than rainbow trout demonstrated that coho and cabezon RBC were sensitive to *A. salmonicida* hemolysin, while largemouth bass and catfish RBC were slightly sensitive or not sensitive, respectively (Figure 4.3).

Physical characterization of the inhibiting factor. The hemolytic inhibitory component of serum was tested for heat lability. Inhibitory effects of serum were augmented with 30 min heating to 40 C or 50 C but at temperatures higher than 50 C the inhibitory effect of the serum decreased (Figure 4.4).

Serum and plasma were equally inhibitory to hemolysis by *A. salmonicida* culture supernatants, indicating that the inhibitor was not an artifact of the preparation of serum (Figure 4.4). Hemolytic inhibition activity was not pelleted from serum by ultracentrifugation at 100,000 x *g*. Serum was subjected to digestion by the proteolytic enzymes proteinase K and papain. These digestions did not remove the HIF activity from serum (Figure 4.4) and the titers from digested serum were equal to the titers of controls incubated without enzyme (not shown).

Figure 4.3. A. Hemolysin inhibition assays using sera from selected species as inhibitor of *A. salmonicida* hemolysin. B. Hemolysin assays with erythrocytes from selected species as target for *Aeromonas salmonicida* hemolysin.


A
300
200
100
0
inhibition titer
RBT
coho
cabezon
bass
catfish
newt
hen
sheep
cow
horse

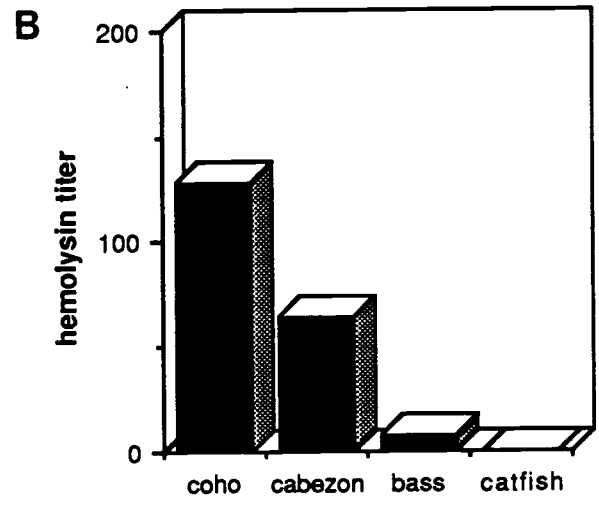
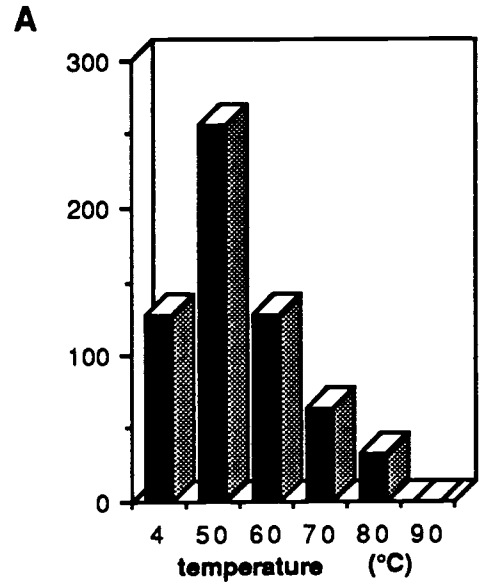

B
200
100
0
hemolysin titer
coho
cabezon
bass
catfish

Figure 4.3

Figure 4.4. A. The sensitivity of rainbow trout hemolysis inhibiting factor to temperature. B. Different treatments and their effects on serum inhibition of *Aeromonas salmonicida* hemolysin. 1. Untreated serum. 2. Untreated plasma. 3. Supernatant of serum after ultracentrifugation. 4. Pellet of serum after ultracentrifugation. 5. Serum digested with proteinase K. 6. Serum digested with papain.


A
300
200
100
0
4 50 60 70 80 90
temperature (°C)

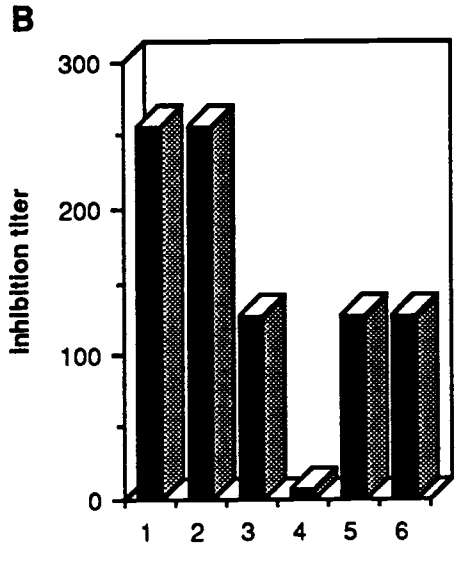

B
300
200
100
0
Inhibition titer
1 2 3 4 5 6

Figure 4.4

Fractionation of serum. Serum digested with proteinase K and subjected to ultracentrifugation was analyzed by preparative isoelectric focusing (Figure 4.5). The inhibitory factor focused at pH 4.5-5.5. After concentration to the original serum volume, the focused material had an inhibition titer of 128, while the material used in the focusing had a titer of 512. Chromatography separated the hemolytic activity as a broad peak just prior to a large OD_{280} peak (Figure 4.6). Samples treated with proteinase K were also chromatographed to determine if protease treatment altered the migration of the hemolytic inhibition in the column. Although the OD_{280} pattern was altered in the proteinase K treated preparations, the hemolytic inhibitor eluted at the same point in the column.

Discussion

A component of piscine serum was capable of protecting homologous RBC against enzymatic or NP-40-mediated lysis. This component was present on unwashed cells, but a single PBS wash removed the inhibitory factor from the RBC. Addition of serum to the washed RBC restored protection to the cells, as previously described (Munro et al. 1980; Titball and Munn, 1981). Serum also protected RBC from lysis by NP-40. Serum had no effect on osmotic stability or pH sensitivity of RBC (not shown).

Figure 4.5. The pH (□) and relative hemolytic inhibition titers (◆) of fractions obtained by isoelectric focusing of rainbow trout serum.

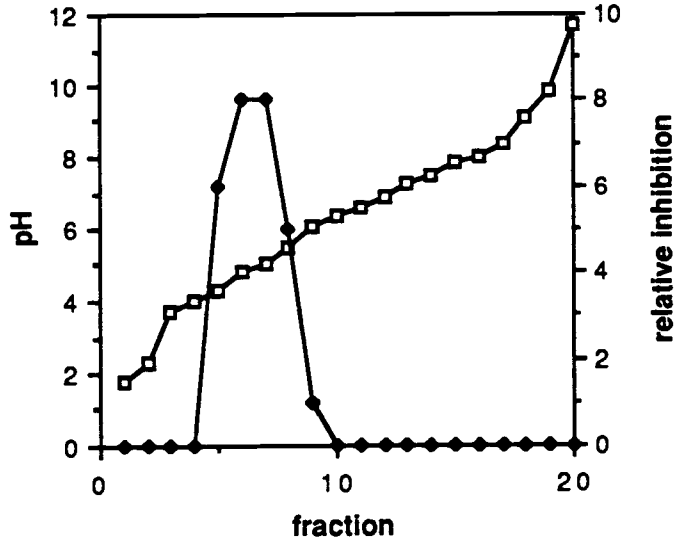


Figure 4.5

Figure 4.6. Optical density at 280 nm (A) and relative hemolytic inhibition (B) of eluted fractions after chromatography of untreated rainbow trout serum (□) and proteinase K treated serum (◆) on Sepharose 6B.

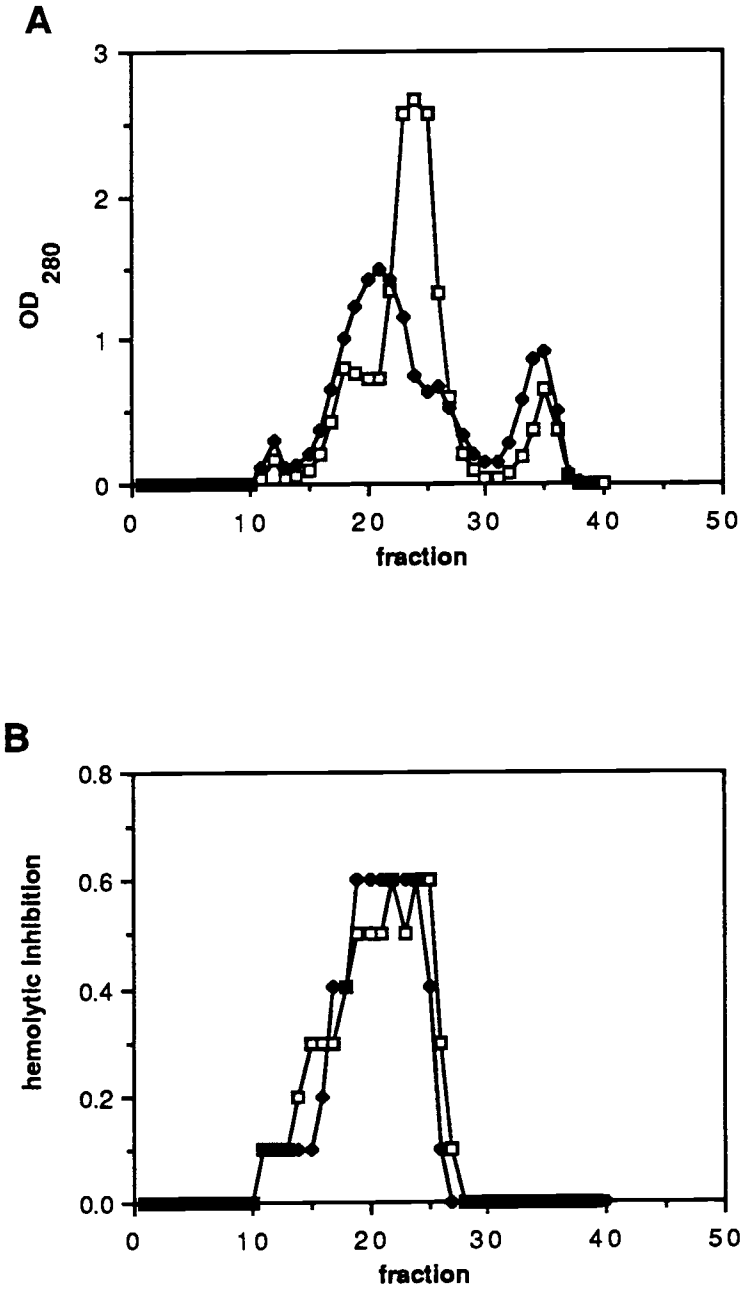

A
3
2
1
0
OD 280
0
10
20
30
40
50
fraction
B
0.8
0.6
0.4
0.2
0.0
hemolytic inhibition
0
10
20
30
40
50
fraction

Figure 4.6

Protection of trout RBC by sera from other fish species was related to the hemolytic activity of *A. salmonicida* supernatant against the respective RBC. As reported by Munro et al. (1980), salmonid sera was inhibitory to hemolysis by culture supernatants. Other species whose erythrocytes were sensitive to *A. salmonicida* hemolysin possessed serum which was protective to trout RBC. This association suggested that either the HIF was a competitive inhibitor, structurally similar to the hemolysin substrate binding site, or that the HIF protected the site recognized by the hemolysin. Because serum also protected RBC against non-enzymatic lysis by NP40 we propose that the HIF blocked sensitive target sites on the RBC and did not specifically inhibit the hemolysin. This is supported by the results of inhibition assays which showed that RBC added first to serum followed by supernatant had higher hemolytic inhibition titers than assays with the supernatant added before the RBC.

Physical characterization of the HIF was performed to determine its molecular nature. Hemolysis inhibition decreased in serum heated above 60 C, but heating serum to 40 or 50 C slightly augmented the HIF activity. This indicated that complement activation was not involved in serum inhibition of hemolytic activity because complement activity is eliminated from serum heated for 20 min at 44 C (Sakai, 1984). Treatment with proteinase K or papain did not remove antihemolytic activity and proteinase K treatment did not alter elution patterns of the inhibitor on Sepharose 6B. Isoelectric focusing of serum

determined a pI for the HIF of 4.5-5.5. In future research, isoelectric focusing and Sepharose 6B chromatography will be combined to purify the HIF from whole serum. This will facilitate the determination of its chemical constituency and its mechanism of interaction with the RBC.

Other authors have determined that injection of a combination of purified hemolysin and purified P1 protease induced the spectrum of clinical signs associated with the disease while injection of either preparation alone did not (Fyfe et al. 1988). This supports the conclusion that hemolysin is responsible for some aspect of pathology associated with the disease. However, the hemolysin is not associated with hemolysis *in vivo* (Ellis, et al. 1981) and although RBC are a convenient target tissue *in vitro*, the *in vivo* target of the enzyme is undetermined. Analyses of the actual concentrations of hemolysin during an infection and alternate tissue target sites are necessary for continued investigation of its role in pathogenicity.

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Chapter 5

Protease and Hemolysin Activity of *Aeromonas salmonicida* Grown in Modified Media or in Salmonid Serum

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Running title: Protease and hemolysin of *A. salmonicida*

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Abstract

The proteolytic and hemolytic activities of *Aeromonas salmonicida* grown in brain heart infusion broth (BHI), supplemented BHI, or in salmonid serum were compared. Ammonium sulfate (3.0%), magnesium sulfate heptahydrate (5.6%), or any of the concentrations of ammonium acetate tested (0.5 -1.5%) reduced the growth rate and eliminated synthesis of the major serine protease (P1 protease) and trout cell specific hemolysin (T lysin). Western blot analysis and substrate gel electrophoresis demonstrated that other proteins and proteases were not affected. Protease activity in culture supernatants from BHI and salmonid serum was qualitatively different and T lysin activity was absent in the serum cultures. Covalent interactions between serum proteins and P1 protease were demonstrated by Western blot analysis and salmonid serum was shown to inhibit different *A. salmonicida* proteases.

Introduction

Aeromonas salmonicida, the causative agent of furunculosis, is an important pathogen of salmon, trout, and other species. Major losses in hatchery-reared and wild salmonids are attributed to this infection (McCarthy and Roberts 1980). Considerable effort has been made to develop methods to prevent or control this disease, including antibiotic therapy, hatchery management techniques for improved fish health, and vaccine preparation. While some of these efforts have helped, the problem of furunculosis remains widespread.

Reports have described both cell-associated and extracellular virulence factors of *A. salmonicida*. The major cell-associated factor is the A layer, an outer membrane protein layer associated with serum resistance and tissue adherance (Munn et al. 1982; Udey and Fryer 1978). Outer membrane lipopolysaccharide (LPS) has also been shown to function in serum resistance (Munn et al. 1982).

Extra-cellular products (ECP) also function in the virulence of *A. salmonicida*. Ellis, Hastings, and Munro (1981) demonstrated that factors secreted by the organism during growth (ECP) were responsible for much of the external and internal signs associated with an infection. Several authors have reported purification and analysis of proteases and cytolytic enzymes from culture

supernatants of *A. salmonicida*. These include a 70 kdal serine protease (P1 protease) (Fyfe et al. 1987; Rockey et al. 1988; Tajima et al. 1983a), a metalloprotease (P2 protease) (Sheeran and Smith, 1981; Rockey et al. 1988), a fibrinolysin (Mellergaard 1983), and a protease with molecular weight 11,000 (Shieh and Maclean 1975). Two hemolysins have been described; one has activity against trout red blood cells and the other lyses horse red blood cells (T lysin and H lysin, respectively) (Nomura and Saito 1982; Titball and Munn 1981, 1983). The leukocidin originally described by Fuller et al. (1977) has not been further characterized. Although the P1 protease and the T lysin have been associated with virulence (Fyfe et al. 1987a, 1988), the function and interaction of this collection of enzymatic activities is unclear (Drinan et al. 1989).

Virulence factors have generally been examined using material harvested from culture supernatants of bacterial cells grown in standard laboratory media in optimal environments. This allows rapid growth of the culture and facilitates the examination of secreted products produced during logarithmic, uninhibited growth. We and others (Fyfe et al. 1986a, 1987b; Titball and Munn 1983) are interested in the production of potential virulence factors in less optimal conditions. In this paper we demonstrated that chemical modifications of medium affect the activity of protease and T lysin in culture supernatants. Growth in serum had qualitative effects on the activity of the proteases and eliminated detectable T lysin activity. We have also

shown that proteases from *A. salmonicida* were differentially inhibited by serum and that P1 protease covalently reacted with normal serum components in vitro.

Materials and Methods

Bacterial strains, salmonid sera, plasma, and media preparation. Lyophilized stocks of *Aeromonas salmonicida* strains RC1, SS70, SS70 A-, and 3.101-2, *Vibrio anguillarum* strain LS 174, and *Yersinia ruckeri* strain HI 70 were rehydrated in brain heart infusion broth (BHI) and grown and maintained on brain heart infusion agar (Difco Laboratories, Detroit, Mich.). Strain SS70 A- was derived from SS70 by growth at 30 C as described by Ishiguro et al. (1981). All *A. salmonicida* strains except SS70 A- were A layer positive (A+).

Rainbow trout (RBT) (*Oncorhynchus mykiss*), cutthroat trout (CT) (*O. clarki*) and coho salmon (*O. kisutch*) were held in specific-pathogen-free water at 12 C. Blood was harvested from anesthetized fish by inserting a needle into the caudal vein just behind the anal fin. After clotting 6 h at 4 C and centrifugation at 2500 x *g*, serum was removed and stored at -20 C. Plasma was obtained by collecting blood using a heparinized needle and syringe (15 I.U./ml whole blood). Blood was centrifuged and plasma removed with a transfer pipet. Red blood cells (RBC) from

heparinized blood were washed in phosphate buffered saline (10 mM PO_4, 0.9% NaCl; PBS) and used for hemolysin assays.

Bacteria to be cultured for supernatant analysis were grown for 24 h in BHI or trypticase soy broth (TSB, Difco), pelleted by centrifugation, resuspended in PBS, and incubated 48 h at 18 C (stationary cells). New cultures were then initiated in fresh BHI (50 ul culture at 10^9 cells/ml into 5 ml BHI) or in filter sterilized (0.22 μ) serum or plasma (33% in sterile PBS) and incubated with shaking for 48 h at 18 C. Supernatants were collected from all cultures after centrifugation, filtered (0.22 μ), and stored at 4 C. Brain heart infusion broth containing ammonium sulfate, ammonium acetate or magnesium sulfate were made from 2X concentrated solutions in water. These were filter sterilized and mixed with equal volumes of 2X BHI. Where indicated, cultures were grown with the following modified serum components; a) complement was inactivated by heating serum at 44 C for 20 min as described by Sakai (1984); b) serum was boiled 5 min and used at 33% in PBS for culture; or, c) media containing salmonid serum and BHI had a 1:2 serum:BHI ratio.

Protease assay and protease inhibition. Quantitative assays of protease activity used gelatin as a substrate. The procedure (Rockey et al. 1988) was a modification of the method for detection of gelatinases described by McDonald and Chen (1965). Briefly, 10 ul samples were incubated with 400 ul 0.2% gelatin for 40 min at room temperature. The reaction was precipitated with 30% trichloroacetic acid (TCA) and the

supernatant brought to neutral pH. This mixture was then tested for total protein using the Lowry Folin reagent (Sigma) and the OD_{525} measured on a Spectronic 20 photospectrometer (Bausch and Lomb). One unit of protease was defined as the amount of activity responsible for an increase of 0.01 OD_{525} in this assay. Protease activities from cultures in BHI supplemented with salts were determined using supernatants concentrated with 45% ammonium sulfate followed by dialysis into PBS. Serum inactivation of proteolytic activity was determined by mixing 20 µl serum with indicated volumes of protease, incubating 20 min at 18 C, and conducting a quantitative protease assay. Inhibition of protease activity was accomplished with phenylmethylsulfonyl fluoride (PMSF) or disodium EDTA pH 8.0 at a final concentration of 5 mM. Positive controls for PMSF inhibitions included the PMSF solvent, 50% isopropanol. Positive controls for EDTA inhibition were EDTA added after the trichloroacetic acid (TCA). All negative controls contained the supernatant tested, plus the inhibitor, added to the gelatin after the TCA.

Separation of P1 and P2 proteases. The PMSF sensitive serine protease (P1) and the EDTA sensitive protease (P2) of *A. salmonicida* culture supernatants were isolated using the protocol of Rockey et al. (1988). Briefly, supernatants from cultures grown 48 h were concentrated with 45% ammonium sulfate. After the pellet was resuspended in water and dialyzed into 20 mM Tris pH 7.9, proteins were chromatographed on DEAE cellulose (DE-23; Whatman Inc., Clifton, NJ). Fractions which had P1 or P2 activity

were independently pooled, dialyzed into phosphate buffer, and separated further using hydroxylapetite chromatography. The P1 and P2 proteases were subsequently concentrated by ultrafiltration over a 10,000 dalton molecular weight cutoff membrane (Amicon Corp., Lexington, Mass.).

Hemolysin assays and inhibition. Hemolysin titers were measured after making twofold dilutions of supernatant in PBS and adding them to equal volumes (50 µl) at 10^6 RBC/ml final concentration. Assays were incubated in 96-well plates at 18 C and observed with an inverted microscope for 30 min. The titer was reported as the last dilution at which 90% of the RBC were lysed. Inhibition of hemolytic activity by RBT serum was tested by incubating equal volumes of supernatant with serum for 15 min at 18 C and subsequent addition of RBC.

Antisera. Polyclonal antiserum was produced in New Zealand white rabbits by injection of ECP concentrated by a 0-45% NH_4SO_4 precipitation (Rockey et al. 1988), mixed with Freund's complete adjuvant (FCA). This was followed two weeks later with an intraperitoneal injection of antigen alone. Monoclonal antibodies against *A. salmonicida* LPS were produced in Balb/C mice using a similar injection scheme with viable *A. salmonicida* strain RC1 mixed with FCA. The fusion with Sp/2 myeloma cells, screening, and harvesting of antibody were conducted as described by Campbell (1984). All antisera were stored at -20 C. Appropriate antibody-peroxidase conjugates for rabbit and mouse primary antibodies were purchased from Boehringer Mannheim

Biochemicals (Indianapolis, Ind.) and Hyclone Laboratories (Ogden, Utah), respectively, and used at a concentration of 1 µl /ml 0.1% Tween-20 in 50 mM Tris pH 8, 150 mM NaCl (TTBS).

Electrophoresis and Western blotting. Discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) using 12% acrylamide gels was conducted as described by Schleif and Wensink (1981) with a Bio-Rad Mini-Protean II electrophoresis unit (Bio-Rad Laboratories, Richmond, Calif.). Polyacrylamide gels containing gelatin for electrophoretic analysis of proteases (G-PAGE) were prepared, electrophoresed, and incubated as described by Rockey et al. (1988). Electrophoretic transfer of PAGE gels to nitrocellulose was conducted in a Mini Trans-blot apparatus (Bio-Rad Laboratories, Richmond, Calif.) following the manufacturer's instructions. Blots were blocked in 3% bovine serum albumin (BSA, Sigma Chemical Co. St. Louis, Mo.) in TTBS for 1 h. This was followed by incubation with antiserum and appropriate conjugate. All antibodies were incubated on blots 1 h and were followed by three 5 min washes in TTBS. Fifteen milligrams 4-chloro-naphthol (Sigma) was dissolved in 5 ml 100% cold methanol, mixed with 25 ml 0.03% H_2O_2 in PBS, and used as substrate. Blots were incubated in substrate for 30 min at room temperature in the dark. Development was stopped by extensive rinsing in distilled water.

Growth curves. Brain heart infusion broth and 33% RBT serum were inoculated with stationary cells and incubated at 18 C with shaking 48 h. Five hundred microliters of each culture

was harvested aseptically at selected intervals and used for determination of cell number by plate counts. The remaining supernatant from each time point was filter sterilized (0.22 μ). Fifty microliters of the supernatant was prepared for denaturing electrophoresis, boiled 2 min, and frozen at -20 C. The remainder of the supernatant was stored in sterile tubes at 4 C for use in G-PAGE and protease or hemolysin assays.

Results

Elimination of proteolytic and hemolytic activity in the presence of chemically modified media. Protease assays, hemolysin assays, and Western blots were conducted on supernatants grown in BHI supplemented with ammonium sulfate (0.6 and 3.0%), ammonium acetate (0.5, 0.75, and 1.5%), or magnesium sulfate heptahydrate (1.1 and 5.6%). As the concentration of each of these three chemicals was increased, growth slowed and at the highest concentrations, proteolytic and hemolytic activity was reduced (Table 5.1). Ammonium acetate was inhibitory at all concentrations. Appropriate controls demonstrated that this reduced activity was not the result of inhibition of the enzymes by the salts. Western blot analysis showed the reduction in protease activity resulted from the lack of detectable concentrations of P1 protease (Figure 5.1) However, other proteins produced by the bacterium were not reduced in

TABLE 5.1. Growth, protease production, and hemolysin production in *Aeromonas salmonicida* cultures grown in chemically modified media.

Medium	h to O.D. 1.0	gelatinase activity[a]	T lysin detected after 48 h	T lysin detected after 72 h
BHI alone	22	38	+	+
BHI +:				
0.6% NH_4SO_4	40	46	+	+
3.0% NH_4SO_4	(0.7)[b]	9	-	-
1.1% $MgSO_4 \cdot 7\ H2O$	36	55	-	+
5.6% $MgSO_4 \cdot 7\ H2O$	42	2	-	-
0.7% NH_4CH_2COOH	(0.6)	8	-	-

a. Units of activity defined in the text.

b. Numbers in parentheses indicate final OD_{520} after 48 h if culture did not grow to OD 1.0.

Figure 5.1. Western blot of culture supernatants of *Aeromonas salmonicida* grown in BHI and modified BHI. Lanes: A, Molecular weights, values in kilodaltons; B, BHI; C, BHI + 0.6% NH_4SO_4; D, BHI + 3.0% NH_4SO_4; E, BHI + 0.75% NH_4CH_2COOH; F, BHI + 1.1% $MgSO_4 \cdot 7\ H_2O$; G, BHI + 5.6% $MgSO_4 \cdot 7\ H_2O$.

Figure 5.2. Gelatin-PAGE of culture supernatants of *Aeromonas salmonicida* grown in BHI and modified BHI. Lanes: A, BHI; B, BHI + 0.6% NH_4SO_4; C, BHI + 3.0% NH_4SO_4; D, BHI + 1.1% $MgSO_4 \cdot 7\ H_2O$; E, BHI + 5.6% $MgSO_4 \cdot 7\ H_2O$; F, BHI + 0.75% NH_4CH_2COOH. P1 protease band is indicated.

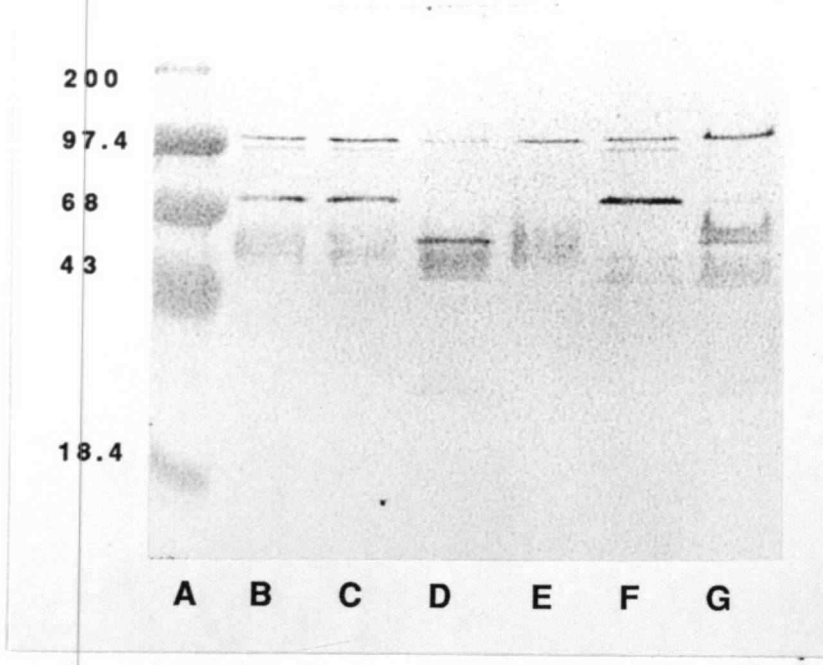

Figure 5.1

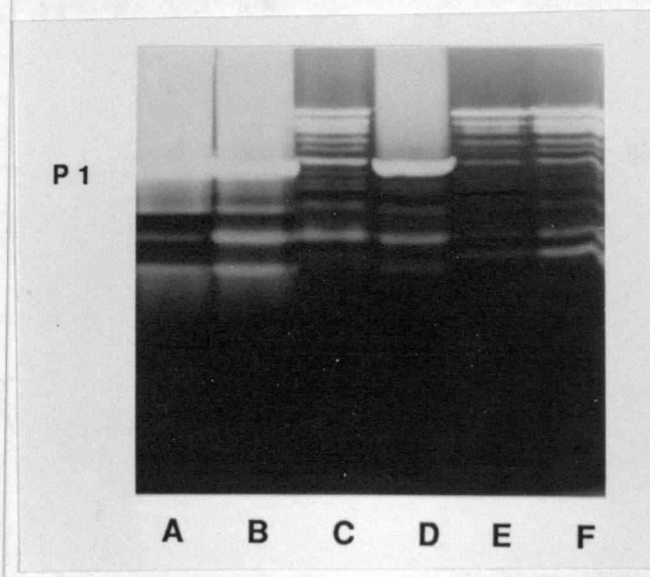

Figure 5.2

these cultures. A protein of approximately 100 kd was constant throughout and other antigenic bands were present in certain cultures. Gelatin-PAGE of supernatants from cultures grown in the modified media also demonstrated that P1 protease was reduced, but other gelatinases, particularly a high molecular weight enzyme, were not (Figure 5.2).

Analysis of protease production in BHI and salmonid serum. Comparison of protease production in cultures grown in BHI or salmonid serum demonstrated that although protease activity was present in both culture conditions, sensitivity to PMSF and EDTA differed. Proteolytic activity in supernatants from BHI cultures (BHI-S) or TSB cultures was sensitive to PMSF while supernatants from serum cultures (sera-S) had a large proteolytic component which was resistant to PMSF (PMSF-RC, Table 5.2). Negative controls with uninoculated serum mixed with BHI-S had no PMSF-RC, indicating that the PMSF-RC did not result from serum interfering with the PMSF and reducing its inhibitory action on *A. salmonicida* protease. The PMSF-RC resulted from the presence of serum and not from the absence of any specific component of BHI. When *A. salmonicida* was cultured in a mixture of BHI and serum, PMSF-RC was produced (Table 5.2). Growth in undiluted serum or 33% serum in water instead of PBS yielded similar results to 33% serum in PBS. Boiling the serum before addition of the bacterium resulted in sera-S lacking PMSF-RC, but heating serum to 44 C for 20 min before inoculation to eliminate serum complement activity did not affect PMSF-RC.

TABLE 5.2. Protease activity in the presence and absence of phenylmethylsulfonyl fluoride (PMSF) of supernatants of cultures of *Aeromonas salmonicida* grown in brain heart infusion broth (BHI), in salmonid serum, or in modified salmonid serum

Culture conditions	+PMSF	-PMSF	% activity in PMSF
Experiment 1			
BHI	1.5	23.5	6.4
TSB	1	22	4.5
33% RBT serum	10	24	42
Experiment 2			
BHI	0	15	0
100% RBT serum	9	17	53
33% RBT serum in PBS	8	22	36
33% RBT serum in BHI	14	26	54
Boiled 33% RBT serum	1	15	6.6
44 C heated 33% serum	3.5	9	36
33% coho serum	4	14	28
33% CT serum	8	18	44

Sensitivity to EDTA of proteases from BHI-S and sera-S was also different. Proteases from organisms grown in serum demonstrated higher EDTA sensitivity than those grown in BHI.

Sera from different species of salmonids were used as media for strain RC1 to determine if PMSF-RC production was unique to *A. salmonicida* cultures grown in RBT serum. Supernatants from cultures grown in coho or cutthroat serum exhibited similar protease activity profiles to that observed when RC1 was grown in RBT serum (Table 5.2).

Gelatin-PAGE analysis showed that the proteases present in BHI-S were also present in sera-S, but a high molecular weight protease band was present in sera-S that was absent in BHI-S (Figure 5.3). Dilutions of BHI-S and sera-S electrophoresed on G-PAGE demonstrated that other high molecular weight proteases similar to those seen in the G-PAGE of supernatants from the modified media were present in cultures grown in both BHI and serum.

Different strains of *A. salmonicida* and *V. anguillarum* and *Y. ruckeri*, two other species which cause gram negative septicemia in salmonid fish, were grown in serum and tested for PMSF-RC. Each of these species causes gram negative septicemia in salmonid fish. *Vibrio anguillarum* and *A. salmonicida* are proteolytic but *Y. ruckeri* is not. All strains, except *A. salmonicida* SS70 A- which is A layer negative and thus significantly more serum sensitive (Munn et al. 1982), grew well in either BHI or serum. All strains of *A. salmonicida* which grew demonstrated a PMSF-RC when

Figure 5.3. Gelatin-PAGE of supernatants from cultures of *Aeromonas salmonicida* grown in BHI (Lanes A-C) or 33% rainbow trout serum (Lanes D-F). Lanes A and D, 20 units of protease; Lanes B and E, 2 units of protease; Lanes C and F, 0.2 units of protease.

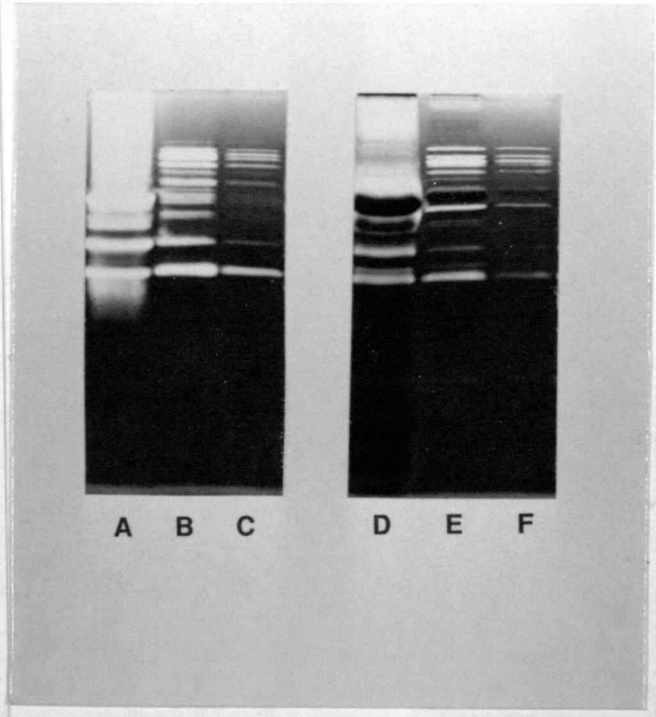

Figure 5.3

cultivated in serum. *Vibrio anguillarum* cultures grown in either medium did not demonstrate qualitative differences in PMSF sensitivity and no protease activity was observed in cultures of *Y. ruckeri* grown in BHI or serum. These results were confirmed with G-PAGE. Proteases of *V. anguillarum* were unique from *A. salmonicida* and *Y. ruckeri* was not proteolytic.

Western blots of supernatants from cultures grown in BHI and serum. Western blots of BHI-S developed with rabbit anti-BHI-S antiserum showed three major groups of bands; a protein band that migrated at 100 kd, the protease band at 70 kd, and a group of bands migrating at approximately 50 kd (Figure 5.4). Monoclonal antibodies against *A. salmonicida* LPS demonstrated that the group of bands at 50 kd were composed of lipopolysaccharide. Western blots from sera-S had a different profile. The major differences were the absence of the 50 kd group, the decreased intensity of the 70 kd band, and the appearance of a collection of larger bands from 70 kd to the top of the blot. The 100 kd protein band was similar in either medium. Western blots of BHI-S which had been incubated with trout serum prior to addition of denaturing dye and subsequent electrophoresis were similar to blots of sera-S. The 70 kd band was diminished, the 100 kd band remained unaffected, and new bands appeared in the blot above 70 kd. Control lanes in these blots had denaturing dye added to BHI-S prior to addition of serum, which eliminated covalent interactions between the serum

Figure 5.4. Western blot of *Aeromonas salmonicida* culture supernatants probed with rabbit anti-extracellular product antiserum. Lane A, Culture grown in brain heart infusion broth (BHI); Lane B, Culture grown in 33% rainbow trout (RBT) serum; Lane C, Uninoculated BHI; Lane D, Uninoculated 33% RBT serum; Lane E, Molecular weight standards, values in kilodaltons.

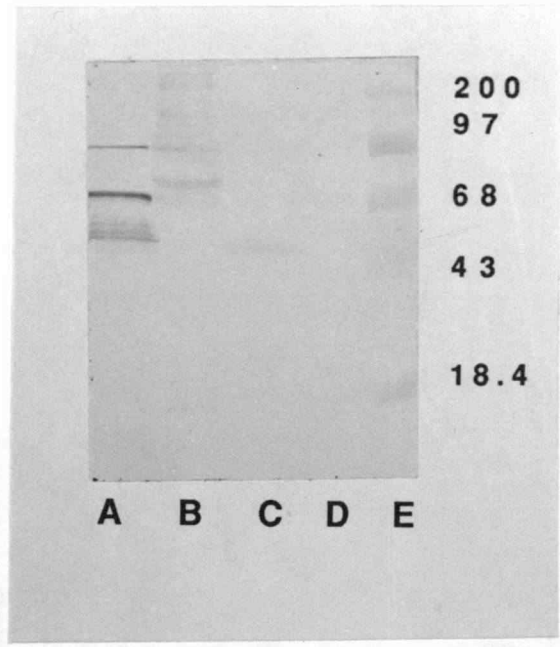

Figure 5.4

and the supernatant. Immunoreactive patterns in these controls were identical to those in BHI-S (Figure 5.4).

Serum inhibition of protease. Preparations of P1 and P2 protease and BHI-S were incubated with normal RBT serum to determine the relative sensitivity of each activity. Both P1 and P2 proteases were inhibited by serum. Thirteen units of P1 protease was 76% sensitive to serum inhibition and 9 units of P2 protease was 35% sensitive. Excessive quantities of either activity were capable of saturating the inhibitory components of serum. Total culture supernatants from BHI were also sensitive to serum and our results agreed with those of Sakai (1984) and Ellis (1987)

Growth curves in serum and BHI. To determine if the kinetics of growth and protease production were affected by culture in BHI or serum, 48 h growth curves were analyzed. After an initial decrease of the bacterial population in the presence of serum, growth was similar in both media (Figure 5.5). However, protease activity at selected time intervals was different; the activity in BHI increased rapidly at 24 h but protease concentrations in the serum cultures were lower and had no rapid increase. During growth, PMSF-RC was detected in sera-S but not in BHI-S. Western blots of these supernatants were similar to the blots of BHI-S and sera-S (Figure 5.3).

Figure 5.5. (A) Growth curve of *Aeromonas salmonicida* in BHI (□) and 33% rainbow trout serum (◆). (B) Protease production assayed in the presence and absence of PMSF at selected time intervals of the growth curve. Supernatant from BHI without PMSF (□), supernatant from BHI with PMSF (■), supernatant from 33% rainbow trout serum without PMSF (◆), supernatant from serum with PMSF (◇).

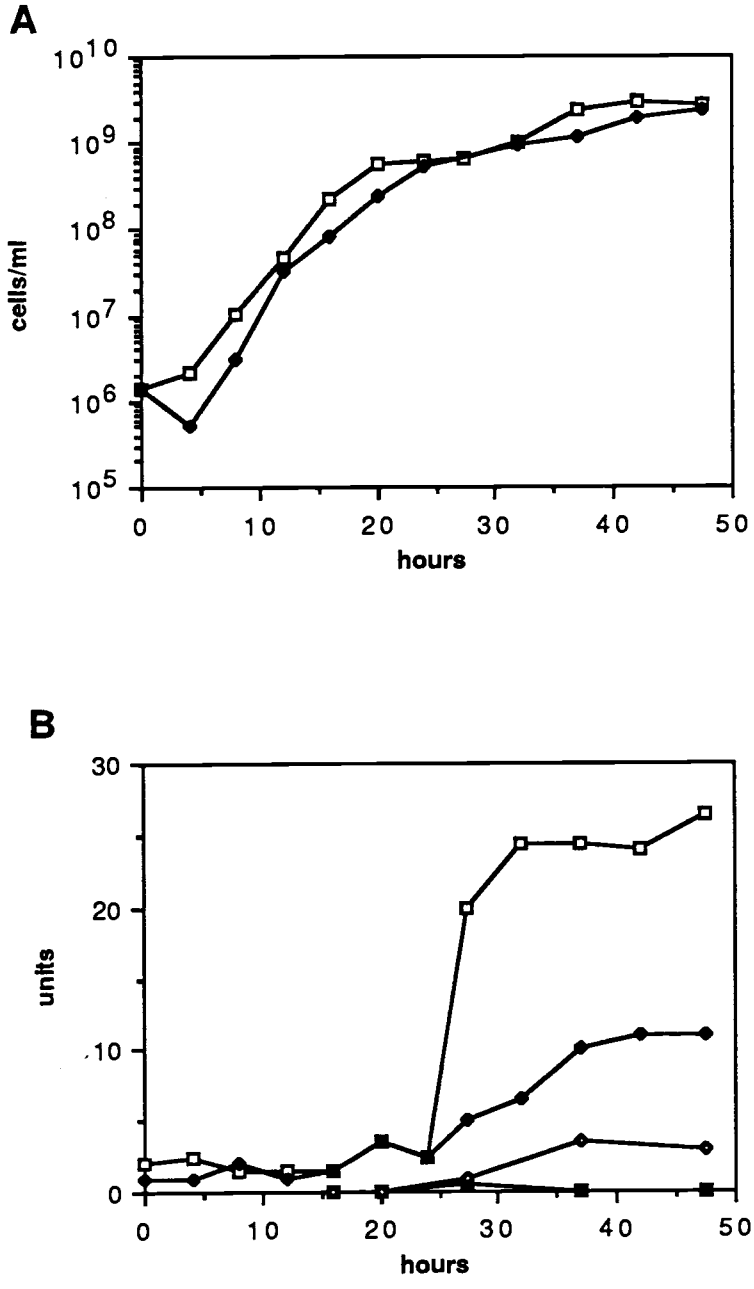
A
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Figure 5.5

Discussion

Other investigators have examined secreted factors of *A. salmonicida* cultured in standard laboratory media under optimal growth conditions. Exceptions to this include work by Fyfe et al. (1986a, 1987b) who examined secretion by bacteria grown in anaerobic conditions and at different temperatures, and a report by Titball and Munn (1983) who used 3% ammonium sulfate to eliminate P1 protease. In this report, we examined production of protease and hemolysin by the bacterium in chemically modified media and in salmonid serum. Gelatin-PAGE, Western blot analysis, and protease and hemolysin assays showed that higher percentages of magnesium sulfate or ammonium sulfate and any tested concentration of ammonium acetate altered P1 protease production and hemolysin activity in supernatants, but other proteins and proteases were not affected. The elimination of enzyme activity in these media was not the result of inhibition of *A. salmonicida* metabolism by a particular element or ion nor was the difference in protease secretion solely a result of slower growth rate. In the case of the 5.6% magnesium sulfate, the growth rate was comparable to the growth in 1.1% magnesium sulfate or 0.6% ammonium sulfate, but P1 protease and hemolysin activities were not detected. Instead, we propose that the decreases in P1 protease and hemolysin activity were caused by a generalized chemical stress imposed upon the bacterium. These results also demonstrated that during this chemical stress, a high

molecular weight protease was still produced. We have shown previously that this protease is a constituent of the P2 protease peak (Rockey et al. 1988) described by Sheeran and Smith (1981). The Western blotting showed that a 100 kd protein was present in all cultures of BHI regardless of the added chemical. Fyfe et al. (1987b) demonstrated a 100 kd protein during growth of *A. salmonicida* at lower temperatures (10 C). It is possible these proteins are the same and represent the high molecular weight P2 protease constitutively expressed in our G-PAGE analysis. Because of the partially denaturing conditions during G-PAGE, we could not accurately determine the molecular weight of the high molecular weight protease.

Growth in serum also affected the proteases present in *A. salmonicida* culture supernatants. In all experiments, protease assays of *A. salmonicida* sera-S resulted in a higher proportion of PMSF resistant, EDTA sensitive protease than BHI-S or TSB-S. This difference was not affected by eliminating serum complement, but it was affected by boiling the serum. The growth curves indicated that, although growth was similar between cultures grown in BHI or in serum, the kinetics of protease production were not. Assays with *Y. ruckeri* and *V. anguillarum* demonstrated that the qualitative differences between sera-S and BHI-S were specific for *A. salmonicida* (not shown). This suggested that although the EDTA sensitive, PMSF resistant P2 proteases were not found in large quantities in cultures grown in BHI or TSB, they may have

been present at higher relative concentrations in cultures grown in sera.

Hastings and Ellis (1985) used isoelectric focusing of bacterial supernatants from cultures grown in trypticase soy broth to demonstrate that only a single peak which contained proteolytic activity was present. These results did not agree with those of Sheeran and Smith (1981) who demonstrated that at least one other protease (P2) was present in spent culture supernatants. The differences in PMSF sensitivities in serum and BHI and the effect of media supplements on protease activities in BHI demonstrated in this paper may account for different patterns observed by the different authors.

We demonstrated that protease secretion can be selectively affected by manipulations of laboratory growth media and that this effect was also seen in cultures grown in serum. We have also shown that serum can interact with *A. salmonicida* proteins after secretion. Western blot data indicated that covalent interactions occur between serum components and P1 protease and protease inhibition assays showed that rainbow trout serum was capable of inhibiting both P1 and P2 protease of *A. salmonicida*. The inhibition data also suggested that P1 protease is inhibited more efficiently than P2 protease. Alpha-macroglobulin proteinase inhibitors are present in trout serum and these may be responsible for the interactions that we report (Ellis and Grisley 1985; Grisley et al. 1984).

Hemolysin assays of supernatants from chemically modified media and from cultures grown in serum demonstrated that hemolytic activity was reduced in certain of these media. Higher concentrations of ammonium sulfate, magnesium sulfate, and any tested concentation of ammonium acetate eliminated detectable hemolytic activity in the supernatant. Furthermore, in our assays hemolysin activity was high in BHI-S, but absent in sera-S. These assays did not measure concentrations of hemolysin present in sera-S and thus we cannot state whether or not hemolysin was released by the organism in serum. Other authors have demonstrated that salmonid serum is inhibitory to the action of *A. salmonicida* protease and T lysin (Munro et al. 1980; Titball and Munn 1981) and that the inhibition of the toxic effects of culture supernatants were generally associated with activation of serum complement (Sakai, 1984). Our assays demonstrated that *A. salmonicida* proteases were detected in sera-S but hemolysin activity was not. Additionally, unpublished data demonstrated that sera-S was inhibitory to the hemolytic activity of BHI-S. This suggested that the two inhibitory mechanisms were distinct.

Based on the results presented, we propose that at least two different patterns for growth of *A. salmonicida* occur. In optimal growth conditions, the organism produced detectable concentrations of P1 protease and T lysin. In modified media, proteolytic activity and synthesis of P1 protease were reduced but P2 proteases were readily detectable. Supernatants from cultures grown in BHI and cultures grown in serum demonstrated similar

profiles on G-PAGE but qualitative differences in protease sensitivity to inhibitors were evident. In all cases, P2 protease was produced but P1 protease and hemolysin were produced only in more optimal growth conditions. Other reports have suggested that P1 protease and possibly host proteases were responsible for the lesion formation during an infection (Hastings and Ellis 1985; Rockey et al. 1988; Sakai 1985a; Tajima et al. 1983a) However, the present report suggests that although P1 protease is associated with visible pathology during infection, P2 protease may be produced during infection as well, possibly at a higher relative rate during physiological stress of the bacterium.

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Chapter 6

Cellular Pleomorphism Induced in Virulent *Aeromonas salmonicida* by Growth in Media Containing High Concentrations of Magnesium Salts

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Abstract

Growth of wild type strains of the fish pathogen, *Aeromonas salmonicida,* in brain heart infusion broth (BHI) plus 5.6% or 8% magnesium sulfate heptahydrate (MgBHI) resulted in bacteria with pleomorphic cellular morphology. Bacteria grown in equimolar concentrations of $MgCl_2$ were also pleomorphic. Cultures of isogenic strains which were deficient in the A layer protein did not produce pleomorphic forms upon culture in MgBHI and incubation of bacteria in MgBHI plus antibiotics or incubation in phosphate buffered saline plus 8% $MgSO_4$ did not result in pleomorphic cells. No differences in the cellular protein constituency or the high molecular weight lipopolysaccharide were observed between *A. salmonicida* grown in BHI or MgBHI. Pleomorphic forms and typical bacteria were equally virulent by LD_{50} and mean day to death analysis in coho salmon (*Oncorhynchus kisutch*).

Introduction

Aeromonas salmonicida is a gram negative coccobacillus which infects salmonids and other species of fish (McCarthy and Roberts 1980). The species causes the disease furunculosis and, in many areas of the world, is a major problem in hatchery populations of fish. A particularly problematic aspect of this disease is that protocols for vaccination against *A. salmonicida* have never been adequately developed while other, similar, bacterial diseases of fish are prevented by simple immersion vaccination (Gould et al. 1979). In attempts to formulate efficacious vaccines against *A. salmonicida* and to understand host-pathogen interactions, detailed analyses of potential antigens and aggressins have been undertaken in many laboratories. Cell-associated antigens and virulence factors include lipopolysaccharide and the A layer, an outer membrane protein layer which covers the surface of the cell (Munn et al. 1982; Udey and Fryer 1978). Secreted aggressins include proteolytic and cytolytic enzymes (Ellis et al. 1981; Titball and Munn 1985).

In our analyses of the aggressins of *A. salmonicida* we determined that proteases produced by the organism were differentially regulated in the presence of high concentrations of ammonium acetate, ammonium sulfate, and magnesium sulfate. The production of the trout erythrocyte specific hemolysin (T-

lysin) was also inhibited by these salts. Here we report that *A. salmonicida* grown in high concentrations of magnesium salts had a pleomorphic morphology and was directly associated with the presence of the A layer.

Materials and Methods

Bacterial strains and culture conditions. Strain RC 1 was isolated from diseased chinook salmon (*Oncorhynchus tshawytscha*) at a hatchery in Oregon, U.S.A. Strain 3173 was isolated from diseased Atlantic salmon (*Salmo salar*) at a production facility in Norway. Isogenic, A layer deficient (A-) derivatives of these two strains were selected by growing the wild type strain at 30 C as described (Ishiguro et al. 1981) and identified by growth on Coomassie Blue agar (Wilson and Horne 1986). For production of pleomorphic forms, bacteria were first grown in 5 ml brain heart infusion broth (BHI) at 18 C for 24 h. These cultures were centrifuged and the bacteria placed in an equal volume of phosphate buffered saline (PBS; 10 mM phosphate, 150 mM NaCl, pH 7.2) and incubated at 18 C for 24 h. Fifty microliters of this suspension was added to BHI containing the selected concentration of salts. These cultures were incubated 48-72 h and examined by gram stain. Cultures were checked for purity on BHI agar and on MgBHI agar. To determine if the pleomorphism occurred in the absence of cell growth or protein

synthesis, cultures grown in BHI were incubated in MgBHI in the presence of 50 mM nalidixic acid or chloramphenicol (Sigma Chemical Corp. St. Louis MO.).

Electron microscopy. Scanning electron microscopy (SEM) was performed on cells washed in PBS, dried on 12 mm glass cover slips, and layered with gold/palladium (60/40). Transmission electron microscopy (TEM) was performed on both whole cells and thin sections. Whole cells were prepared for TEM by negative staining with uranyl acetate. Cells to be sectioned were washed in water, fixed in 2.5% glutaraldehyde and embedded using the method of Spurr (1969). Eight hundred angstrom sections were cut and stained with lead citrate (Reynolds 1963).

Electrophoretic analyses. Cellular proteins were examined using discontinuous polyacrylamide gel electrophoresis as described by Schleif and Wensink (1981). Cellular lysates were produced by sonication (2 x 10 s, 60 W) of cultures pelleted and washed in PBS. High molecular weight lipopolysaccharide (LPS) of these lysates was examined with Western blots using a monoclonal antibody against LPS as probe. Outer membrane extracts were prepared using 2 M guanidium chloride as described by Ward et al. (1985).

Pathogenicity and immunogenicity analyses. Virulence of pleomorphic forms was examined using LD_{50} and mean time to death analyses in juvenile coho salmon (*O. kisutch*) cultured in a 12° C pathogen-free water system. Ten fish were

challenged with each concentration of microorganism. Vaccines were prepared using cultures grown in BHI or in 5.6% MgBHI. Bacterial cells from these cultures were washed in PBS and killed with 0.6% formalin. Vaccines were administered to juvenile coho salmon (20 salmon in each group) by immersion in bacterin at 5 x 10^7 cells/ml for 2 min. Vaccinated fish plus mock vaccinated control fish were challenged 21 d later by 2 min waterborne exposure to 5 x 10^8 bacteria/ml from a culture grown in BHI broth. Mortality was monitored daily and dead fish examined for presence of the pathogen.

Results

Differences between cells grown in MgBHI and in BHI were observed in gram stains and by TEM of whole bacteria. Wild type A layer positive (A+) cells grown in BHI were typical coccobacilli while cells from MgBHI were elongated and sigmoid shaped. Scanning electron microscopy further demonstrated these traits (Figure 6.1). Cells from MgBHI were thinner and longer than cells grown in BHI. The A layer was associated with the formation of pleomorphic cells, because isogenic A- cells grown in MgBHI had typical morphology (Figure 6.1). The results were similar for both isolates of *A. salmonicida* tested. Other authors have demonstrated that the A layer is responsible for the aggregating characteristic of the species (Udey and Fryer 1978). Scanning electron micrographs showed this aggregating characteristic was

Figure 6.1. Scanning electron micrographs of *Aeromonas salmonicida* grown in selected media. (A) A layer + bacteria grown in BHI; (B) A layer - bacteria grown in 8% MgBHI; (C) A layer + bacteria grown in 8% MgBHI. Bar is 5 μm.

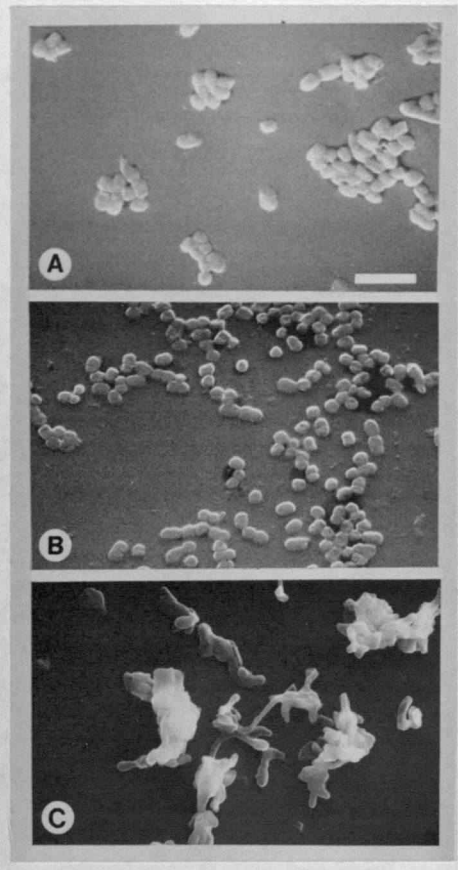

Figure 6.1

present in the pleomorphic forms. Examination with TEM of whole cells from cultures grown in BHI demonstrated the repeating outer membrane A layer lattice sloughing off of the cells as reported by other authors (Ishiguro et al. 1981). Cells grown in MgBHI did not have this sloughing outer layer and no regular lattice was observed (Not shown). Observation of thin sections revealed that the A layer was distinct on the surface of cells grown in BHI but was difficult to observe on the surface of cells grown in MgBHI (Figure 6.2). These micrographs also demonstrated the pleomorphy of cells from MgBHI and the relative uniformity of cells grown in BHI. Micrographs of sections of bacteria from A- cultures grown in MgBHI confirmed that pleomorphy was associated with the presence of the A layer. Examination of cellular proteins and lipopolysaccharide revealed that no differences between bacterial cells grown in BHI and MgBHI. This included comparisons of total cellular proteins, guanidium chloride extracted outer membranes, and Western blots of lipopolysaccharide.

Growth was required for the formation of pleomorphic forms and also for the return to their typical morphology. Bacteria incubated in appropriate media plus either chloramphenicol or nalidixic acid did not transform from typical morphology to pleomorphic forms, nor did they revert back to regular coccobacilli forms from pleomorphic forms. Further, bacteria grown in BHI prior to incubation in PBS plus 8% magnesium sulfate did not form pleomorphic cells.

Figure 6.2. Transmission electron microscopy of thin sections of: (A and B) A layer + *Aeromonas salmonicida* grown in BHI, (C and D) A layer -*A. salmonicida* grown in 8% MgBHI, (E and F) A layer + *A. salmonicida* grown in 8% MgBHI. Magnification: A, C and E x 45000; B, D and F x 12500.

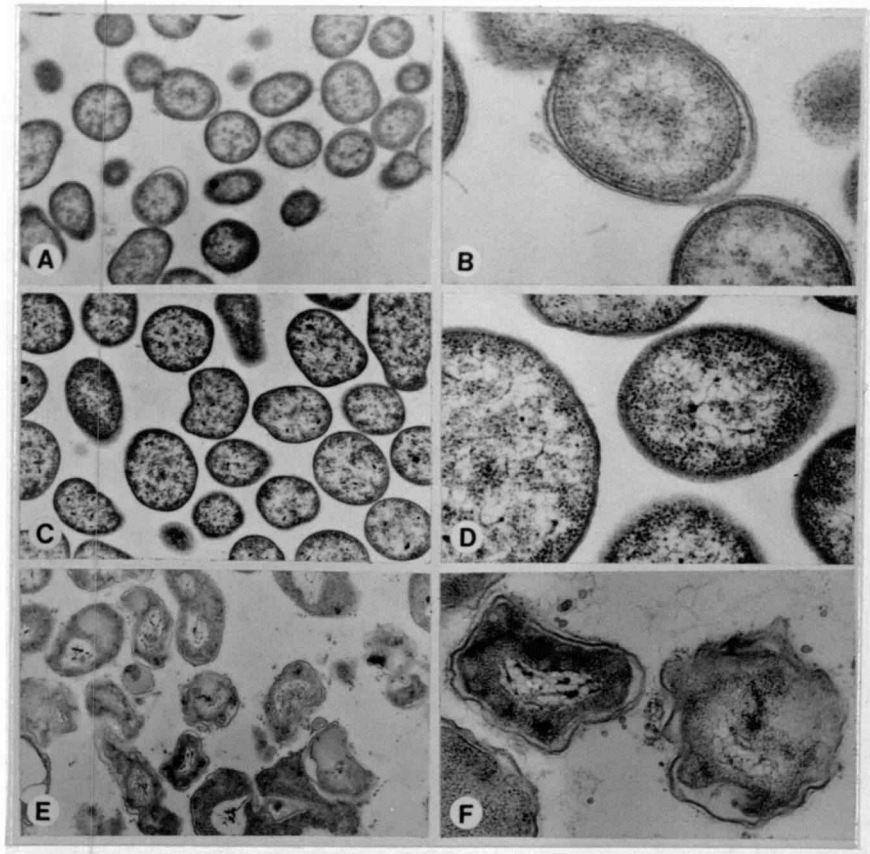

Figure 6.2

The virulence of the pleomorphic forms in coho salmon was examined by LD_{50} analysis. Bacteria from MgBHI and from BHI had LD_{50} values less than 1000 and mean time to death values of 6 days after intrasmuscular injection of 10^3 cells. Protection of coho salmon with bacterins produced from cells grown in 5.6% MgBHI was examined relative to bacterins from cells grown in BHI. In two separate trials, relative percent survival values of fish vaccinated with bacterins from MgBHI were lower but not significantly different than relative percent survival values provided by bacterins produced in BHI (Data not shown).

Discussion

Morphological variations of otherwise uniform bacteria resulting from modified culture conditions or exposure to antibiotics have been described for several different bacterial species. These include small cell formation in starved gram negative species, morphological variation associated with growth, and L-forms of bacterial cells exposed to penicillin or osmotic stabilizers (Novitsky and Morita 1976; Ensign and Wolfe 1964; Luscombe and Gray 1974; Madoff 1986). There are also reports of morphological variants induced by changes in salt concentration of culture media. For example, *Streptococcus mutans* switches from coccoid to baccillary morphology when the relative concentration of potassium and bicarbonate in culture media are manipulated

(Tao et al. 1987). We do not believe that the pleomorphic forms described in this paper parallel any of these examples. Certain images of L-forms in the literature generally resemble the ultrastructural morphology seen in figure 2E, and L-forms can be induced and stabilized by high concentrations of salts (Madoff 1986; Ryter and Landman 1964). But, the elongated shape of the *Aeromonas salmonicida* pleomorphic forms and dependence on an extracellular protein layer for formation are not traits shared by other species producing L-forms. Additionally, there are no descriptions of L-forms of the genus *Aeromonas* (Madoff 1986).

The physical cause of the pleomorphy is unclear. These forms were present only in cultures possessing the outer membrane protein A layer. However, we did not find qualitative or quantitative differences in the A protein between A+ cells grown in BHI or MgBHI. Pleomorphic forms observed with transmission EM of whole cells and sections demonstrated that the A layer, if present on the surface of the cells, was deficient in its characteristic lattice formation. The electrophoresis and Western blot data indicated that A protein and lipopolysaccharide were present in outer membrane extracts of cells grown in MgBHI.

Because of the association of morphologic variants and the induction of disease in some species (Pachas 1986) we examined the effects of the pleomorphy on the interaction of the bacteria with its fish host. Pleomorphic forms and normal cells had similar virulence properties as determined by LD_{50} and mean time to death analyses in coho salmon. Additionally, preliminary

evaluation of protection did not establish a difference between the two forms. Although relative percent survival values were lower in each of two vaccination trials, vaccination of coho salmon with bacterins produced from cells grown in MgBHI was not significantly different than vaccination with bacterins produced from cells grown in BHI. Therefore there was no evidence for differences in host-pathogen interaction between bacteria grown in either media.

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Chapter 7

Monoclonal Antibodies Against *Aeromonas salmonicida* Lipopolysaccharide Identify Differences Among Strains

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Abstract

Monoclonal antibodies (Mabs) directed against *Aeromonas salmonicida* lipopolysaccharide (LPS) were produced and characterized. The specificity of the antibodies to LPS was determined by examination of Western blots of protease treated antigens and of antigens deficient in a major outer membrane protein layer, the A layer. Two sets of anti-LPS Mabs were produced. The first set reacted with all but a unique group of *A. salmonicida* strains tested and had binding properties that were insensitive to periodate oxidation of antigen. The second set, represented by a single IgM clone, reacted with a more limited collection of bacterial isolates and the binding was sensitive to periodate treatment of antigen. Western blot analysis using a collection of *A. salmonicida* isolates as antigen resulted in three different reactivity groups. The Mabs were used in competitive ELISA assays comparing polyclonal antisera produced against formalin-killed whole *A. salmonicida* cells from rainbow trout (*Oncorhynchus mykiss*) and rabbits. These assays demonstrated that qualitative differences in antibody specificities were present between these species.

Introduction

Aeromonas salmonicida, the etiologic agent of furunculosis and ulcer disease of salmonids and other fish, causes major losses to hatchery salmonid populations in many parts of the world (McCarthy and Roberts 1980; Snieszko et al. 1950; Bootsma et al. 1977). Although protective furunculosis vaccines have been reported, the ability of reported vaccines to stimulate a protective specific immune response is debated (Ellis 1988). Therefore, these diseases are principally controlled by antibiotic therapy and pathogen avoidance.

Both cellular and secreted antigens of *Aeromonas salmonicida* have been investigated as possible immunogens (Hastings and Ellis 1988; Munro 1984). Immunologically important antigens associated with the cell surface are the outer membrane protein A layer (McCarthy et al. 1983) and lipopolysaccharide (LPS; Paterson and Fryer, 1974). A high degree of uniformity of these two components has been demonstrated within the species (Kay et al. 1984; Chart et al. 1984).

Three subspecies of *Aeromonas salmonicida* are currently recognized. These are *A. salmonicida salmonicida*, *A. salmonicida achromogenes*, and *A. salmonicida masoucida* (Popoff 1984). However, many isolates do not fit into any of these categories and

modifications to the current classification scheme have been proposed (McCarthy and Roberts 1980; Belland and Trust 1988). These modifications include the addition of a new subspecies, *A. salmonicida nova*, and the combining of most of the isolates from *A. salmonicida masoucida* and *A. salmonicida achromogenes* into a single subspecies with the latter epithet.

The present paper describes the production and characterization of monoclonal antibodies (Mabs) against *Aeromonas salmonicida* LPS which identify antigenic differences among different strains. The Mabs were also used to demonstrate qualitative differences between the antibody response of rainbow trout (RBT, *Oncorhynchus mykiss*) and rabbits towards *A. salmonicida* LPS.

Materials and Methods

Bacterial isolates and antigen preparation. All isolates were grown on brain heart infusion (BHI, Difco Laboratories, Detroit, MI) agar for isolation and were stored at 4 C on BHI agar slants. Isogenic A layer negative (A-) mutants were selected by growing wild type (A+) *Aeromonas salmonicida* strains on Coomassie Blue agar at 30 C and recovering white colonies (Ishiguro et al. 1981; Wilson and Horne 1986). Whole cell antigen for Western blots was prepared from cultures grown in BHI broth for 24-48 h at 17 C. These cultures were centrifuged and washed

in phosphate buffered saline (PBS; 10 mM PO_4, 150 mM NaCl, pH 7.0) and resuspended in PBS at approximately 10^9 cells/ml. The cultures were kept on ice and sonically lysed with two 10-second bursts using a probe sonicator with power level at 60 W. Aliquots of these sonicates were mixed with equal volumes of electrophoresis dye (Schleif and Wensink 1981), boiled 2 min, and stored at -20 C. The remaining sonicate was stored at -70 C.

Whole cell extracts were treated with proteinase K (Sigma Chemical Co. St Louis, MO.) at 0.5 mg/ml for 4 h at room temperature. Digestion was confirmed using SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie Blue staining. These proteinase K preparations were used as antigen in Western blots. Outer membrane extracts of whole *A. salmonicida* cells were prepared using guanidine hydrochloride (GCl) as described by Ward et al. (1985) and used as antigen in ELISA assays. Protein concentrations were measured using the Lowry method of protein determination (Lowry et al. 1951).

Production of monoclonal antibodies. Viable strain RC 1 bacterial cells harvested from a BHI broth culture were washed in sterile PBS and adjusted to an optical density of 1.0 at 525 nm (A_{525}). This was mixed with an equal volume of Freund's complete adjuvant (FCA) and 0.5 ml was injected subcutaneously into female Balb/C mice. One hundred microliters of a similar antigen preparation without adjuvant was injected intraperitoneally 21 d after the primary injection. Three days after the secondary injection spleen cells were harvested and

fused with Sp2/0 myeloma cells as described by Campbell (1984). Clones producing antibodies against wild type *Aeromonas salmonicida* cells were selected using an antigen enzyme-linked assay with whole *A. salmonicida* cells as antigen (Campbell 1984), expanded, and minicloned twice by limiting dilution. All antibodies were harvested from tissue culture. Heavy and light chain isotypes and subisotypes were determined using a commercial kit (Bio-Rad Laboratories, Richmond CA).

Polyclonal antisera. Antisera were produced in New Zealand White rabbits with two different antigens; A+ strain RC 1 and A- strain RC 1. The cellular antigens were prepared as described for production of monoclonal antibodies. One milliliter of antigen emulsified in FCA was injected subcutaneously followed by injection of 200 ul of antigen without FCA after 28 d. Blood was collected by cardiac puncture 14 d after the secondary injection and serum was collected after overnight clotting at 4 C. Rainbow trout antisera were produced against whole A+ and A- bacteria in RBT using a similar injection protocol. Fish were bled from the caudal vein. All antisera were stored at -20 C.

Agglutination assays. Bacterial agglutination tests were performed using formalin-killed A- RC 1 as antigen. Rabbit and RBT antisera were incubated at 45 C for 20 min to inactivate the complement in the sera. Doubling dilutions of serum samples and monoclonal antibody preparations were performed in 'V-bottomed' 96-well plates with PBS as a diluent. Equal volumes of formalin-killed A- *Aeromonas salmonicida* at an A_{525} of 0.85

were added and the reaction incubated 4 h at room temperature. Titers were reported as the highest initial dilution with no cell pellet visible in the bottom of the well. Normal rabbit serum, normal RBT serum, and unrelated monoclonal antibodies with the same subisotype as the tested antibodies were used as negative controls in these and all immunological methods.

Electrophoresis and Western blots. Polyacrylamide gel electrophoresis was performed on each isolate used for antigenic analysis. Unless otherwise indicated, approximately 3 x 10^6 bacteria were mixed with denaturing dye (Schleif and Wensink 1981) and electrophoresed using a Bio-Rad Mini-Protean II electrophoresis apparatus. Gels were stained with Coomassie Blue. Western blots were performed with identical gels transferred to nitrocellulose using a Bio-Rad Laboratories Mini-Transblot apparatus. Blots were also performed with tenfold dilutions of cell sonicates electrophoresed as antigen. After transfer, blots were blocked with 3% bovine serum albumin (BSA) in tris-buffered saline (TBS, 150 mM NaCl, 50 mM tris pH 8.0) plus 0.1% tween-20 (TTBS). Blots were then washed once in TTBS and the primary antibody applied. All blots were incubated at room temperature for 1 h. After incubation, the blots were washed three times in TTBS and incubated in appropriate horseradish peroxidase-conjugated second antibodies (Hyclone Laboratories, Logan, UT). This was incubated and then washed three times in TTBS. Blots were developed with 4 chloro-naphthol (4CN) and hydrogen peroxide (0.5 mg/ml 4CN and 0.03% H_2O_2). Periodate

oxidation of blots to determine epitope sensitivity to oxidation was performed as described by Woodward et al. (1985). Briefly, blots were incubated after transfer in TTBS for 30 min and then incubated in 10 mM periodic acid in 50 mM sodium acetate pH 4.5 for 1 h in the dark. The blot was rinsed in 50 mM sodium acetate and incubated in 50 mM sodium borohydride in PBS. Control blots were incubated in TTBS instead of periodate. After treatment with borohydride, the primary antibody was added to all blots and incubations proceeded as with untreated blots.

Examination of bacterial virulence. Bacterial isolates were cultured in BHI broth for 48 h at 17 C prior to centrifugation and resuspension in sterile PBS. These bacteria were enumerated and 2 x 10^4 - 4 x 10^4 colony forming units were injected intramuscularly into juvenile coho salmon (*Oncorhynchus kisutch*). These fish were kept in pathogen-free water at 12 C and examined daily for 3 weeks to determine if the isolate caused a lesion. Tissue from developing lesions was inoculated to BHI agar for reisolation of the pathogen.

ELISA assays. Competetive ELISA assays were conducted by coating a 96-well immunoassay plate (Costar, Cambridge, MA.) with 50 µl of a GCl extract of A+ or A- cells at 1.4 mg protein/ml. After overnight incubation, the plate was blocked with 200 ul 1% BSA in TTBS for 1 h. After one rinse in TTBS, wells were incubated with twofold dilutions of blocking antibody (RBT or rabbit) for 1 h. After three washes with TTBS, a constant concentration of Mab was added and incubated for 1 h. Three

washes followed this incubation and peroxidase-conjugated goat anti-mouse immunoglobulin (Hyclone) was added and incubated 1 h. After three more washes, reactions were developed with 2 2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS, 75 μg/ml) and hydrogen peroxide (0.015%) in 0.2% sodium citrate pH 4.5. Optical densities were measured at 405 nm after 20 min incubation in substrate.

Results

Characterization of monoclonal antibodies. Four monoclonal antibodies were produced and characterized. Three were of the IgG_{2b} subisotype with κ light chains (Mab 1, C, and D). One was of the IgM isotype with a κ light chain (Mab 6). Two of these antibodies (D and 6) were reacted with selected antigen preparations to examine their binding specificity. Western blots using proteinase K treated antigens and blots using A- derivatives of isolate RC1 showed that both antibodies bound *Aeromonas salmonicida* LPS (Figure 7.1). Western blots were also performed using different concentrations of antigen. These blots demonstrated that the localization of antigen on the blot was dependent upon the concentration of antigen electrophoresed and transferred (Figure 7.2). Periodate treatment prior to Western blotting demonstrated that the epitope bound by Mab D was periodate-insensitive and the epitope bound by Mab 6 was

Figure 7.1. Western blot of *Aeromonas salmonicida* isolate RC 1 cell preparations using Mab D as probe. (Std) Protein molecular weights, values in kilodaltons. (1) A layer - cells. (2) Proteinase K-treated A layer + cells. (3) Untreated A layer + cells.

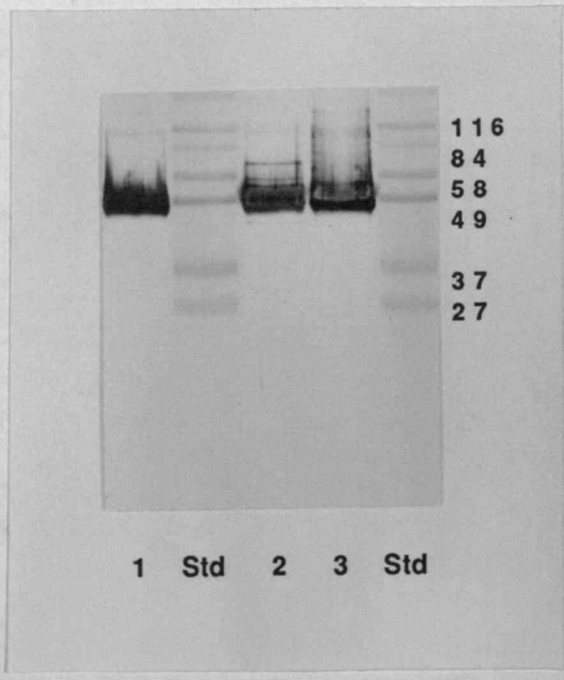

Figure 7.1

Figure 7.2. Western blot of dilutions of *Aeromonas salmonicida* isolate RC 1 cells. Mab D used as probe. (1) 3 x 10^6 cells. (2) 3 x 10^5 cells. (3) 3 x 10^4 cells.

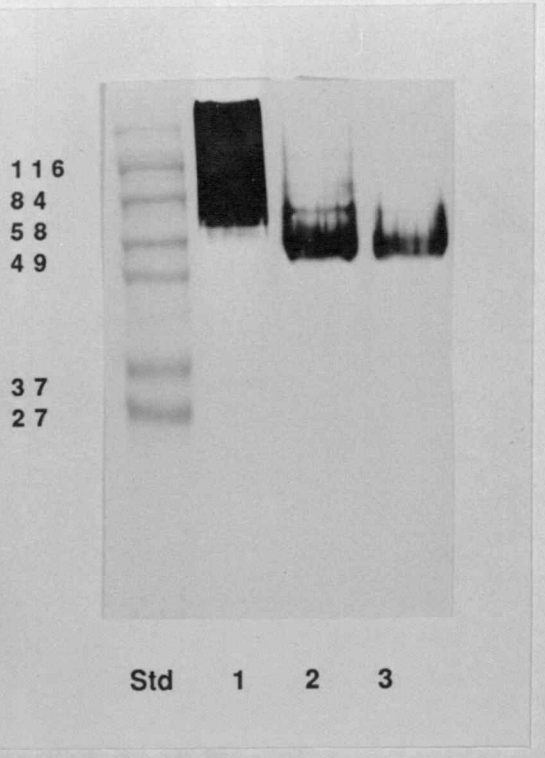

Figure 7.2

periodate-sensitive. Examination of the other IgG Mabs demonstrated that their reactivity was similar to Mab D.

Three groups of antibody reactivity patterns were observed after examination of different isolates by Western blot (Figure 7.3). Isolates reacted with both Mab D and Mab 6 (+/+ reaction), or with Mab D but not Mab 6 (+/- reaction), or the strains reacted with neither antibody (-/- reaction). Isolates that did not react with Mab D also did not react with rabbit antiserum directed against LPS from *A. salmonicida salmonicida* strain RC 1. Lipopolysaccharide from all isolates reacting with Mab D were recognized by this rabbit antiserum. All isolates that reacted with Mab 6 also reacted with Mab D.

The results demonstrated that reactivity varied among isolates (Table 7.1). Comparisons among previously classified subspecies of *Aeromonas salmonicida* demonstrated that all tested *A. salmonicida salmonicida* were +/+, the type culture of the subspecies *A. salmonicida achromogenes* was +/-, and the two *A. salmonicida masoucida* isolates were +/+. Unclassified or atypical *A. salmonicida* were +/+, +/-, or -/-.

Antibodies were also used as probes in Western blots of sonicates of other bacterial fish pathogens. This examination included strains of *Aeromonas hydrophila*, *Yersinia ruckeri*, *Pseudomonas fluorescens*, *Edwardsiella sp.*, *Vibrio anguillarum*, *Lactobacillus piscicola* and *Renibacterium salmoninarum*. Neither antibody D nor 6 reacted with these bacteria in Western blots (Data not shown).

Figure 7.3 (A) Polyacrylamide gel of selected *Aeromonas salmonicida* isolates; (B) Western blot of these isolates using Mab D as probe; (C) Western blot of these isolates using Mab 6 as probe. In each photograph the order is the following: (Std) Molecular weight standards in kilodaltons; (1) A+ RC1; (2) A- RC 1; (3) 3173; (4) 2764; (5) 2779.

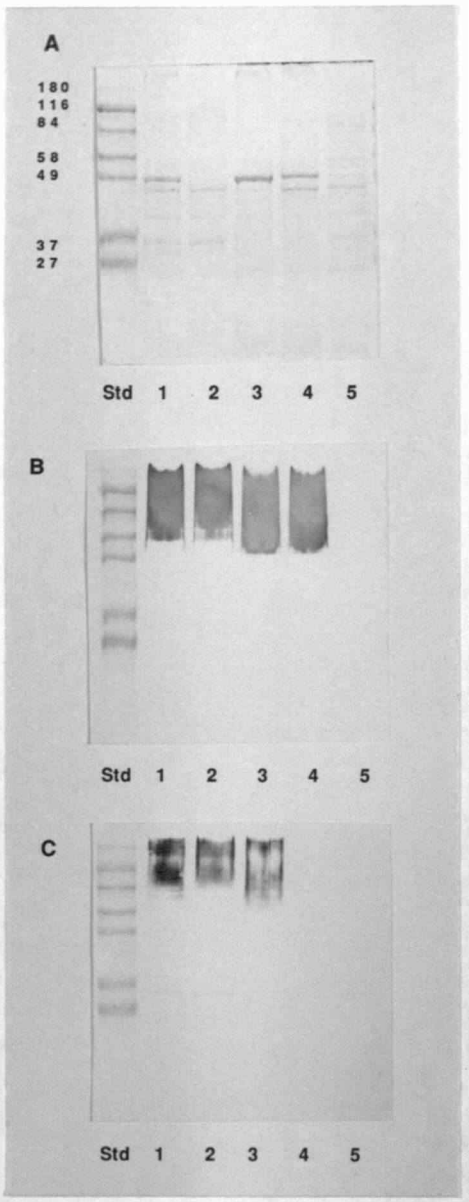

Figure 7.3

Table 7.1. Reaction of Mabs D and 6 with selected isolates of *A. salmonicida*.

Isolate	subspecies[∞]	Host	Rx with Mab D	Rx with Mab 6	Source
ATCC 14174	*sal* T †	BT*	+	+	A. T. C. C.
RC 1	*sal*	Ch	+	+	Rockey et al. 1988
SS 70	*sal*	Ch	+	+	Udey and Fryer 1978
MLE-2	*sal*	MS	+	+	Fryer et al. 1988
567	*sal*	AS	+	+	N. V. I.**
2683	*sal*	AS	+	+	N. V. I.
3173	*sal*	AS	+	+	N. V. I.
77/88	*sal*	Br	+	+	E. M. Bernoth
MT 518	*sal*	AS	+	+	T. Hastings
MT 194	*sal*	AS	+	+	T. Hastings
NCMB 1110	*ach* T	Br	+	-	N. V. I.
MT 533	*ach*	AS	+	-	T. Hastings
ATCC 27013	*mas* T	MS	+	+	Kimura 1969
2b1	*mas*	MS	+	+	M. Yoshimizu
865	atyp	Br	+	+	N. V. I.
1391	atyp	WF	+	+	N. V. I.
2221	atyp	BT	+	+	N. V. I.
1977	atyp	AS	+	+	N. V. I.
362	atyp	AS	+	-	N. V. I.
937	atyp	AS	+	-	N. V. I.
797	atyp	AS	+	-	N V I
2120	atyp	Br	+	-	N. V. I.
2764	atyp	AS	+	-	N. V. I.
2778	atyp	AS	+	-	N. V. I.
3020	atyp	AS	+	-	N. V. I.
02	atyp	Ayu	+	-	M. Yoshimizu
561	atyp	Ayu	+	-	M. Yoshimizu
5606	atyp	GF	+	-	E. Shotts
As 7	atyp	GF	+	-	Elliott and Shotts 1980

Table 7.1 continued

Isolate	subspecies	Host	Rx with Mab D	Rx with Mab 6	Source
Ar 57	atyp	GF	-	-	Tajima et al. 1987
06	atyp	Ayu	-	-	M. Yoshimizu
2013	atyp	AS	-	-	N. V. I.
2779	atyp	AS	-	-	N. V. I.
8060	atyp	AS	-	-	Ward et al. 1985
5602 (A405)[a]	atyp	GF	-	-	Trust et al. 1980
5603 (A404)	atyp	GF	-	-	Trust et al. 1980

* Host codes. Ch: chinook salmon (*Oncorhynchus tchawytscha*). MS: Masou salmon (*Oncorhynchus masou*). BT: Brook trout (*Salvelinus fontinalis*). AS: Atlantic salmon (*Salmo salar*). Br: Brown trout (*Salmo trutta*). WF: Whitefish (*Coregonus* sp.). Ayu: (*Plecoglossus altivelis*). GF: Goldfish (*Carrasius auratus*).

∞ subspecies codes: *sal*; *A. salmonicida salmonicida. ach*; *A. salmonicida achromogenes. mas*; *A. salmonicida masoucida.* atyp; atypical *A. salmonicida.*

† indicates type strain.

** National Veterinary Institute, Oslo, Norway.

a Other common designations are indicated in the parentheses.

Examination of isolate virulence. Selected isolates from subspecies other than *Aeromonas salmonicida salmonicida* were examined for their ability to cause pathology in coho salmon injected intramuscularly with 2 x 10^4 to 4 x 10^4 bacterial cells. Previous investigation determined that this number of *A. salmonicida salmonicida* was the equivalent of 10 to 40 LD_{50}'s. Isolate 2221 caused lesions in 10 % of the fish injected (4 of 40 fish) and the pathogen was recovered from these lesions. Isolate 1977, a catalase negative, non-pigmented *A. salmonicida*, caused a lesion in 1 of 20 fish examined, but the pathogen was not recovered. Both of these isolates were +/+. Six isolates (362, 561, 797, 2013, 2120, and 2779) from the +/- group or the -/- group did not cause lesions or other pathology under these conditions.

Competetive ELISA assays. Immune sera from RBT were compared with immune sera from rabbits for their ability to block epitopes recognized by Mab D or Mab 6. Agglutinating titers were $\geq$ 2048 for all RBT and rabbit immune sera tested, but titers for the monoclonals were low; 2 for Mab D and 32 for Mab 6. Antiserum from RBT was more efficient at blocking the binding of Mab 6 than Mab D. Conversely, antibodies from two different individual rabbits immunized with A+ or A- whole cells were capable of blocking Mab D but not capable of blocking Mab 6 (Figure 7.4). Controls for these assays included normal RBT or rabbit sera to block binding of the Mabs, or unrelated monoclonal antibodies of the appropriate subisotype to examine non-specific

Figure 7.4. Competetive ELISA assays comparing the ability of rabbit or rainbow trout (RBT) immune sera directed against A-*Aeromonas salmonicida* cells to block the binding of Mab D or Mab 6. Well C is monoclonal antibody in the absence of blocking antisera. Well 7 is rabbit or RBT serum diluted 1/10. Blocking antisera is subsequently diluted 1:1 through the remaining wells. Negative control blocking serum (□); immune serum (◆). (A) Rabbit serum followed by Mab D; (B) Rabbit serum followed by Mab 6; (C) RBT serum followed by Mab D; (D) RBT serum followed by Mab 6.

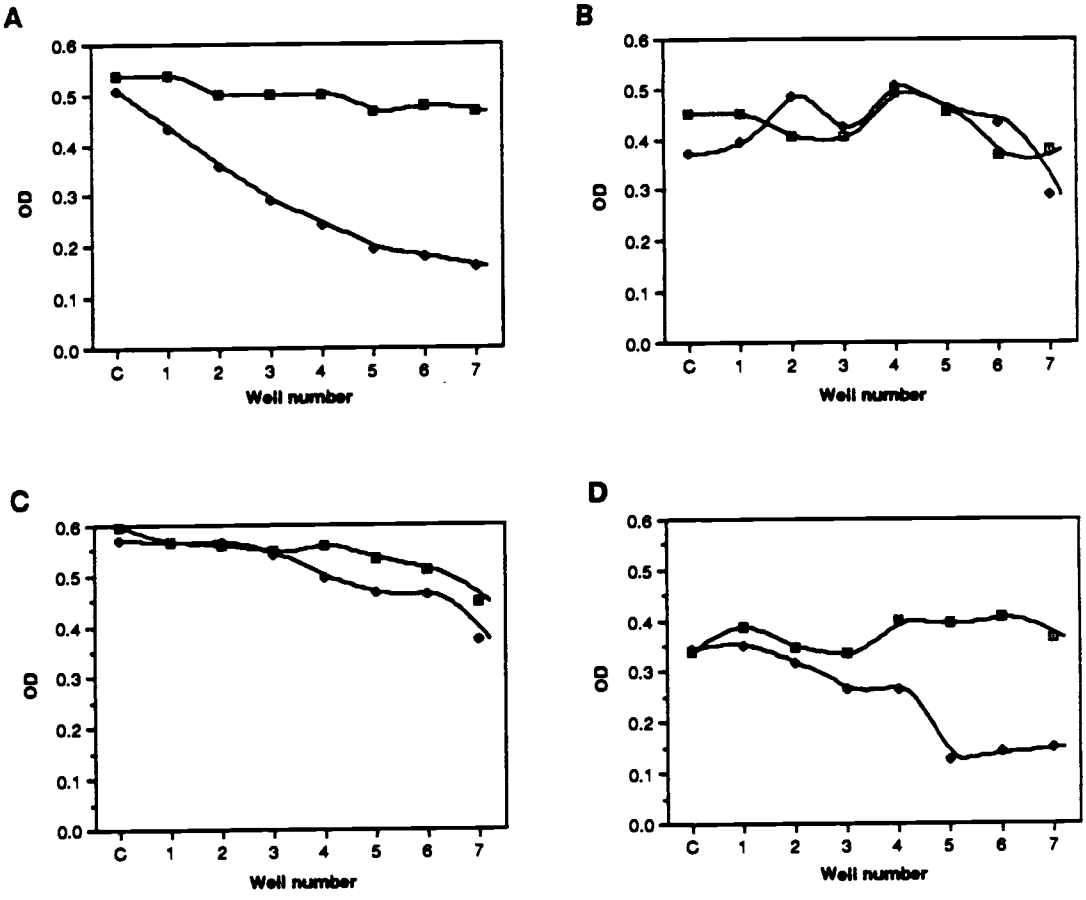

Figure 7.4

interactions of the mouse antibody or the antibody conjugate with the antigen.

Discussion

We have produced monoclonal antibodies against *Aeromonas salmonicida* LPS which differentiate among isolates of *A. salmonicida*. These Mabs reacted specifically with *A. salmonicida* LPS and did not cross-react with other bacterial fish pathogens tested. The Mabs were poor agglutinating antibodies but were useful in Western blot and ELISA assays. The Mabs were differentiated on the basis of their isotype, binding sensitivity to periodate oxidation of antigen, and reactivity with selected *A. salmonicida* isolates. The binding of set 1 antibodies (3 independent γ_{2b}, κ clones) was insensitive to periodate oxidation of antigen, while the binding of set 2 antibody (1 μ, κ clone) was sensitive. The reactivity of the Mabs with different isolates showed the majority of isolates reacted with the set 1 antibodies, while a subset of these isolates reacted with the set 2 antibody. All isolates which reacted with set 1 antibodies also reacted with rabbit antiserum produced against a strain of *A. salmonicida salmonicida* and those isolates that did not react with set 1 antibodies did not react with the rabbit antiserum. All isolates which reacted with set 1 antibodies also reacted with rabbit antiserum produced against a strain of *A. salmonicida salmonicida*

and those isolates that did not react with set 1 antibodies did not react with the rabbit antiserum. No isolates were discovered that reacted with set 2 antibodies but not set 1 antibodies. Certain isolates did not react with either set. Chart et al. (1984) use a monoclonal antibody in their comparison of the structure and immunogenicity of different isolates of *A. salmonicida*. They showed that all strains with high molecular weight lipopolysaccharide reacted with the monoclonal antibody. They used these and other data to support the conclusion that *A. salmonicida* lipopolysaccharide is structurally and immunochemically homogenous. Our data support most of their conclusions except that we showed that lipopolysaccharide has antigenic variation among isolates. We suggest that the monoclonal antibody discussed in their paper is similar to the antibodies in our set 1.

The classification of isolates into specific subspecies of *Aeromonas salmonicida* may be facilitated by examination with the Mabs. In our analyses, all isolates of the subspecies *A. salmonicida salmonicida* were +/+. These included isolates from North America, Europe, and Korea. The type strain of *A. salmonicida achromogenes*, NCMB 1110, was +/-, and the ATCC type strain of *A. salmonicida masoucida* was +/+. Isolates referred to as atypical had no consistent reactivity pattern. The atypical isolates were taken from fish in Europe, Japan, North America, and Australia.

The classification of atypical isolates may be facilitated by the correlation of pigment production with antibody binding group. Tested slow-pigmenting isolates of *A. salmonicida* were +/- and all typical isolates or isolates producing no pigment were +/+ (T. Lunder, unpublished data). Exceptions to this generalization included the isolates which did not react with either antibody and strain MT 533.

The Mabs were examined for correlation between reactivity patterns and the virulence of clinical isolates for salmonids. In our assays using coho salmon, pathology was produced only by +/+ isolates and not by +/- or -/- isolates. Our results however are based on a limited number of strains, a single host species, and infection by injection. Clinical results from hatcheries in Norway indicate that +/- *A. salmonicida*, such as 2778 and 937, were isolated from populations undergoing epizootics. Additionally, strain 8060, which we type as -/-, is capable of causing epizootics in salmonids (Ward et al. 1985). Future research is needed to support the use of these Mabs as an indicator of virulence.

We also used these Mabs to compare the specific immune response of rabbits and RBT to whole *Aeromonas salmonicida*. Other authors have demonstrated that salmonids produce a more limited repertoire of antibodies than rabbits when injected with either *A. salmonicida* extracellular product or *Renibacterium salmoninarum* soluble antigen (Hastings and Ellis 1988; Kaattari et al. 1988). Our results supported these conclusions. There was, however, an epitope that was more efficiently recognized by the

trout than the rabbit. Trout antiserum was more efficient at blocking Mab 6 while rabbit antiserum was more efficient at blocking Mab D. Results were consistent when RBT or rabbit sera from animals injected with either A+ or A- bacteria were used. These results are based on a limited number of test animals (two pools of three trout each and two rabbits) and further experimentation with different salmonid species or antigen preparations is needed.

Acknowledgements. We are indebted to the individuals referenced in Table 1 who contributed isolates for this study. We also are indebted to Dr. J. L. Fryer for direction and critical review of the manuscript. Principal funding for this research was received from USDA Science and Engineering grant no. 85-CRSR-2-2578. Oregon Agriculture Experiment Station Technical Paper No. 8936.

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Conclusions

1. A protocol is presented for the separation of proteolytic and hemolytic enzymes from culture supernatants of *Aeromonas salmonicida*.

2. Distinct proteases, labeled P1 and P2, are secreted by *Aeromonas salmonicida* during in vitro culture in either media or salmonid serum. P1 protease is a single protein with activity sensitive to phenylmethylsulfonyl fluoride while P2 protease consists of a group of proteins of different molecular weight with sensitivity to ethylenediaminetetraacetic acid. P1 but not P2 was detected in coho salmon (*Oncorhynchus kisutch*) infected by injection. Other unidentified proteases, possibly of host origin, were detected in lesions of these fish.

3. The interaction of the T-lysin with the P1 protease in the complete in vitro lysis of trout erythrocytes involves two separate events. The T-lysin has activity against the outer membrane and P1 protease has activity against the nuclear membrane.

4. Activity of the T-lysin is inhibited by salmonid serum. This was demonstrated by mixing serum with actively hemolytic culture supernatants, by growing *Aeromonas salmonicida* in

salmonid serum, by examination of the hemolytic activity of cell-free exudate from *A. salmonicida* lesions, and by mixing cell-free exudate with actively hemolytic culture supernatants.

5. The serum component responsible for inhibition of the T-lysin activity was a protease insensitive, high molecular weight compound with a pI of 4.5-5.5. This component is inhibitory to the lytic effects of *Aeromonas salmonicida* and *A. hydrophila* hemolysins, and the detergent Nonidet P40. The inhibitor protects the erythrocyte from lysis without inhibiting the hemolytic enzymes or detergent. Serum from other families of fish and orders of vertebrates are less active against the hemolytic inhibitor in assays using salmonid erythrocytes as targets.

6. High concentrations of selected salts included in the culture medium are inhibitory to the production of P1 protease and T-lysin but have no effect on the production of P2 protease.

7. Culture supernatants from *Aeromonas salmonicida* grown in salmonid serum have a higher relative amount of protease activity which is resistant to the inhibitor phenylmethylsulfonyl fluoride than supernatants from cultures grown in laboratory medium such as brain heart infusion broth and tryptic soy broth. This suggests that P2 proteases are more active in serum than in laboratory medium and that these proteases may also be more active in vivo.

8. Both P1 and P2 protease activity against gelatin are inhibited by salmonid serum. Covalent interactions between *Aeromonas salmonicida* extracellular products and serum proteins were demonstrated with Western blots. These interactions were observed in supernatants of cultures grown in salmonid serum as well as in mixtures of supernatants from cultures grown in brain heart infusion broth which were subsequently mixed with uninoculated serum. These interactions may involve P1 protease and host serum proteinase inhibitors.

9. Growth of *Aeromonas salmonicida* in high concentrations of magnesium salts produces pleomorphic cellular morphology. This pleomorphy is associated with the presence of the A layer and requires growth for formation. Pleomorphic forms have similar LD_{50} and mean day to death values as wild type *A. salmonicida*, but bacterins produced from pleomorphic cells are less protective than bacterins from wild type *A. salmonicida*

10. Monoclonal antibodies against *Aeromonas salmonicida* lipopolysaccharide differentiate among different strains of the organism. These antibodies also demonstrate epitope specificity differences between salmonids and rabbits.

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