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Evaluation of the Protective Antigens of  
Aeromonas salmonicida

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DISSERTATION

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Evaluation of the Protective Antigens of Aeromonas salmonicida.

Abstract

The development of an effective mass application vaccine against furunculosis of fish has been hampered by an incomplete knowledge of the protective antigens of Aeromonas salmonicida, the causative agent. Three antigens have been identified as either immunogenic and/or protective in previous studies, but no comparative tests of these purified antigens have been conducted.

In this study these three major antigens, A-protein (AP), lipopolysaccharide (LPS) and fraction 4 (F4) were examined by passive administration of rabbit and trout hyperimmune sera to juvenile coho and chinook salmon, which were subsequently challenged with A. salmonicida. Rabbit and rainbow trout antisera against live and formalin-killed whole cells, AP, LPS and F4 was characterized by agglutination titer, counterimmunoelectrophoresis and immunodiffusion prior to use in passive immunization tests. Rabbit antisera against AP+ cells (which contained antibodies against AP and LPS), and absorbed antisera (which contained only antibodies against AP), were protective in passive tests. Trout antisera against AP+ cells, or absorbed sera, was protective, although antibodies against AP were not detected. Rabbit antisera against AP- cells lacked antibodies against AP, and provided less and inconsistent protection compared to antisera against AP+ cells. Trout antisera against AP- cells also lacked antibodies against AP, and failed to protect passively immunized fish from mortality due to furunculosis. These results demonstrated that AP is a

protective antigen of A. salmonicida. However, purified AP was poorly immunogenic in rabbits and trout, and antisera was not protective. Lipopolysaccharide was poorly immunogenic in rabbits and strongly immunogenic in trout, but neither antisera was protective. Fraction 4 was immunogenic in both rabbits and trout, however, both antisera provided equivocal protection in passively immunized fish.

Passively immunized salmon surviving challenge with A. salmonicida were injected with an anti-inflammatory drug and the carrier rate determined at three time intervals. The carrier rate for fish injected with antisera from AP+ or AP- strains was 99.2% and 99.0%, respectively, 14 d after injection. This rate declined to 0.0% and 42.1% for AP+ and AP- antisera injected salmon respectively, 56 d after injection. These data suggested a role of anti-AP antibodies in the elimination of bacteria from fish tissues.

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## Introduction

Furunculosis, caused by the gram-negative bacterium Aeromonas salmonicida, is the oldest known and most studied bacterial disease of fish. Its cosmopolitan distribution, wide host range, and ability to cause high mortality were once considered to be limiting factors to the successful culture of salmonid fish.

The presence of the bacterium in wild populations, antibiotic resistance, and the failure to develop an effective vaccine have made control difficult. Although attempts to develop a furunculosis vaccine have been pursued since the 1940's, no successful oral or immersion type commercial vaccine exists. Enteric redmouth and vibriosis diseases of salmonids have been controlled using immersion bacterins of formalin-killed Yersinia ruckeri and Vibrio anguillarum / V. ordali, respectively. However, furunculosis bacterins prepared and delivered by these methods have not provided reliable protection.

The failure of these vaccines stimulated investigations to identify virulence factors and protective antigens of A. salmonicida. A better understanding of the bacterium's structure, host interactions, and pathogenic mechanisms has resulted. This knowledge has allowed a more rational approach to vaccine development.

Three bacterial antigens have been proposed to confer protective immunity. Two are cell associated antigens: lipopolysaccharide (LPS), and A-layer protein (AP), and one is an extracellular product: fraction four (F4).

The purpose of the following study was to investigate each of these antigens, in purified form, for their immunogenicity in rabbits and

trout, by the ability of passively transferred serum to confer protection to juvenile salmon against furunculosis.

## Literature Review

History. There are more published reports on furunculosis than any other fish disease (Herman 1968). The disease and its causative agent have been reviewed by McCraw (1952), Herman (1968), Bullock et al. (1971), Snieszko and Bullock (1975) and McCarthy and Roberts (1980). It has been called "the great red plague" by hatchery personnel, and historically has been one of the most serious constraints of salmonid culture (McCraw 1952).

Furunculosis has been known by descriptive accounts in continental Europe since 1898 (McCarthy and Roberts 1980). Emmerich and Weibel (1894) isolated the agent, Bacterium salmonicida, from diseased hatchery brown trout in Germany, and provided the first descriptive account of the etiological agent and the disease. From 1901 to 1914 furunculosis was detected in trout farms and streams in many European countries including England, Ireland, Bavaria, Austria, France, Belgium and Switzerland (McCarthy 1975a; McCarthy and Roberts 1980). In England it was first reported in 1911 (McCarthy 1975a; Mackie and Menzies 1938), and by 1928 furunculosis was so widespread that a special committee was assembled to study the disease (Mackie and Menzies 1938).

The first North American isolation of A. salmonicida was from hatchery brook trout (Salvelinus fontinalis) in Michigan (Marsh 1902). It was not found in wild salmonids of North America until Duff and Stewart (1933) isolated it from dolly varden (Salvelinus malma) and cutthroat trout (Salmo clarkii) in British Columbia. Later, Fish (1937) isolated the bacterium from wild brown trout (Salmo trutta) in

Wyoming and Montana. Furunculosis has also been observed in Pacific salmon, Oncorhynchus spp. (Shapovlov and Taft 1954, Wood 1959 and Miyazaki and Kubata 1975). By the mid 1900's the disease existed in nearly all species of salmonids in the western USA (McCarthy and Roberts 1980).

The current distribution of furunculosis in salmonid fishes is, with the possible exception of New Zealand and Tasmania, worldwide. Its absence from these countries is due to strict regulations on the importations of fish (Snieszko and Bullock 1975; Herman 1968). Matthey (1963) and Leibman (1963) reported the eradication of furunculosis from Switzerland and Germany, respectively. However, the disease was subsequently reintroduced into Germany (Herman 1968). Further details on the geographical distribution of furunculosis are presented in Table 1.

While most work has concentrated on furunculosis of freshwater salmonids, the disease has been reported from fish and invertebrates from diverse origins and habitats (Table 2). Additionally, A. salmonicida may survive in mud and sediments of fish ponds following epizootics (McCarthy and Roberts 1980 and Sakai 1987). In recent years more attention has been given to A. salmonicida infections of non-salmonid freshwater fishes, and also of marine species. These isolates are often referred to as atypical, having unique characteristics that differentiate them from the typical strain isolated from salmonid fishes.

Furunculosis has undoubtedly spread due to shipments of diseased or carrier fish. Fish (1937) believed rainbow trout (Oncorhynchus mykiss)

Table 1. Geographical distribution of furunculosis.<sup>1</sup>

EUROPE	ASIA
Austria	Japan <sup>2</sup>
Bulgaria	Korea <sup>3</sup>
Czechoslovakia	
Denmark	AUSTRALIA <sup>4</sup>
England	
Finland	NORTH AMERICA
France	Canada
Germany	United States
Hungary	
Ireland	
Italy	
Poland	
Rumania	
Scotland	
Sweden	
Ukraine	
Wales	
Yugoslavia	

<sup>1</sup> Reported from Herman, 1968, except as otherwise indicated.

<sup>2</sup> Kimura, 1969.

<sup>3</sup> Fryer et al., 1988.

<sup>4</sup> Trust et al., 1980.

Table 2. Species susceptible to infection by *Aeromonas salmonicida*.

CLASSIFICATION	COMMON NAME	STRAIN <sup>1</sup>	REFERENCE
INVERTEBRATES <i>Strongylocentrus purpuratus</i>	sea urchin	NR	Gilles and Pearse 1986
VERTEBRATES			
AGNATHA			
PETROMYZONTIDAE <i>Ichthyomyzon castaneus</i>	lamprey	NR	Hall 1963
OSTEICHTHYES			
AMMODYTIDAE			
<i>Ammodytes lancea</i>	sand eels	A	Dalsgaard and Paulsen 1986
<i>Hyperoplus lanceolatus</i>	sand eels	A	Dalsgaard and Paulsen 1986
ANGUILLIDAE <i>Anguilla rostrata</i>	cultured eels	NR	Hayasaka and Sullivan 1981
ANOPLOPOMATIDAE <i>Anoplopoma fimbria</i>	sablefish	A	Evelyn 1971
CATOSTOMATIDAE			
<i>Catostomus commersoni</i>	white sucker	T	Ostland et al. 1987
<i>Carrassius auratus</i>	goldfish	A	Williamson 1929 Shechmeister et al. 1962 Mawdesley-Thomas 1969 Saito et al. 1975 Trust et al. 1980 Shotts et al. 1980

Table 2. (cont..)

CLASSIFICATION	COMMON NAME	STRAIN	REFERENCE
<i>Phoxinus phoxinus</i>	minnow	NR	Williamson 1929
NR	minnow	A	Hastein et al. 1978
<i>Cyprinus carpio</i>	carp	A	Bootsma et al. 1977
<i>Rutilus rutilus</i>	roach	NR	Bucke 1979
<i>Scardinius erythrophthalmus</i>	rudd	NR	Bucke 1979
<i>Notropis cornutus</i>	common shiner	T	Ostland et al. 1987
<i>Notomegonus crysoleucus</i>	golden shiner	T	Ostland et al. 1987
<i>Semotilus atromaculatus</i>	creek chub	T	Ostland et al. 1987
 GADIDAE			
<i>Gadus morhua</i>	Atlantic cod	A	Cornick et al. 1984
 PERCIDAE			
<i>Perca fluviatilis</i>	perch	A	McCarthy 1975a
<i>Blicca bjoerkna</i>	silver bream	A	Bucke 1979
<i>Abramis brama</i>	common bream	A	McCarthy 1975a
 PLEURONECTIDAE			
<i>Solea solea</i>	common sole	T	Flutcher 1979
 SALMONIDAE			
<i>Coregonus pollan</i>	pollan	NR	Vickers and McCleean 1961
<i>Salmo salar</i>	Atlantic salmon	A	Paterson et al. 1980
		T	Bruno 1986
	trout	T	Novotny 1978
	<i>Salmo</i> spp.		

Table 2. (cont.)

CLASSIFICATION	COMMON NAME	STRAIN	REFERENCE
<i>Salvelinus fontinalis</i>	brook trout	T	McCarthy 1975a
<i>Salvelinus namaycush</i>	lake trout	T	McCarthy 1975a
<i>Salvelinus pulvius</i>	iwana	NR	Hara et al. 1976
<i>Oncorhynchus</i> spp.	salmon	NR	Novotny 1978
<i>O. mykiss</i>	rainbow trout	A	Bulllock and Roberts 1980
<i>O. tshawytscha</i>	chinook salmon	NR	Wood 1959
<i>O. kisutch</i>	coho salmon	NR	Shapovlov and Taft 1954
<i>O. gorbuscha</i>	pink salmon	NR	Kimura 1969
<i>O. masou</i>	masou salmon	NR	Kimura 1969
<i>O. rhodurus</i>	amago	NR	Hara et al. 1976
<i>O. nerka</i>	sockeye salmon	NR	Hara et al. 1976
 SERRANIDAE			
<i>Roccus mississippiensis</i>	yellow bass	NR	Bowen 1965

<sup>1</sup> *Aeromonas salmonicida* strains were reported as typical (T), atypical (A) or were not reported (NR) at the subspecies level.

from western USA (Mount Shasta) were the original source, and shipment of diseased fish to Europe and other areas disseminated furunculosis. He believed the resistance of rainbow trout, compared to other salmonid species, had resulted from a long relationship with the pathogen. Others believe the disease originated in continental Europe and was disseminated to England and other areas by fish shipments (Mackie and Menzies 1938).

Etiological Agent. Originally described as Bacterium salmonicida by Emmerich and Weibel (1894) the characteristics and taxonomy of A. salmonicida were further described by Griffin et al. (1953), Smith (1963), Eddy and Carpenter (1964), McCarthy (1977), MacInnes et al. (1979) and Popoff (1984).

Popoff (1984) described A. salmonicida as a gram-negative, oxidase-positive, facultatively anaerobic coccobacillus existing in short chains or clumps. Optimum growth occurs between 22 and 25 C, producing round, translucent, friable colonies on agar. A brown, water soluble pigment is produced on media containing tyrosine or phenylalanine. Biochemically, A. salmonicida utilizes gelatin, arginine, glucose, maltose, arabinose, galactose, dextrin, mannose and trehalose.

Although DNA hybridization studies do not justify strain separation into subspecies categories, the subspecies nomenclatures suggested by Schubert (1967a and 1967b) have been retained. These divisions are based on biochemical differences between isolates, and are :

- 1) A. salmonicida subsp. salmonicida (Lehman and Neuman 1896)  
produces brown pigment, is indole negative, and utilizes both

mannitol and esculin.

- 2) *A. salmonicida* subsp. *achromogenes* (Smith 1963) does not produce pigment, is indole variable, and utilizes neither mannitol nor esculin.
- 3) *A. salmonicida* subsp. *masoucida* (Kimura 1969) does not produce pigment, is indole positive, and utilizes both mannitol and esculin.

The typical strain, *A. salmonicida* subsp. *salmonicida*, is usually associated with septicemic disease. In contrast, atypical strains (subspecies *achromogenes* and *masoucida*) produce skin ulcerations, or infrequently, septicemic infections.

#### Forms of the Disease.

Typical furunculosis in salmonid fishes. Typical strains of *A. salmonicida* (subsp. *salmonicida*) cause bacterial hemorrhagic septicemias, similar to those caused by other gram-negative bacterial pathogens of fish (Hill et al. 1974). In salmonid fishes the disease may occur in one of several forms, depending on such factors as host susceptibility, bacterial virulence, and environmental conditions (Bullock et al. 1971). Herman (1968) has suggested the following disease categories for typical salmonid furunculosis :

Acute - a rapid increase in mortality within a population of fish with few or no external signs of disease.

Subacute - a gradual increase in mortality with the formation of furuncles in the musculature and hemorrhaging at the base of fins.

Chronic - a low, constant rate of mortality with occasional hemorrhaging and intestinal inflammation.

Latent - no mortality or clinical signs, however, the bacteria can be isolated from internal organs.

An additional category of carrier could be added, in which infected fish lack clinical symptoms and bacteria cannot be isolated from fish tissues by standard methods.

McCarthy and Roberts (1980) proposed similar disease categories, with more extensive descriptions of the typical pathology seen in each category. Mackie and Menzies (1938) and others, have observed that within a single population of diseased fish individuals may be found exhibiting signs of several disease categories.

The presence of furuncles on diseased fish is not a consistent feature of the disease (Snieszko and Bullock 1975; McCraw 1952; Mackie and Menzies 1938). Furuncles are rarely present in acutely infected fish, being more characteristic of subacute to chronic forms of the disease (Herman 1968; Mackie and Menzies 1938). However, their presence is highly variable (Mackie and Menzies 1938). When furuncles exist, they may be overgrown by aquatic fungi (Fish 1934), and upon rupturing may leave a crateriform lesion (Hill et al. 1974).

Typical furunculosis in non-salmonid fishes. More recently, typical strains of A. salmonicida have caused disease in non-salmonid fishes. A disease called post traumatic septicemia of centrarchids was observed in yellow bass (Bulkey 1969) and in smallmouth bass (Le Tendre et al. 1972). Septicemic disease and mortality occurred during springtime,

following stressful situations such as net capture, increased water temperature, sexual maturation, poor nutrition, and increased fish density. Typical furunculosis has been transmitted from infected baitfish (common and golden shiners, creek chub, and white sucker) to salmonids (Ostland et al. 1987).

Atypical furunculosis. Atypical strains of A. salmonicida are responsible for a variety of diseases in salmonid as well as non-salmonid fishes. The atypical strain A. salmonicida subsp. achromogenes is the agent usually isolated from clinical cases of atypical furunculosis. Pathology is usually limited to surfaces, rarely penetrating beyond the dermis to become systemic. Atypical strains are unable to survive systemically due to poor iron sequestering abilities (Chart and Trust 1983). Three forms of atypical furunculosis have been reported in the literature, in salmonids, carp and goldfish. 1). Bullock and Roberts (1980) experimentally infected rainbow trout by swabbing scarified skin with an atypical strain of A. salmonicida. They observed shallow circular hemorrhagic skin ulcerations similar to pathology seen in cyprinid fishes infected with atypical A. salmonicida. Paterson et al. (1980) reported that an atypical strain of A. salmonicida was responsible for 50% mortality among hatchery-reared Atlantic salmon (Salmo salar) postyearling parr in Nova Scotia. External signs included badly eroded dorsal fins and external body lesions, typical of ulcer disease caused by Hemophilus piscium previously seen in Nova Scotia. Paterson et al. (1980) further showed that previous outbreaks of ulcer disease were actually caused by

atypical strains of A. salmonicida, and not H. piscium. These bacteria were cultured on enriched media (trypticase soy agar, TSA, with 5% sheep blood), and identified as A. salmonicida subsp. achromogenes. However, atypical strains are not always limited to external surfaces. Ljungberg and Johansson (1977) observed systemic infections caused by A. salmonicida subsp. achromogenes in cultured salmonids in Sweden. Disease occurred at temperatures above 10 C, and caused 10 to 90% mortality.

2). Fijan (1972) identified two agents from carp exhibiting clinical signs of carp dropsy syndrome. One was identified as Rhabdovirus carpio, the etiological agent of carp viral hemorrhagic septicemia, and the second was an unidentified bacteria. Bootsma et al. (1977) later identified it as an atypical A. salmonicida, and the disease as carp erythrodermatitis. The disease is characterized by shallow, circular skin ulcerations with hemorrhagic centers and dark borders. Lesions enlarge laterally, rarely penetrating the stratum spongiosum, developing necrotic centers and often causing scale loss. Mortality is often associated with secondary pathogens such as Aeromonas hydrophila or fungi, but septicemic furunculosis may occur if lesions expand inwardly (McCarthy and Roberts 1980).

3). Mawdesley-Thomas (1969) described a similar disease in goldfish and called it goldfish ulcer disease (GUD). The etiological agent was later identified as A. salmonicida subsp. achromogenes by Elliott and Shotts (1980). Experimental transmission of GUD in goldfish was accomplished by Shotts et al. (1980).

Other fish species infected with atypical A. salmonicida. Numerous other species have been reported to be infected with atypical strains of A. salmonicida. These include Atlantic cod (Cornick et al. 1984), sand eels (Dalsgaard and Paulson 1986), sablefish (Evelyn 1971), minnows (Hastein et al. 1978), cultured eels (Kitao et al. 1984), perch, common bream, and silver bream (McCarthy 1977).

Pathology. The pathology induced by A. salmonicida is similar to that caused by other gram-negative fish pathogens (Hill et al. 1974). Fish with subacute to chronic forms of the disease may have furuncles as a clinical sign. These, however, differ from true furuncles, as seen in higher vertebrates, by lacking pus or notable neutrophil infiltration. In contrast, fish furuncles consist of bacteria, liquefied necrotic tissues, and a serosanguinous fluid (McCraw 1952; McCarthy and Roberts 1980).

The pathology associated with furunculosis has been reviewed by Klontz et al. (1966), Ferguson and McCarthy (1978), and McCarthy and Roberts (1980). Further studies on the role of extracellular growth products in inducing pathological changes have been conducted by Sakai (1977 and 1978), Ellis et al. (1981), Nomura and Saito (1982), Tajima et al. (1983), Ellis and Grisley (1985) and Fyfe et al. (1986).

Several theories have been proposed as to the ultimate cause of death. The Furunculosis Committee (Mackie et al. 1935) believed that rapid bacterial growth within blood capillaries obstructed blood flow causing ischemic necrosis to affected tissues. Field et al. (1944), observed a hypoglycemia in the blood of infected fish, and suggested

that these fish died from hypoglycemic shock. The results of Field et al. (1944) were contradicted by the studies of Sheih and MacLean (1976) who found increased levels of blood glucose in experimentally-infected brook trout. They found reduced levels of total serum proteins, particularly hemoglobin, which they attributed to either hemorrhage or erythrocyte hemolysis associated with the disease. In addition, elevated serum levels of amino acids and nitrogen compounds were found, indicative of muscular degeneration which is usually found in diseased fish. Klontz et al. (1966) observed a diminished inflammatory response, and suggested that *A. salmonicida* may produce a leukocidin. They suggested the absence of phagocytic leukocytes allowed bacteria to multiply rapidly to high levels, with death occurring by a fatal bacteremia. Fuller et al. (1977) isolated a leukocytolytic factor from broth cultures of *A. salmonicida*. This glycoprotein was cytolytic for rainbow trout leukocytes in vitro, and produced a pronounced leukopenia in vivo. Ferguson and McCarthy (1978) performed histopathological examinations on subacutely-infected brown trout. Although acute inflammatory responses were lacking, there was a cellular response to the bacterium. Also, the lack of necrosis in tissues surrounding bacterial foci suggested a lack of cytotoxic substances. They observed liquefactive necrosis of splenic ellipsoids leading to collapse of this organ, and notable cardiac involvement, which they suggested led ultimately to death.

Control. The most effective means of reducing furunculosis among cultured fish populations is avoidance. This may be accomplished

through the use of pathogen-free water combined with sanitation methods to prevent bacteria from entering the facility with eggs, fish or equipment. O'Donnell (1947) has described methods of hatchery disinfection. McFadden (1969), Snieszko (1978), and others have outlined methods for disinfection of eggs. The availability of certified furunculosis-free stocks of fish also facilitates control of the disease (Snieszko and Bullock 1975).

However, it is not always possible to avoid the pathogen. In these cases, management practices to increase fish resistance to the disease must be employed (McCarthy and Roberts 1980). This involves minimizing stress (reduction of crowding; providing good water quality with adequate oxygen; temperature regulation), use of disease resistant strains of fish (eg. rainbow trout) when possible, sanitation of holding areas, culling of poor fish, and vaccination (Bullock et al. 1971).

The last resort, if disease should occur, is antibiotic therapy. This should be combined with efforts to improve the fish's environment, thereby minimizing the possibility of disease recurrence (Snieszko 1978).

Chemotherapy. Furunculosis was the first fish disease treated with antibiotics (Snieszko and Bullock 1975; Austin 1985). Although many antibiotics are effective against A. salmonicida (Snieszko and Bullock 1975), only three have been approved for use on food fish in the United States. These include Romet-30 (sulfadimethoxine and ormetoprim), sulfamerazine, and terramycin (oxytetracycline) (Schnick et al. 1987).

Since many disadvantages exist with the use of antibiotics, it should be the last resort in treatment (McCarthy and Roberts 1980).

Recurrence of the disease following antibiotic therapy is common (McCarthy and Roberts 1980), and may occur for several reasons. Antibiotics are bacteriostatic (except Romet-30), therefore, as drug levels in the tissues drop, surviving bacteria may begin multiplying, especially if a poor environment remains. Numerous drug-resistant strains of A. salmonicida have been reported (Snieszko and Bullock 1957; Aoki et al. 1971; Drinan et al. 1978; Hahnel and Gould 1982; Ledo et al. 1987; Ganzhorn 1987; Tzoumas et al. 1987), making antibiotic therapy ineffective. Grondel et al. (1987) observed uneven tissue distribution and low bioavailability of oxytetracycline given orally to Cyprinus carpio. Drug levels were below therapeutic doses for many bacterial pathogens. The potential development of drug-resistant strains, existence of carriers among fishes, and subsequent reinfection of hatchery fish receiving untreated river waters has been postulated by Wood (1968).

Undesirable side effects of sulfa drugs were observed by Guttell and Snieszko (1949) and Wood and Snieszko (1955) who found decreased growth rates, kidney damage and sterility in trout fed prophylactic doses of sulfamerazine. Johnson and Brice (1953), and Wood and Johnson (1957) observed lethal sulfa toxicity in Pacific salmon fed therapeutic doses of sulfamerazine. DeCew (1972) observed teratogenic effects in progeny of chinook salmon injected with a complex of penicillin G, dihydrostreptomycin sulfate and oxytetracycline-HCl to control furunculosis and bacterial kidney disease. Additionally, McCarthy and

Roberts (1980) suggested that reliance on chemotherapy may promote poor management practices.

Immunosuppressive and immunotoxicological effects have recently been associated with terramycin therapy. Van Muiswinkel et al. (1985) observed immunosuppression in rainbow trout fed oxytetracycline. Grondell and van Muiswinkel (1986) commented on immunotoxicity of some antibiotics in fishes, resulting in impairment of immune mechanisms required to eliminate disease agents. They remarked that antibiotic therapy should work in concert with host immune mechanisms, resulting in elimination of pathogenic agents. It is therefore necessary to chose carefully the drugs for therapy.

In the United States, federal regulations require a 21 to 42 d depuration period after drug therapy prior to marketing or planting of food fish (Schnick et al. 1987). This allows for residual drug levels in the tissues to fall to a safe level for human consumption. This causes economic loss to fish growers in two ways : 1) markets may be lost if fish are not available on scheduled delivery dates; 2) fish must be fed and cared for past the anticipated market date, resulting in increased costs for food, labor and holding.

#### Active Immunization

Since control using management practices and chemotherapy is difficult, a vaccine to control furunculosis is very desirable. Attempts to develop vaccines by oral, injection, and immersion delivery methods have had mixed results. Early attempts often failed due to the use of avirulent strains of A. salmonicida in bacterins, and reliance

upon natural epizootics for challenge. The discovery by Udey and Fryer (1978) that virulent strains were autoaggregating due to the presence of an additional protein layer, (A-protein, AP), allowed new insights into vaccine development. The development of standardized challenge methodologies by Michel (1980), Cipriano (1982a), and McCarthy (1983), and of standardized methods for vaccine testing and evaluation by Amend (1981) greatly added to our ability to test and evaluate experimental furunculosis vaccines.

Oral Vaccines. Duff (1942) was the first to immunize salmonids against furunculosis. The remarkable success of his oral vaccine has not been improved upon in spite of many attempts to produce oral vaccines. Duff mixed chloroform-killed *A. salmonicida* cells into hatchery food (1 part raw beef liver:3 parts canned salmon) and fed this to one and two-year-old cutthroat trout daily or on alternate days for up to 70 d. Water temperature averaging 8 C (2.2 to 14.4 C) was increased to 19 C for challenges by immersion (addition of bacterial cultures to the water), cohabitation (addition of heavily infected fish to the tank), or intramuscular (i.m.) injection. Statistical analysis indicated fish fed 60 d or more were highly protected from mortality following immersion or cohabitation challenge, with vaccinees experiencing 24 to 26% mortality compared to 71 to 75% among controls. Development of serum agglutinating antibodies was not significantly different between vaccinees and controls, being either absent or at very low levels in all cases.

Further efforts to develop an oral vaccine by Snieszko and Friddle

(1949), Krantz et al. (1964b), Spence et al. (1965), Udey and Fryer (1978) and Michel (1979) have failed. Others have had marginal success in developing oral furunculosis vaccines. Klontz and Anderson (1970) and Hara et al. (1976) immunized salmonid fishes with a water soluble extract (furunculosis soluble antigen, FSA) from sonicated live *A. salmonicida* cells. This antigen was precipitated onto alum and mixed into food. Although preliminary experimental results indicated excellent protection, further field tests (Klontz and Anderson 1970) at three Washington state hatcheries failed to protect fish. Smith et al. (1980) mixed formalin-killed *A. salmonicida* cells into food and observed protection from furunculosis in brown trout fry. No antibodies were detected in serum or gut mucus, but protection was correlated with a cell-mediated immune (CMI) response. Austin and Rodgers (1981) and Rodgers and Austin (1985) immunized brown trout fry with whole-cell, toxoid, and whole-cell/toxoid/AP vaccines mixed into food. Whole-cell vaccines were protective, and the level of protection was increased by addition of toxoid and AP to the vaccine. Toxoid alone was not protective.

Injection Vaccines. Numerous investigations on the efficacy of injected *A. salmonicida* vaccines have been conducted (Table 3). Most studies have immunized fish with bacterins rather than purified antigens, which makes identification of the protective antigens difficult. In addition, variability in bacterial strains used in vaccines and challenges, culture methods, vaccine preparation, the use of adjuvants, and fish size and species used may have affected the

Table 3. A summary of injectable furunculosis vaccines, their characteristics and efficacy.<sup>1,2</sup>

Investigators	Vaccine Strain	Inact.	Adj.	Major Antigen	Challenge	Test Species	Prot.	Comments
Krantz et al. 1963 and 1964a	AP-	F	FCA	LPS	inad.	BRK, BRN	NA	An avirulent strain was used for vaccine and challenge.
Hara et al. 1976	AP+	F	FCA	AP	epiz.	PS	+	
Fryer et al. 1976 and Udey and Fryer 1978	AP+	F	FCA	AP	epiz.	SS	+	
Antipa and Amend 1977	BI	F	none		inad.	KS, SS	NA	Challenge depended on an epizootic which failed to occur.
Michel 1979	AP+ AP+ AP- AP-	F F F F	none none none none	AP AP LPS LPS	1.m. i.p. 1.m. 1.p.	RBT RBT RBT RBT	++ - + -	Fish challenged by i.m. injection were slightly protected.
Palmer and Smith 1980	AP+	F	FCA + Tween	AP	epiz.	AS	-	
Paterson 1981	AP+ AP-	NR NR	NR NR	AP LPS	NR NR	BRK, AS BRK, AS	++ +	No details on vaccine preparation and challenge were given. AP+ vaccine was much more protective than AP- vaccine.

Table 3 (cont.)

Investigators	Vaccine Strain	Inact.	Adj.	Major Antigen	Chall.	Test Species	Prot.	Comments
Cipriano 1982c	AP+ AP-	none none	none none	F4 F4	1mm. 1mm.	BRK BRK	+	Column fractions F1, F2 and F3 tested concurrently were not protective.
Cipriano 1982b	AP+ AP+ AP+	F F none	none none none	AP/ECP AP ECP	1mm. 1mm. 1mm.	BRK BRK BRK	- - +	Three vaccines were prepared from 5 d broths: 1) FK broth 2) washed cells 3) ECP precipitate from cell-free broths.
Cipriano and Starliper 1982	AP-	none	none	LPS/ECP	1mm.	BRK, AS	+	The vaccine was a live, attenuated broth culture.
Cipriano 1983b	AP+ AP+ AP+	none none none	none FIA Sephadex Beads	F1 F1 ECP	1mm. 1mm. 1mm.	BRK BRK BRK	- + +	F1 was protective only if injected with adjuvant. ECP (140 ug/fish) link- ed to sephadex was protective.

Table 3 (cont.)

Investigators	Vaccine Strain	Inact.	Adj.	Major Antigen	Chall.	Species	Test Prot.	Comments
McCarthy et al. 1983	AP+	F	none	AP	i.m., i.p., cohab.	KS, SS	+	AP+ vaccines were treated to extract/solubilize AP.
	AP-	F	none	LPS	i.m., i.p., cohab.	KS, SS	-	
Shieh 1984	AP+	none	none	purified protease	i.m.	AS	+	
Olivier et al. 1985a	AP+	F	+	FCA	AP	i.p.	SS	
	AP+	none	+	FCA	ECP	i.p.	SS	
	AP-	F	+	FCA	LPS	i.p.	SS	
	AP-	none	+	FCA	ECP	i.p.	SS	
Olivier et al. 1985b	AP+	F	MFIA	AP	i.p.	SS	+	First record of the protective ability of adjuvant without antigen in fish.
	AP+	F	none	AP	i.p.	SS	+	
	none	NA	MFCA	MYCO.	i.p.	SS	+	
Paterson et al. 1985	NR	NR	NR	NR	i.m.	AS, SS, BRK	+	Commercially available vaccine (FURONG).
Michel 1985	AP+	F	none	AP	i.m., 1mm.	RBT	-	The second vaccine was a cell-free extract of AP+ cells containing soluble AP as the major antigen.
	AP+	none	none	AP	i.m., 1mm.	RBT	-	

Table 3 (cont.)

Investigators	Vaccine Strain	Inact.	Adj.	Major Antigen	Chall.	Species	Test Prot.	Comments
Newman and Majnarich 1985a	AP+	F	none	AP	imm.	AS, BRK, RBT	-	Bacterial cells were extracted with EDTA to solubilize AP.
Sakai 1985a	AP+	none	none	AP	i.p.	SOS	-	An avirulent live strain was used as a vaccine.
Evelyn (unpubl.)	AP+	none	NL	purified LPS	inject.	SS	-	High agglutinating antibody titers.

<sup>1</sup> Abbreviations : inact., inactivator; adj., adjuvant; chall., challenge; prot., protection; AP+, having AP; AP-, lacking AP; AP+, both AP+ and AP- strains were tested; BI, bivalent bacterin (composed of *A. salmonicida* and *V. anguillarum*); F, formalin; +FCA, with and without Freund's Complete Adjuvant; MFCA, modified FCA; MFIA, modified Freund's Incomplete Adjuvant; LPS, lipopolysaccharide; AP, A-protein; F4, fraction 4; F1, fraction 1; ECP, extracellular product; MYCO., Mycobacterium; inad., inadequate; epiz., epizootic; imm., immersion; cohab., cohabitation; i.m., intramuscular; i.p., intraperitoneal; inject., injection; AS, Atlantic salmon; KS, chinook salmon; SS, silver salmon; SOS, sockeye salmon; PS, Pacific salmon (amago, yamame); BRK, brook trout; BRN, brown trout; RBT, rainbow trout; NR, not reported; NA, not applicable; NL, used but not listed; +, positive protection; -, no protection.

<sup>2</sup> All vaccines were delivered by i.p. injection except Shieh (1984) who injected i.m.

outcome of any individual study.

Most of the protective vaccines have utilized AP+ strains of A. salmonicida mixed in Freund's complete adjuvant (FCA) (Fryer et al. 1976; Hara et al. 1976; Udey and Fryer 1978; Olivier et al. 1985a and 1985b), however, McCarthy et al. (1983) and Olivier et al. (1985a) have demonstrated protection using AP+ strains without adjuvant. A lack of protection has been observed in fish immunized with AP+ strains without adjuvant, and with AP- strains with or without adjuvant (Michel 1979; Cipriano 1982b; McCarthy et al. 1983; Olivier et al. 1985a; Michel 1985; Newman and Majnarich 1985; Sakai 1985a). These results indicated AP as a protective antigen, whose immunogenicity in fish was improved by administration in adjuvant.

Contradictory to this, Cipriano and Starliper (1982) demonstrated AP- strain bacterins were protective to brook trout and Atlantic salmon. Michel (1979) and Paterson (1981) also observed a low level of protection using AP- bacterins. Therefore, additional immunogen(s) may exist on AP- cells. In support of this, Olivier et al. (1985a) observed that rabbit antisera against AP- cells passively protected coho salmon. But they also found that coho salmon sera against AP- strains was not protective in passive tests, suggesting that fish were inefficient in responding to immunogens on AP- cells. In comparison to AP+ cell bacterins, the protective nature of AP- bacterins has been much less reproducible, possibly due to the low immunogenicity of antigens on AP- cells to fish.

Evelyn (unpublished data, cited in Olivier et al. 1985a) found purified lipopolysaccharide (LPS) from a virulent strain of A.

salmonicida to be antigenic in coho and sockeye salmon. An elevated level of serum agglutinins was observed, but vaccinated fish were not protected from furunculosis.

In the only study using a purified component from extracellular products (ECP), Shieh (1984) injected sublethal doses of purified protease from a virulent strain of A. salmonicida into juvenile Atlantic salmon. Vaccinates were highly protected, experiencing only 5.0% mortality compared to 100.0% among control fish injected with phosphate buffered saline (PBS).

The protective nature of ECP and components of ECP derived from avirulent strains of A. salmonicida has been emphasized by Cipriano (1982b; 1982c; 1983b). Extracellular products were separated into four fractions (F1, F2, F3 and F4) by anion exchange chromatography (Cipriano et al. 1981). Cipriano (1982c) found F4 from virulent, avirulent and atypical A. salmonicida strains was protective to brook trout. He characterized F4 as a 67 kdal glycoprotein common to all A. salmonicida strains. Cipriano (1983b) observed that F1 (primarily endotoxin) was protective to brook trout when injected with Freund's incomplete adjuvant (FIA). Crude ECP was protective to brook trout when injected i.p. in soluble form (Cipriano 1982b) or when linked to Sephadex A-25 beads (Cipriano 1983b).

Olivier et al. (1985a) injected ECP from AP+ and AP- strains of A. salmonicida into coho salmon. Only fish vaccinated with the virulent (AP+) strain ECP were protected from challenge with a virulent strain of A. salmonicida. The protective antigen was not identified, but was believed to be AP present in the ECP. McCarthy et al. (1983) also

reported an extracellular immunogen (AP) present in young (less than 32 h old) broth culture fluids.

High serum agglutinating antibody titers (titrated against AP<sup>+</sup>, non-aggregating strains of *A. salmonicida*) have been observed in vaccinated fish (Krantz et al. 1963 and 1964a; Hara et al. 1976; Udey and Fryer 1978; Michel 1979; Palmer and Smith 1980; McCarthy et al. 1983; and Michel 1985). However, high titers do not correlate with protection from disease (Michel 1979; Cipriano 1983a). McCarthy et al. (1983) titrated immune fish sera against both AP<sup>+</sup> and AP<sup>-</sup> *A. salmonicida* cells. They found no correlation between protection from furunculosis and antibody titer against AP<sup>-</sup> cells, however, a positive correlation existed between serum titer against AP<sup>+</sup> cells and protection. This suggested that antibodies against AP were protective, while antibodies against antigens on AP<sup>-</sup> cells (LPS and OM proteins) were not protective.

Tatner et al. (1984) noted the contradictory results achieved by various investigators using oral, injection and immersion vaccines. They stressed the need for a basic understanding of the mechanisms of antigen processing by fish to better understand how to elicit protective immune responses. They administered live, AP<sup>+</sup> *A. salmonicida* and formalin-killed (FK), radiolabelled cell bacterin to rainbow trout by oral, injection and immersion methods. The route travelled by live and killed cells was monitored over 5 d. They found that live or killed cells given orally localized in the gut; given by injection bacteria localized in reticuloendothelial (RES) tissues (liver, spleen, kidney, heart and gut); and given by immersion only low

levels were detectable, with localization initially in gills and skin, followed by localization in RES tissues. They observed that killed vaccines localized primarily in the kidney, where the primary antibody producing cells exist, whereas live vaccines were absent from kidney at the end of the study, but tended to localize in the spleen. Since others have noted a lack of correlation between antibody titer and protection, Tatner et al. (1984) speculated that the localization of live antigen in the spleen may induce another type of immunity, possibly protective. They further speculated that oral vaccines have failed because vaccine is rapidly eliminated through the gut after only minor local antigenic stimulation. Injectable vaccines present the most amount of antigen to lymphoid organs, and are good candidates for vaccines. Immersion vaccines deliver a very low dose of vaccine to the fish, however, this may still provide protective immunity. Others (Rijkers et al. 1980) have observed that both route and dose of primary immunization are important in creating immunological memory.

The importance of CMI in protection against furunculosis has been addressed by Smith et al. (1980) and Olivier et al. (1985b). Smith et al. (1980) were the first to use CMI (as measured by a leukocyte migration inhibition, LMI, test) to assess vaccine efficacy. They found that brown trout fry orally immunized with FK A. salmonicida were protected against furunculosis. No agglutinating antibodies were detected in serum or gut mucus, but a positive LMI response against A. salmonicida was observed among vaccinated fish. Unvaccinated control fish lacked a significant LMI response.

Olivier et al. (1985b) investigated the role of adjuvants and

immunomodulating agents in providing protection from furunculosis and other fish diseases. They observed that juvenile coho salmon (8 to 20 g) immunized with FK *A. salmonicida* mixed in either saline or modified Freund's incomplete adjuvant (MFIA) were protected at approximately the same level as those receiving modified Freund's complete adjuvant (MFCA) without bacteria. This was the first report of protective immunity in fish induced by adjuvants.

Juvenile coho salmon were injected i.p. with either saline or one of four adjuvants (MFCA, MFIA, levamisole, and muramyl dipeptide) and challenged after 30 d by i.p. injection with several different levels of *A. salmonicida*, *A. hydrophila*, or *V. ordalii*. Protection was observed against all three diseases, and was best in fish injected with MFCA. An estimate of efficacy was made by recording LD<sub>50</sub> increase values compared to saline injected fish. Respective values were 450x, 5.3x, and 560x for *A. salmonicida*, *A. hydrophila*, and *V. ordalii*.

Various doses of immunomodulating agents (glycogen, killed *Mycobacterium butyricum*, silica, latex beads, and killed *Renibacterium salmoninarum* cells) were injected i.p. into coho salmon, which were challenged after 1 to 4 d by i.p. injection with *A. salmonicida*. Fish receiving low doses of glycogen or *M. butyricum* displayed increased resistance, while those injected with silica, latex beads, *R. salmoninarum*, or high levels of *M. butyricum* had increased susceptibility to furunculosis. Olivier et al. (1985b) believed the observed protection was associated with macrophage activation by *M. butyricum* or glycogen. The rapidity of the response (1 to 4 d), and non-specific nature of the protection support this conclusion.

Particulate preparations, such as immunomodulators, are known to affect macrophage function. Silica, or high doses of immunomodulators, caused macrophage death or blockade and resulted in decreased protective immunity. Low doses resulted in macrophage activation and increased cellular and protective immunity. The authors suggested further research on adjuvants may result in preparations providing broad based protective immunity against many fish diseases, some of which are non-treatable by currently available methods.

Immersion Vaccines. Smith et al. (1980) prepared disrupted whole-cell vaccines using sodium dodecyl sulfate (SDS) and ultrasonication. Brown trout fry were immunized using the hyperosmotic-infiltration (HI) method of Amend and Fender (1976). Fish challenged by exposure to a naturally occurring epizootic were marginally protected (62% mortality among vaccinees compared to 86% among controls). Serum or secretory agglutinating antibodies were not detected, however, a positive CMI response was measured by LMI. An oral vaccine tested concurrently gave better protection and a stronger CMI response.

Palmer and Smith (1980) prepared bacterins from FK 48 h cultures of virulent, aggregating strain A47R. Bacterins diluted in sterile PBS had final concentrations of 5.3% NaCl, 0.2 M phosphate buffer, and  $5.3 \times 10^8$  cfu/ml bacterial cells. Hyperosmotic-infiltration vaccination of 15,000 Atlantic salmon presmolts (40 g) was by a 1.5 min dip in aerated vaccine. After 10 wk, fish were transferred to net pens in sea water, where an ongoing low grade natural epizootic was occurring. Significant protection ( $p < 0.001$ ) was observed after 7 wk, with

vaccinates experiencing 0.5% specific mortality, compared to 2.5% among controls. Agglutinating antibody titer was not significantly different between vaccinates and controls, and was not correlated with protection. Because only a low level of mortality was observed among controls, the authors cautioned that these results may not apply to high-challenge situations.

Cipriano and Starliper (1982) vaccinated juvenile brook trout and Atlantic salmon by immersion for 60 s in a live avirulent strain of *A. salmonicida*. Low serum agglutinating antibody titers (averaging 330) were measured 14 d after immunization. Fish were challenged after 28 d by immersion in approximately  $10^7$  to  $10^9$  cfu/ml. Both fish species were protected. Cumulative mortalities after 14 d were 44% in brook trout, 12.5% in Atlantic salmon, and 87 and 94% respectively for each species, in controls. The authors caution, however, on the potential of reversion to virulence, and suggest further research on the use of live vaccines.

Cipriano et al. (1983) prepared three immersion vaccines from avirulent *A. salmonicida* strain 3.95. Bacterial cultures grown for 48 h were inactivated with 0.3% chloroform, which was later removed by aeration. Vaccines were : 1) whole-cell vaccine (WCV), consisting of inactivated cells suspended in PBS to approximately  $10^9$  cfu/ml; 2) soluble antigen vaccine (SAV), which was undiluted spent culture media; and 3) whole-cell - soluble antigen vaccine (WC-SAV), consisting of WCV and SAV combined. Preliminary tests using brook trout (3.6 g) immunized by a 60 s immersion in vaccine and challenged after 30 d by immersion, indicated that only WC-SAV was protective. Cumulative

mortality during the 14 d monitoring period was 15.7% among fish receiving WC-SAV compared to 41.7% in controls. Further tests using WC-SAV on Atlantic salmon (62.0 g) confirmed the efficacy of this vaccine. Control mortality was 95.8% compared to 6.9% among fish receiving WC-SAV.

Field tests with this vaccine using brown trout were conducted at a local hatchery. Vaccinated fish (7.0 g) were challenged after 30 d by exposure to a natural epizootic. Control fish experienced 28.9% mortality, compared to 2.1% in vaccines. Cipriano et al. (1983) believed the protective nature of this vaccine was associated with endotoxin (the primary component of the WCV) combined with a cell wall glycoprotein, F4 (believed to be the major immunogenic component of SAV).

Cipriano (1983b) compared the humoral immune response of brook trout immunized with ECP by three routes. Fish were either injected with 140 ug ECP-Sephadex, fed ECP mixed in food at 140 ug ECP/g food for 10 d at 3% body weight/d, or immersed in 140 ug ECP/ml water for 2 min. After 60 d serum agglutinating antibodies were measured. The following mean titers were observed: control fish, 14; injected, 2458; oral, 154; immersed, 512. Since injectable vaccines are not economical, and reasonably high titers were achieved with the immersion vaccine, Cipriano (1983b) suggested that efforts to develop a furunculosis vaccine should concentrate on immersion delivery methods.

Johnson and Amend (1984) used an EDTA (ethylene-diamine-tetra-acetic acid) extracted FK bacterin prepared from virulent cells grown in casein yeast broth (CYB) to immunize juvenile chinook (4.0 g) and coho

(8.1 g) salmon. They compared i.p. injection and immersion methods using single and multiple vaccine doses. Fish were challenged by bath immersion (McCarthy 1983) in approximately  $10^4$  cfu/ml for 20 min with concurrent heat stress. They found that a single immunization by either i.p. injection or immersion provided significant protection, however, i.p. injected vaccine was significantly ( $p = 0.050$ ) better than immersion bacterins. Fish receiving multiple vaccinations were better protected than singly vaccinated fish, with no significant difference existing between multiple i.p. injection or multiple immersion vaccination protection levels. Immersion bacterins were more efficacious when given at initial bacterin dilutions of 1:2 or 1:4 followed in 14 d by a booster in 1:2 diluted bacterin. Specific cumulative mortality 14 d after challenge was 19% among vaccinees compared to 54% in controls.

They observed that Vibrio and Yersinia bacterins were effective at 20 s immersions, with protective immunity associated with heat-stable LPS antigens. In contrast, A. salmonicida bacterins were ineffective at immersion times less than 2 min. They theorized that the protective antigen of A. salmonicida may therefore be something other than LPS.

Amend and Johnson (1984) investigated the use of multivalent bacterins of A. salmonicida, V. anquillarum, Y. ruckeri and R. salmoninarum administered by immersion or i.p. injection to provide protection from their respective diseases. Bivalent A. salmonicida / R. salmoninarum bacterins provided better protection against furunculosis than the monovalent A. salmonicida bacterin. Juvenile chinook and coho salmon vaccinated with monovalent A. salmonicida

bacterin had increased resistance to both vibriosis and enteric redmouth (ERM) disease. They believed this cross protection was associated with shared common antigens between the pathogenic agents. Since antigenic interference did not decrease protection provided by multivalent vaccines (in contrast, a synergistic or potentiating effect was observed), they proposed multivalent vaccines could be useful in the fish health field.

Newman and Majnarich (1985) prepared a vaccine from a virulent, autoaggregating strain of *A. salmonicida*, AS-1. Bacteria were grown in brain heart infusion broth (BHIB) supplemented with glucose and oxygen for 24 h at 20 C in a fermentor. Pelleted cells were suspended to 17.5 g/L in sterile water, pH 8.0, then 5 mM EDTA was added for 1 h. Cells were inactivated with 0.3% formalin and stored at 4 C until used.

Vaccines were tested on three species of salmonids, rainbow trout (3.5 to 10.0 g), brook trout (3.5 to 5.0 g), and Atlantic salmon (approximately 35 g). Initially fish were immersed once in bacterin diluted 1:5 or 1:10 for 5 min, then challenged after a suitable period (approximately 30 d) by immersion in the homologous strain, AS-1. Significant protection was not observed in these fish.

Subsequently, fish were immunized by a 5 min immersion in bacterins diluted 1:5 or 1:10, followed by an equal booster vaccination after 14 d. Significant protection was observed in Atlantic salmon and rainbow trout, with mortalities among vaccinees ranging from 0 to 13% compared to 70 to 76.9% in controls. Concurrent mortality in Atlantic salmon immunized only once was 36.7%.

Because multiple vaccinations seemed uneconomical, they investigated

the use of hyperosmotic-infiltration to enhance vaccine efficacy.

Rainbow trout were immunized for 5 min in bacterin diluted 1:5 with 5% salt. Significant protection was observed in each of three trials. In trials 1 and 2 mortality among vaccinees was 7.4 to 43.3% compared to 72.7 to 74.2% for controls. In trial 3 mortality among vaccinees was 0 to 12.5% compared to 28.1% in controls.

Newman and Majnarich (1985) also investigated the use of cell-free supernatants of these bacterins to protect fish. Rainbow trout were immunized for 5 min in undiluted supernatant, and later challenged by immersion, i.p. injection, or immersion with heat stress. In four separate trials significant protection was observed, with average mortality among vaccinees of 33.6% compared to 76.4% among controls.

Aqua Health, LTD, has tested an immersion type vaccine (EUROGEN) for furunculosis (Zinn 1986). A group of 8,600 Atlantic salmon (3.0 g) were vaccinated by a 60 s immersion, then placed in raceway tailwaters below unvaccinated controls. An epizootic caused 36% mortality among controls, compared to 10% mortality among vaccinees.

Cox and Hedrick (1987) prepared five bacterins from an avirulent, autoaggregating, AP+, protease positive strain, 3.101-2. Vaccines prepared from 24 h BHIB cultures were: 1) live, saline washed cells; 2) alkaline hydrolysis (ALKOH) extracted cells plus supernatant, with 0.3% formalin; 3) ALKOH extracted cells plus supernatant, without formalin; 4) the cell fraction of bacterin 3; and 5) the supernatant of bacterin 3. Chinook salmon (8.7 g) were immersed for 5 min in 1:4 diluted bacterin on day 1, followed by a second immunization in 1:2 diluted bacterin on day 14. After 28 d fish were challenged by

immersion for 20 min in  $1.0 \times 10^4$  cfu/ml virulent strain AS-1R. Fish were monitored for 28 d and results were evaluated as recommended by Amend (1981). Bacterins 4 and 5 failed to protect fish, while bacterins 1, 2 and 3 provided statistically significant ( $p < 0.100$ ) protection. Best protection was observed with the ALKOH extracted live cell bacterin, with vaccines experiencing 71.4% mortality compared to 96% among controls. Serum antibody titers of 80 to 160 existed in all groups 21 d after vaccination, but titer was not correlated with protection.

Combination Vaccines. Only one report exists on the use of combination vaccination methods for control of furunculosis. Udey and Fryer (1978) injected juvenile coho salmon i.p. with a FK AP<sup>+</sup> strain of A. salmonicida, then fed fish for 45 d with the same bacterin mixed into food. They observed much lower serum agglutinating antibody titers in these fish than in fish receiving the injection without further oral vaccination. Titers in fish receiving the dual vaccine treatment were 32 to 128 compared to 1024 to 2048 in fish receiving only injected vaccine. They suggested a possible immune suppression.

#### Passive Immunization

Spence et al. (1965) were the first to use passive immunization to protect against furunculosis. Hyperimmune sera was prepared in adult rainbow trout injected with FK A. A. salmonicida mixed in FCA. Sera with agglutinating titers exceeding 320 were pooled, filter sterilized, and injected i.p. into yearling coho salmon (19.3 g) held in 17 C water.

Passively immunized fish were challenged by applying a concentrated broth of virulent A. salmonicida to a small patch of abraded skin, followed at 24 and 48 h by a 60 min immersion in approximately  $3.0 \times 10^7$  cfu/ml. During the next 7 d they observed lower cumulative mortality and a delay in the average time of death in passively immunized fish compared to controls. The serum component providing protection was not identified, but was believed to be antibody.

In a separate study Udey (1977) developed hyperimmune sera against A. salmonicida strain SS-70 (AP<sup>+</sup>, virulent) and strain Sil-67 (AP<sup>-</sup>, avirulent) in coho salmon. These sera were injected i.p. into 10 g coho salmon fingerlings, with each fish receiving 2000 agglutinating units. Negative controls were injected with preimmune sera, while positive controls were actively immunized by i.p. injection of FK SS-70 mixed in FCA. Fish were challenged by a thermal stress (raising the water temperature from 12 to 18 C), followed after 5 h by immersion in  $10^6$  cfu/ml virulent strain SS-70 A. salmonicida for 15 min. Specific mortality among actively immunized controls was 4% compared to 42% among fish injected with preimmune sera. Fish receiving anti SS-70 sera experienced 16% mortality, while those injected with anti Sil-67 sera had 38% mortality. Protection with the anti-virulent sera was associated with cell wall antigens other than LPS O-antigen.

A lack of passive protection using rabbit antisera was observed by Cipriano (1981). He passively immunized Atlantic salmon with either rabbit or rainbow trout antisera developed against typical and atypical strains of A. salmonicida. Fish were challenged by immersion (Cipriano 1982a) and cause of death was confirmed by fluorescent antibody

technique. He observed no protection with rabbit antisera, but good protection with rainbow trout antisera.

Antisera to both virulent and avirulent strains of A. salmonicida provided passive protection to brook trout (Cipriano 1982b). Rainbow trout were injected with sublethal doses of either avirulent or virulent A. salmonicida, inducing an active infection in the latter. Antisera recovered from these fish (titer averaged 512) was injected into juvenile brook trout, which were challenged by immersion after 72 h. Each antisera was equally protective, suggesting that the protective antigen existed on both virulent and avirulent strains. Mortality in fish passively immunized with anti-virulent sera was 15 to 20%; anti-avirulent sera, 20%; preimmune sera, 60 to 70%; non-injected controls, 70 to 90%.

The ability of normal fish sera to neutralize the cytotoxicity of F4, *in vitro*, correlated with protection against A. salmonicida (Cipriano 1983a). He studied the ability of normal sera from rainbow trout (furunculosis resistant fish) and Atlantic salmon (furunculosis susceptible fish) to protect brook trout fingerlings from mortality following experimental challenge. Fish were injected i.p. with 0.1 ml of sera, then challenged after 72 h by a 60 s immersion in  $1.2 \times 10^9$  cfu/ml of A. salmonicida. Cumulative mortality after 14 d was 75% in controls, 70% in fish injected with Atlantic salmon sera, and 50% in fish receiving rainbow trout sera. Protection afforded by rainbow trout sera was correlated with the ability to neutralize cytotoxicity of F4 (i.e. serum neutralizing ability), and not with serum agglutinating antibody levels. The neutralizing serum component was identified as an

alpha-migrating protein (Ellis et al. 1981, Cipriano 1983a). Cipriano (1983a) also tested 11 different strains of rainbow trout for protection from experimental challenge with A. salmonicida. He found strain differences in susceptibility, and this also correlated with ability of sera to neutralize F4.

Additional tests were conducted which confirmed the protective nature of F4 antisera (Cipriano 1983b). He injected rainbow trout three times at monthly intervals with a sublethal dose of virulent A. salmonicida, thereby generating antisera to both cell-associated and extracellular or secreted bacterial antigens. Antisera was fractionated by anion exchange chromatography (DEAE Sephadex A-25) into three activities, as measured by immunoelectrophoresis, counterimmunoelectrophoresis, passive hemagglutination and neutralization tests. These were precipitating, agglutinating and neutralizing activities. He found that agglutinins were immunoglobulins of beta mobility, which reacted against his F1 (endotoxin). Serum precipitins were immunoglobulins of beta to gamma mobility, and were reactive against F2 (a mixture of at least six proteins, including protease and leukocytolytic activities). Serum neutralizing factor was not an immunoglobulin, had alpha mobility, and reacted against F4. Cipriano (1983b) injected these serum fractions into brook trout, which were challenged by immersion after 72 h. Serum agglutinating and neutralizing factors provided the best protection. Fish injected with agglutinating or neutralizing fraction had a 25% mortality, compared to 72% among those injected with the precipitin fraction and 38% among those injected with normal (preimmune) sera.

A protein was found to be a protective antigen by McCarthy et al. (1983). They prepared rabbit antisera against AP<sup>+</sup> and AP<sup>-</sup> cells, and boiled AP<sup>+</sup> cells (this method removed AP). Using cross absorbed antisera, they prepared a mono-specific anti-AP serum. Absorbed and unabsorbed antisera were injected i.p. into sockeye salmon fingerlings (5.7 to 9.0 g), which were challenged by immersion in approximately 10<sup>5</sup> cfu/ml for 20 min. Since only AP<sup>+</sup> antisera, and the mono-specific antisera were protective, they concluded that AP was the protective antigen.

Better protection was observed using virulent cells compared to avirulent cells by Olivier et al. (1985a). They produced antisera against FK virulent (VFK) and non-virulent (NVFK) A. salmonicida cells, and ECP derived from broth cultures of each of these (VECP, NVECP) in subadult (350 g) coho salmon. Rabbit antisera were prepared against live virulent (VL) and avirulent (NVL) cells, formalin and heat-killed (VFK, VHK) cells, and ECP from both virulent and avirulent broth cultures (VECP, NVECP). These antisera were injected i.p. into juvenile (7 g) coho salmon, which were challenged after 2 h by i.p. injection of one LD<sub>50-90</sub> of virulent A. salmonicida. Results indicated moderate protection with NVL or NVECP antisera. Among the rabbit antisera, VL, VFK, VECP and NVL provided strong protection. Fish receiving VHK had much reduced protection, while those immunized with NVECP were not protected.

Olivier et al. (1985a) believed the protection afforded was associated with an additional antigen present on virulent cells and in young broth culture supernatants, probably AP. Passive immunity tests

using cross absorbed antisera also validated this supposition. Rabbit VL and NVL sera were absorbed with virulent or avirulent cells. Agglutination titers were reduced from 640 and 1280 respectively, to zero in all absorbed sera. Only fish receiving VL antisera absorbed with avirulent cells were protected. This suggests that VL sera contained an antibody against an additional antigen (possibly AP) associated with protective immunity.

#### Virulence Mechanisms

Virulence mechanisms of *A. salmonicida*, a very homogeneous species (McInnes et al. 1979; Paterson et al. 1980; McCarthy and Roberts 1980; Hahnel et al. 1983) and a highly evolved and successful pathogen, have been extensively reviewed (McCarthy and Roberts 1980, Paterson 1981, Michel 1982, Cipriano 1983b, Trust et al. 1983 and Munro 1984).

Virulence has been attributed to AP and ECP, and to a lesser degree LPS.

A remarkable degree of homogeneity has been observed for LPS, AP and F4. Chart et al. (1984) analyzed LPS from 16 typical and 14 atypical *A. salmonicida* isolates of diverse origins and found O-polysaccharide side chain lengths and sugar composition to be unusually homogeneous. Additionally, LPS from different strains were immunologically cross reactive, and LPS was observed to traverse the AP surface layer. A protein derived from typical and atypical isolates is virtually identical structurally, immunologically, and in amino acid composition (Hubbert and Brain 1980, Evenberg et al. 1982, Trust et al. 1983, and Kay et al. 1984). Cipriano (1983b) reported that F4 antigens from

virulent typical and atypical isolates, and F4 from avirulent derivatives of these were identical. Although ECP consists of many components, there is agreement that the pathology associated with furunculosis is reproducible by injecting ECP into fish (Fuller et al. 1977; Sakai 1977; Ellis et al. 1981; Pol et al. 1980; Sheeran et al. 1983; Munro et al. 1980; Cipriano 1980; Cipriano et al. 1982b; Shieh 1982; Finley 1983), and that the pathology is associated with protease activity (Mackie et al. 1935; Griffin 1954; Klontz et al. 1966; Shieh and MacLean 1975; Sakai 1977 and 1978; Ellis et al. 1981; Munro et al. 1980; Shieh 1982; Sheeran et al. 1983; Finley 1983; and Rockey et al. 1988).

A-protein. A-protein, a cell bound protein present on the outer surface of virulent strains of A. salmonicida, was first observed by Udey (1977). He observed that virulent isolates ( $LD_{50} < 8 \times 10^3$  cfu/fish) differed from avirulent isolates ( $LD_{50} > 1 \times 10^8$  cfu/fish) of A. salmonicida by possession of the following characteristics : 1) autoaggregation in broth cultures; 2) enhanced adherence to fish and mammalian cell monolayers in vitro; 3) existence of few receptors for A. salmonicida specific bacteriophage, and resistance to phage lysis; and 4) presence of an additional protein layer (AP) detectable by electron microscopy exterior to an otherwise typical gram-negative cell wall. In contrast, avirulent strains of A. salmonicida lack these features. Udey (1977) characterized the AP as an 8 to 9 nm wide electron-dense layer separated from the outer membrane (OM) by an electron-translucent zone (assumed to be the O-side chain of LPS). A-

protein was firmly attached to the cell wall, existing as an interrupted periodic pattern, or tetragonal array. Udey (1977) demonstrated that autoaggregation of AP<sup>+</sup> A. salmonicida strains was a reversible process dependent on the presence of multivalent cations. The proposed mechanism of aggregation was bridging between divalent cation binding sites of adjacent cells, and the same mechanism was assumed to be responsible for bacterial cell adhesion to eukaryotic cells.

Protein surface layers (S-layers) have since been reported on other gram-negative bacterial pathogens, including Brucella (Every and Sherman 1980), Salmonella (Dubray and Berzard 1980), and Campylobacter fetus (Winter et al. 1978). Autoaggregating, highly virulent strains of A. hydrophila pathogenic to fish have recently been reported to possess a tetragonal surface array (Murray et al. 1988; Dooley et al. 1988). Although structurally very similar to the S-layer of A. salmonicida, differences were apparent in its amino acid composition, molecular weight, and biological activities.

Although Udey (1977) found AP to be requisite for virulence, it did not confer virulence. He noticed the existence of an AP<sup>+</sup> avirulent strain, and concluded that additional virulence mechanisms beside AP must also contribute to the disease process. Although AP<sup>+</sup> avirulent strains are not common, they have been reported by Sakai (1977), Trust et al. (1983), McCarthy et al. (1983), Cox and Hedrick (1987) and Olivier (1988).

Udey and Fryer (1978) proposed that the function of AP was similar to the adhesive pili of K-antigens of other gram-negative bacterial

pathogens. This belief was substantiated by the studies of Evenberg and Lugtenberg (1982) who found the amino acid composition and sequence of AP to be similar to that of enteropathogenic E. coli K88 fimbria.

A-protein from typical and atypical strains exists in a tetragonal pattern, as observed by electron microscopy (Udey and Fryer 1978, Hubbert and Brain 1980, Trust et al. 1980, and Kay et al. 1981). A-protein has been purified by various methods (Trust et al. 1980; Kay et al. 1981; Evenberg and Lugtenberg 1982; Phipps et al. 1983) and its molecular weight as determined by SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) was approximately 50 kdal (45 to 54 kdal). Amino acid analysis of typical and atypical isolates from various geographical locations revealed nearly identical composition. Most importantly, the peptides exposed to the surface had identical amino acid sequences, and were of a hydrophobic nature (Kay et al. 1981 and 1984).

The proposed functions of AP are : 1) cell attachment via either cationic binding and/or hydrophobic interactions, 2) facilitated entry into the host, likely mediated by hydrophobic interactions, 3) action as a physical barrier to complement mediated lysis, 4) resistance to degradation by phagolysosomal enzymes after internalization by macrophages, and 5) sequestering of ferric iron from host lactoferrin and transferrin.

The observations of Udey (1977) on enhanced cell adhesion by AP<sup>+</sup> strains of A. salmonicida have been corroborated by the studies of Sakai (1985b) and Trust et al. (1983). Although Udey (1977) believed this binding was mediated by cationic binding sites (supported by

observed inhibition of binding in the presence of EDTA), Trust et al. (1983) proposed that enhanced binding to trout and mouse macrophages was mediated by hydrophobic cell-surface interactions. They observed that AP inhibited binding of bacteria to trout erythrocytes by masking OM protein receptors, however, an enhanced association with macrophages was observed. They observed internalization of A. salmonicida by macrophages and theorized that bacteria survive as facultative intracellular parasites.

A-protein presents a physical barrier essential for virulence (Phipps et al 1983) which apparently protects A. salmonicida from host defense mechanisms. Resistance to complement mediated lysis of AP<sup>+</sup> cells in the presence of either normal or immune sera was observed by Munn et al. (1982). Isogeneic AP<sup>-</sup> cells, however, were susceptible to complement lysis in immune sera, and slightly resistant in normal sera. Similar results have been observed by others (Sakai 1985b; Munn and Trust 1984). Munn and Trust (1984) have suggested that AP confers resistance to phagocytic killing, allowing intracellular proliferation of the bacteria.

The availability of free iron is a limiting factor for microbial growth. Bacteria causing infectious diseases must successfully compete with host iron binding mechanisms to survive (Stanier et al. 1976). Chart and Trust (1983) examined six typical and three atypical strains of A. salmonicida for their ability to acquire ferric iron from host iron chelators transferrin and lactoferrin. They found that typical strains successfully sequestered iron by the production of low molecular weight siderophores, and by direct stripping of iron from

host chelators using a cell-bound molecule. Atypical strains had poorer iron-sequestering abilities. Kay et al. (1985) found that purified AP bound directly to protoporphyrin IX, and speculated that this was the initial step in sequestering iron. Crosa et al. (1977 and 1980) described a 50 megadalton plasmid coding for iron-sequestering proteins in the fish pathogen *V. anguillarum*. Loss of this plasmid resulted in both attenuation of virulence and loss of iron-sequestering abilities (Crosa et al. 1980, Crosa 1980).

The synthesis, export and assembly of *A. salmonicida* AP was investigated using transposon mutagenesis (Belland and Trust 1985). They found that AP was the product of a single gene, and was transported across the periplasmic space with the aid of at least one other protein. It required a homogeneous O-side chain LPS structure for assembly in the typical tetragonal array. If O-side chains were heterogeneous, AP was not assembled, but was exported to the culture media. Similarly, the S-layer protein of *A. hydrophila* fails to assemble on the surface of "rough" LPS mutants (those lacking O-polysaccharide side chains and core oligosaccharides), being exported to the culture media. In contrast, rough mutants of *A. hydrophila* which lacked the O-side chain but possessed core oligosaccharides were able to assemble the S-layer, suggesting that these core sugars of the LPS molecule are anchorage sites for S-layer proteins (Murray et al. 1988).

Stewart et al. (1986) using computer image processing of electron micrographs observed two distinct structural patterns in AP on whole cells of *A. salmonicida*. The AP layer had previously been

characterized as a refractive and possibly impermeable layer. Stewart et al. (1986) speculated that one of the two observed patterns was a structural transformation which altered permeability of the AP layer, facilitating release of ECP virulence factors.

Isogenic AP<sup>-</sup> mutants are easily produced from virulent AP<sup>+</sup> *A. salmonicida* strains. Culture in tryptic soy broth (TSB) at 30 C caused 90% mutation to an AP<sup>-</sup> avirulent strain after only one passage (Ishiguro et al. 1981). McCarthy et al. (1983) observed that culture in certain media (TSB) resulted in spontaneous mutation to the AP<sup>-</sup> form, whereas CYB or BHI broths did not cause mutation. Serial passage on artificial media and prolonged storage at -80 C, or at 4 C on BHI slants caused decreased virulence (McCarthy and Roberts 1980 and Cipriano 1983b). A-protein deficient mutants have been produced by adding 0.25% lithium chloride to broth cultures during growth (Duff 1939 and Hamilton et al. 1981).

Others have reported loss of virulence without loss of AP. Sakai (1977) produced an AP<sup>+</sup> avirulent mutant from an AP<sup>+</sup> virulent strain by treatment with N-methyl, N'-nitro, N-nitrosoguanidine and EDTA. Trust et al. (1983) have recovered an AP<sup>+</sup> virulent strain from an AP<sup>-</sup> avirulent strain. Cox and Hedrick (1987) have recovered an AP<sup>+</sup> avirulent strain from fish injected with an AP<sup>-</sup> avirulent strain. Olivier (pers. comm.) observed loss of virulence but retention of AP after prolonged storage at -70 C.

A-protein was considered a very important component of furunculosis vaccines (McCarthy et al. 1983). They investigated the effects of culture media on AP production. Optimal yields of AP<sup>+</sup> cells were

observed using CYB and BHI broths, however, low yields were seen using TSB. They extracted AP from whole cells by sonication, EDTA, pH lysis, and ALKOH, but were unable to extract AP using proteolytic enzymes (pepsin, trypsin, and chymotrypsin) or detergents (SDS and Triton X-100). A-protein was found to be heat-stable at 100 C for 15 min, but not at 50 C for 60 min.

Lipopolysaccharide (LPS). The endotoxin (LPS) of *A. salmonicida* is similar to that of other gram-negative bacteria (Paterson and Fryer 1974a), and although highly toxic to mice (LD<sub>50</sub> values of 232 to 536 ug/mouse following i.p. injection) it is not toxic to fish even when injected with up to 700 mg/kg body weight (Paterson 1972; Pol et al. 1980; Wedemeyer et al. 1968). Lipopolysaccharide was therefore assumed to be relatively unimportant in the pathology of furunculosis (Shieh 1982; McCarthy and Roberts 1980). Fish injected with LPS developed high titered antisera (Paterson and Fryer 1974b; Paterson 1972) but these antibodies were not believed to be associated with protective immunity (Michel 1982; Cipriano 1983a; Amend and Johnson 1984; Olivier et al. 1985a and 1985b).

The importance of LPS to the virulence of *A. salmonicida* lies in providing a substrate upon which AP is assembled (Chart et al. 1984 and Belland and Trust 1985). Lipopolysaccharide of various *A. salmonicida* isolates was structurally analyzed and found to be extremely homogeneous, with a long O-polysaccharide side chain, characteristic of "smooth" LPS. The uniformity of this side chain length was shown to be requisite for assembly of AP. Mutants with heterogeneous O-

polysaccharide side chains failed to develop an S-layer, but produced and exported AP to the culture media. Strains with homogeneous O-polysaccharide side chain lengths developed S-layers composed of AP, typical of virulent strains of A. salmonicida.

Extracellular Products (ECP). The pathology associated with furunculosis is consistent with pathological effects caused by proteolytic enzymes present in ECP (Mackie et al. 1935; Griffin 1954; Klontz et al. 1966; and Mawdesley-Thomas 1969). When injected into fish, crude preparations of ECP reproduced the pathology of furunculosis (Fuller et al. 1977; Sakai 1977; Cipriano 1980; Munro et al. 1980; Ellis et al. 1981; Pol et al. 1981; and Fyfe et al. 1986). If crude ECP is injected into rainbow trout gross pathological changes consistent with the disease (a darkening in color, lethargy, tachybranchia, rectal inflammation, enlargement and inflammation of the spleen, and hemorrhage and tissue liquefaction at the injection site) are seen (Ellis et al. 1981). Gross observations are consistent with histopathological changes, including a lymphocytic infiltration of the gills, and lesions in muscle, liver, and kidney tubules. Behavioral and histopathological changes are consistent with systemic anaphylaxis, and they proposed that ECP interacted with eosinophilic granular cells (EGC) in the intestinal connective tissue causing histamine release, vasodilation, and acute shock resulting in death. This belief was further studied by Ellis (1985) who observed EGC degranulation, increased blood levels of histamine, and widespread vasodilation 45 min following i.p. injection of ECP in rainbow trout fingerlings. Fuller

et al. (1977) observed that ECP injected concurrently with live *A. salmonicida* cells enhanced the virulence of these cells.

In vitro effects of crude ECP preparations have been described by Ellis et al. (1981), Munro et al. (1980), MacIntyre et al. (1979 and 1980), and Buckley et al. (1982). Proteolytic (caseinase and gelatinase), hemolytic (blood agar, trout erythrocytes), leukocytolytic (trout leukocytes), and lecithinase activities of ECP have been shown (Munro et al. 1980). These activities were neutralized by treatment with fish serum. MacIntyre et al. (1979 and 1980) and Buckley et al. (1982) identified phospholipase, lysophospholipase, and cholesterylacyltransferase activities from a single enzyme derived from ECP.

Dahle (1971a and 1971b) first isolated an extracellular protease (Proteinase B) from *A. salmonicida*. He purified it from ECP of an avirulent strain by ammonium sulfate precipitation followed by column chromatography. Its molecular weight was 43.6 kdal, with a pH optimum of 9.0. Media composition affected production of proteinase B, being inhibited by casein, and enhanced by trypsinized casein, gelatin and neopeptone.

Shieh and MacLean (1975) purified an 11 kdal serine protease from ECP by ammonium sulfate precipitation, acetone precipitation and column chromatography. Temperature and pH optima were 60 C and 10.5, respectively.

Fuller et al. (1977) isolated a leukocytolytic factor (LF) from ECP of virulent and avirulent strains by ammonium sulfate precipitation, ethanol precipitation, and column chromatography. This 10 to 300 kdal

glycoprotein was cytolytic in vitro to rainbow trout leukocytes, and produced a profound leukocytopenia in vivo in adult rainbow trout.

Sheeran and Smith (1981) produced a protease deficient mutant by the method of Sakai (1977). They recovered two proteases (P1 and P2) from ECP by ammonium sulfate precipitation and column chromatography. P1 was a serine protease with caseinase, gelatinase, and collagenase activities, and was believed to be the same as the 11 kdal protease of Shieh and MacLean (1975). P2 was a metallo-enzyme with gelatinase and collagenase activities. Sheeran et al. (1983) found that P1 (caseinase<sup>+</sup>) was toxic when injected i.m. into fish, whereas P2 (caseinase<sup>-</sup>) was not. Studies by Drinan and Smith (1985), however, indicated that caseinase activity was not essential for inducing disease. They injected a caseinase<sup>-</sup> mutant of *A. salmonicida* into Atlantic salmon and followed the histopathological changes. They observed typical virulence and pathology with the mutant strain, and concluded that P1 activity was not essential for virulence. These results are in contrast to Sakai (1977) who found a caseinase<sup>-</sup> mutant to be avirulent.

Rockey et al. (1988) purified P1 and P2 proteases, and hemolysin or T-lysin (Titball and Munn 1983) from broth culture supernatants. In vitro activities of P1 and P2 were as described by Sheeran and Smith (1981). In addition, P1 lysed nuclear membranes of rainbow trout erythrocytes in vitro. T-lysin acted independently of P1, lysing the outer membrane of erythrocytes. However, in vivo hemolysis of erythrocytes was not observed in infected fish, and it was believed that erythrocytes were not the target tissue for T-lysin. Lesions from

infected tissue lacked P2 and T-lysin, but contained the P1 protease. The authors concluded that the P1 protease was the major proteolytic factor produced by A. salmonicida.

Mellergaard (1983) purified an 87.5 kdal serine protease from ECP of a virulent strain by ammonium sulfate precipitation and column chromatography. Temperature and pH optima were 48 C and 9.0, respectively. Its virulence in fish was not tested.

Finley (1983) purified a 70 kdal protease from ECP of virulent A. salmonicida by column chromatography. Temperature and pH optima were 50 C and 9.0, respectively. When injected into rainbow trout fingerlings typical pathology and mortality was observed. Fyfe et al. (1986) compared the pathologies induced by this protease with that of crude ECP. They found significant hemorrhaging and tissue liquefaction in Atlantic salmon fingerlings injected with purified protease, however, crude ECP effects were more profound. They suggested that protease acted synergistically with other ECP components to produce pathological changes.

For purposes of vaccination, it is important to inactivate virulence factors. Protease activity was effectively inactivated with 2.0% formalin for 24 h, at a pH of 2.0 for 30 min, or by heating to 60 C for 30 min (Pol et al. 1981). Shieh (1982) inactivated protease by heating to 100 C for 20 min.

Purified ECP factors were immunogenic in rabbits and fish (Fuller et al. 1977; Shieh 1982), eliciting a humoral antibody response. However, ECP may detrimentally affect the immune system. Fuller et al. (1977) and Munro et al. (1981) also observed that low amounts of ECP inhibited

pseudopod formation by macrophages. Sakai (1985b) observed protease production and a concurrent fall in antibody titer when protease\* *A. salmonicida* cells were grown in media supplemented with immune sera. He postulated that protease may degrade serum antibodies in vivo resulting in impaired immune defenses.

Hastings and Ellis (1985) analyzed six *A. salmonicida* isolates from different geographical regions for differences in the protein and protease composition of their ECP's. They detected differences in caseinase, gelatinase, and hemolysin activities, and suggested that different strains may produce different virulence factors and have varying modes of pathogenesis. The serological homogeneity seen with AP and LPS may, therefore, not exist with ECP (Munro 1984).

Fraction 4 (F4). Cipriano (1980) and Cipriano et al. (1981) concentrated ECP from 4 to 7 d old BHIB cultures of virulent and avirulent typical and atypical strains of *A. salmonicida* using ammonium sulfate and ethanol/sodium acetate precipitation. Concentrated ECP was separated into four fractions by anion exchange chromatography on DEAE Sephadex A-25. Column fractions were characterized by their in vivo and in vitro pathological effects, immunogenicity and protective abilities, and physical properties (Cipriano 1980; Cipriano et al. 1981; Cipriano 1982b and 1982c; Cipriano and Starliper 1982; Cipriano 1983b; and Cipriano et al. 1983).

Fraction 1 (F1), which eluted from Sephadex columns with 0.01 M K<sub>2</sub>HPO<sub>4</sub>, pH 8.0, was composed mainly of sloughed endotoxic material from bacterial cellular autolysis. Although it lacked proteolytic or

leukocytolytic activities, it was cytotoxic to RTG-2 monolayers in vitro, and toxic to juvenile rainbow and brook trout and Atlantic salmon in vivo. Fraction 1 failed to provide protective immunity alone, but when mixed in FIA fish were protected from mortality following immersion challenge.

Fraction 2 (F2) eluted from columns with 0.3 M NaCl, and contained the LF previously described by Fuller et al. (1977). Cipriano (1983b) detected at least six different proteins in F2 by SDS-PAGE. Fraction 2 was proteolytic, leukocytolytic, and cytotoxic to RTG-2 and CHSE monolayers in vitro. In vivo, F2 injected i.m. or i.p. into fish caused lesions and mortality. In contrast to Fuller et al. (1977), Cipriano (1980) found no differences in the amount of LF produced by virulent or avirulent strains.

Fraction 3 (F3), eluted with 0.6 M NaCl, was hemolytic to sheep red blood cells. It also contained the diffusible water soluble melanopigment of chromogenic strains of A. salmonicida. It was cytotoxic to RTG-2 cells, and toxic to brook trout and Atlantic salmon, but not to rainbow trout.

Fraction 4 eluted from columns with 1.2 M NaCl. It contained no proteolytic or leukocytolytic components, was weakly cytotoxic to RTG-2 monolayers, and caused no mortality or pathological effects in fish.

Whereas F1, F2 and F3 provided no, or inconsistent, protection against furunculosis, F4 was shown to provide consistent and significant protection when injected at 5 ug/fish. This protection was not enhanced by mixing in FIA, however, particulation onto Sephadex beads increased protection.

Fraction 4 was a glycoprotein (1.0 : 1.2, protein : carbohydrate) with a 67 kdal protein subunit and a 45 kdal carbohydrate moiety. It was immunologically distinct from AP and LPS by immunodiffusion studies. Since F4 was not a virulence factor, and because F4 from many diverse A. salmonicida isolates all provided protective immunity, Cipriano (1982c) suggested F4 was a common antigen among all A. salmonicida isolates, and an important antigenic component of furunculosis vaccines.

## Materials and Methods

### Bacterial Cultures

Sources and Descriptions. Six strains of *A. salmonicida* were used in this study. Their origins and characteristics are summarized in Table 4. Strains AS-1R, 3.101-1 and 3.95 were originally isolated as virulent, AP<sup>+</sup> isolates, however, the latter two strains lost both virulence and AP (A-protein) after prolonged storage at -80 C in brain heart infusion broth, BHIB, (Cipriano, pers. comm.). Strain AS-1R was isolated from healthy coho salmon (*Oncorhynchus kisutch*) carriers, and is a highly virulent strain, requiring as few as 5 colony forming units (cfu)/fish by i.m. injection to cause mortality. Strains AS-1S and 3.101-3 are AP<sup>-</sup> mutants derived from AP<sup>+</sup> strains AS-1R and 3.101-2, respectively, by culture at 30 C, as described by Ishiguro et al. (1981). Strain 3.101-2 is an avirulent, AP<sup>+</sup> isolate derived by serial passage of strain 3.101-1 through chinook salmon (*O. tshawytscha*) fingerlings.

Growth of Cultures. Cultures were grown on BHIA for 2 to 3 d at 20 C. Two to five colonies from BHIA plates were used to inoculate broth cultures in BHIB of 50 ml. After 20 to 24 h at 20 C and 200 rpm on an orbital shaker the cultures were examined microscopically for purity and either used directly or to inoculate larger broth volumes.

Strains of *A. salmonicida* were identified by the following characteristics: production of a brown diffusible pigment; gram-negative; non-motile; oxidase positive; and agglutination with specific antisera. Additionally, AP<sup>+</sup> strains produced friable colonies on agar

Table 4. Sources and characteristics of *Aeromonas salmonicida* strains used in this study.

Strain	AP	Virulence	Year Isolated	Location	Host Species	Source
AS-1R	+	+	1978	Manchester, WA	coho salmon	Tavolek Labs, WA
AS-1S	-	-	1986	Davis, CA		W. Cox
3.101-1 <sup>a</sup>	-	-	1978	Kearneysville, W VA	brook trout	R. Cipriano
3.101-2	+	-	1984	Davis, CA	chinook salmon	W. Cox
3.101-3	-	-	1986	Davis, CA		W. Cox
3.95 <sup>a</sup>	-	-	1977	Huntsdale, PA	trout	R. Cipriano

<sup>a</sup> Original isolates of *A. salmonicida* strains 3.101-1 and 3.95 were virulent, having LD<sub>50</sub> values of 14.4 and 105.3 cfu/fish by injection into brook trout (Cipriano 1980).

and autoaggregated in 0.85% saline. Strains lacking AP produced butyrous colonies on agar and uniform suspensions in saline. Biochemical characterizations were conducted on API-20E test strips (Analytab Products) as described by Kent (1982) and reactions are listed in Appendix A. Antibiotic sensitivities were determined by the method of Bauer et al. (1966) and are listed in Appendix B.

Storage. Broth cultures were centrifuged at 5000 xg for 15 min at 4 C. Pellets were resuspended in nine volumes of Mist desiccans (6.9% glucose, 0.3% TSB, and 69.6% fetal bovine serum), and 1.0 ml aliquots were stored at -80 C.

Virulent Strains. Virulence of strain AS-1R was maintained as described by Michel (1980). Briefly, kidneys from experimentally infected chinook or coho salmon fingerlings were aseptically removed, suspended in 0.85% sterile saline, and injected i.m. into uninfected fingerlings. After three or more fish passages bacteria were reisolated on BHIA, cultured once in BHIB, and aliquots were stored as described. This stock of virulent *A. salmonicida* was used for challenging fish.

Production. Initial 50 ml broths were used to inoculate larger volumes of BHIB. A 2% inoculum (McCarthy et al. 1983) was used, and growth was as previously described. Broths were harvested at 20 to 120 h, depending upon their ultimate use.

Other Media. Differential media and BHIA were used to reisolate *A. salmonicida* from infected fish tissues and for plate counts. Congo red agar (TSA-CR) was prepared by adding 30 ug congo red (Sigma) per ml of TSA. *Aeromonas salmonicida* AP<sup>+</sup> colonies appeared as red, while AP<sup>-</sup> strains and most non - *A. salmonicida* bacterial contaminants produced pink to white colonies (Ishiguro et al. 1985). Coomassie blue agar (TSA-CB) was prepared by adding 10 ug of Coomassie blue R-250 (BioRad Laboratories) per ml of TSA. *Aeromonas salmonicida* AP<sup>+</sup> colonies were blue, while AP<sup>-</sup> colonies were white (Cipriano 1987).

#### Fish Sources and Holding Facilities

The fish species, sources, sizes of individuals and experimental use are summarized in Table 5. Rainbow trout obtained from the University of California, Davis (UCD) trout hatchery were bled and sera tested for antibody titers against *A. salmonicida* strain AS-1S. Furunculosis has never occurred at this hatchery, and all sera tested negative. Chinook salmon from the Mad River hatchery were not tested for antibodies, however, no furunculosis had existed at this hatchery for at least 2 yr. Chinook salmon from the Moccasin Creek hatchery also had no history of furunculosis. Serum from a sample of 55 fish tested negative for agglutinating antibodies, and no bacteria were cultured from kidney samples.

Fish used in passive immunization tests were obtained from the Iron Gate hatchery, which was free of furunculosis. Water hardened coho salmon eggs were transported to the UCD Fish Pathology Laboratory and treated with 100 ppm iodophor (Prepodyne, WestAgro) for 10 min (Herwig

Table 5. Species, sizes and sources of fish used in this study.

Species	Date obtained	Size	Source	Experimental Use
Rainbow trout	09-87	500 g	UCD hatchery	Antisera production for use in passive immunization tests.
Chinook salmon	09-84	25.4 g	Mad River hatchery	Virulence testing of <i>A. salmonicida</i> strains
Chinook salmon	06-86	8.7 g	Moccasin Creek hatchery	Test ability of <i>A. salmonicida</i> strains AS-1R, AS-1S and 3.101-2 to survive in vivo within host tissues.
Coho salmon	12-86	8.4 g	Iron Gate hatchery	Passive immunization test 1. Obtained as water hardened eggs and hatched in the laboratory.
Chinook salmon	01-88	12.4 g	Iron Gate hatchery	Passive immunization test 2. Obtained as fry prior to first feeding.

1979) prior to placement in a vertical flow Heath incubator supplied with 5 gal/min of 15 C well water. Daily flush treatments with 1.1 % malachite green were administered to control fungus (Leitritz and Lewis 1980). Fry were placed in 711 L circular tanks and reared to 8 to 30 g on a commercial trout diet. Chinook salmon used in passive immunization tests were obtained from incubator trays prior to feeding, and transported to the laboratory. They were placed in circular tanks and reared to 12.4 g on a commercial trout diet.

Fish were held at the UCD Fish Pathology Laboratory, Davis, CA. The laboratory is supplied with pathogen-free well water and has equipment for water temperature regulation. Stock fish were held at 15 C  $\pm$  2 C. Experiments were conducted at either 15 or 18 C  $\pm$  2 C. Three tank designs were used, and are summarized in Table 6. Each tank was supplied with bubbled air. Fish were fed daily rations of a commercial trout diet.

#### Quantification of Virulence : LD<sub>50</sub> Methods

Aeromonas salmonicida cells were grown for 20 to 24 h in BHIB, pelleted by centrifugation at 5000 xg for 15 min at 4 C, then resuspended in cold sterile 0.85% saline. Aggregates of AP<sup>+</sup> cells were dispersed by shaking until no clumps were visible, then for an additional 5 min. Cell suspensions were then adjusted to OD<sub>520</sub> = 0.9 (Spectronic 20, Bausch and Lomb) by addition of sterile saline. Ten fold serial dilutions in 0.85% sterile saline were prepared, and viable cell counts were made by plating 100  $\mu$ l aliquots onto TSA-CR, TSA-CB, and BHIA plates by the drop plate method of Miles and Misra (1938).

Table 6. Fish tank designs at the UCD Fish Pathology Laboratory.

Tank Design	Dimensions	Capacity (L)	Waterflow (L/min)	Turnover (min)
Circular	48 in. diam. 24 in. deep	711	18.9	37.6
Rectangular	17 x 28 in. 16 in. deep	130	3.8	34.4
Circular	11 in. diam. 11 in. deep	15.5	0.5	34.0

Three methods were used to challenge fish: 1) fish were anaesthetized in 50 ppm tricaine methanesulfonate, MS-222 (Argent Chemical Laboratories) and injected i.m. anterior to the dorsal fin with 0.1 cc of graded doses of bacteria (Michel 1980), 2) bacterial dilutions were added directly to fish tanks for 20 min, after which water flow was resumed (McCarthy 1983), 3) sodium chloride was added to 0.9% (wt/vol) 10 min prior to the 20 min immersion challenge. Fish in some challenges were also stressed by a 0.1 cc i.p. injection of sterile saline 24 h prior to challenge, and/or a 3 C heat stress (water temperature increased from 15 to 18 C). Groups of 10 or 30 fish were used, and replicates were run when enough fish were available.

Fish were monitored for 10 to 14 d following challenge, and dead fish removed twice daily. Aseptic kidney samples were struck onto TSA-CR, TSA-CB or BHIA, and specific mortality determined by reisolation in pure culture of *A. salmonicida*. The LD<sub>50</sub> values were calculated by the method of Reed and Meunch (1938).

#### In Vivo Survival Tests

The ability of *A. salmonicida* strains AS-1R, AS-1S and 3.101-2 to survive within host tissues was investigated using the methods of Munn and Trust (1984). Groups of 20 chinook salmon fingerlings (8.68 + 1.96 g) were injected i.m. with approximately  $1.0 \times 10^4$  cfu/fish of each strain of *A. salmonicida*. At 24 h intervals two fish from each group were removed and euthanized in 100 ppm MS-222. Blood was collected after caudal severance into heparinized capillary tubes. Tissues (heart, liver, spleen and kidney) were removed, surface disinfected in

75 ppm iodophor for 2 to 3 min, and rinsed three times in sterile 0.85% saline. Tissues and blood were placed in sterile 50 ml polypropylene tubes, weighed or measured and diluted to a 10% suspension in sterile 0.85% saline. Tissue suspensions were homogenized (Brinkman Polytron) and 100  $\mu$ l seeded onto BHIA plates. Bacterial colonies were examined and identified by cultural characteristics and slide agglutination with specific rabbit antisera.

#### Electrophoresis in Polyacrylamide Gels

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for two purposes : analytically to test the purity of the bacterial antigens AP, LPS and F4, and preparatively to purify the AP from autoaggregating strains of *A. salmonicida*.

Analytical Gels. A discontinuous buffer system using 0.75 mm thick 10% acrylamide slab gels was used for SDS-PAGE (Laemmli 1970). Whole bacteria or bacterial products were solubilized in sample buffer, heated to 100 °C for 2 min, cooled on ice, and 50  $\mu$ l loaded into each lane of the gel. Molecular weight standards (Bio Rad Laboratories) were included in all gels. Electrophoresis was completed in about 5 h at 12 mA/gel and gels were fixed and stained in 0.125% Coomassie blue R-250, or in silver nitrate (Merrill et al. 1981).

Preparative Gels. Preparative slab gels were used to purify AP from an ammonium sulfate precipitate of alkaline hydrolysis (ALKOH) treated cells. Samples were adjusted to approximately 5.0 mg/ml in sample

buffer, heated to 60 C for 10 min, and proteins were separated by electrophoresis in 3.0 mm thick polyacrylamide gels at 48 mA/gel.

Gels were stained in 0.1% Coomassie blue R-250 in distilled water for 60 min then destained in distilled water for approximately 30 min or until the AP band was easily visualized. The AP band was excised with a clean scalpel and processed for AP recovery.

#### Purification Procedures

A-protein Purification. Cells from AP<sup>+</sup> strains AS-1R or 3.101-2 were washed 3x in cold sterile 0.85% saline and suspended to 10 g/L. A-protein was extracted by the ALKOH method of McCarthy et al. (1983). Briefly, the pH of a continuously stirred cell suspension was raised to 11.0 by addition of 5 M NaOH. After 30 min the pH was lowered to 7.0 by addition of 5 M HCl. The solution was centrifuged at 10,000 xg for 15 min (4 C) and supernatant decanted into a sterile 2.0 L flask. Ammonium sulfate (Fisher Scientific Co., purified grade) was slowly added to 80% SAS (saturated ammonium sulfate) and equilibration occurred over the next 4 to 12 h at 4 C, with slow stirring. This solution was centrifuged as before, and the pellet dissolved in 50 ml of cold, sterile 15mM Tris-HCl buffer, pH = 8.0 (Tris buffer). It was then dialyzed against 6.0 L Tris buffer at 4 C. The resulting contents of the dialysis tubing were 1) an upper clear solution and 2) a lower insoluble flocculated material. The clear solution was recovered and sterile Tris buffer was added to a final volume of 100 ml. Further protein fractionation was done at 0 to 35%, 35 to 50% and 50 to 80% SAS intervals. Conditions were as above except equilibration occurred over

a shorter interval (2 to 4 h) and centrifugation was at 20,000 xg for 15 min (4 C).

After dialysis, the protein concentration of each fraction was determined (Bradford 1976), and samples were adjusted to 10 mg/ml for analysis by SDS-PAGE. Prior to electrophoresis sample concentration using PEG (polyethylene glycol) 20,000 was sometimes required (Cooper 1977). Protein samples were prepared in sample buffer to final concentrations of 1.25 to 5.00 mg/ml, heated to 60 C for 10 min, and separated by electrophoresis as previously described. Results of analytical gels indicated that the majority of AP precipitated at 35 to 50% SAS, therefore this fraction was used for AP purification in preparative gels.

A-protein was recovered from polyacrylamide gels following the procedures of Evenberg and Lugtenberg (1982). Briefly, gel slices containing AP were homogenized in 25 ml of elution buffer (25 mM Tris-HCl pH = 6.8, 0.2% SDS, 5.0% glycerol, and 0.1% 2-mercaptoethanol) and eluted at room temperature at 150 rpm for 4 h. The solution was centrifuged at 1000 xg and the supernatant was removed and saved. An additional 25 ml elution buffer was added to the remaining gel particles for further extraction. This procedure was repeated for a total of three extractions. Supernatants were pooled and centrifuged at 10,000 xg for 15 min (4 C) to remove residual gel particles, then dialyzed in three changes of 5 mM ammonium bicarbonate, 0.025% SDS, pH = 7.7, at 4 C. The dialysate was lyophilized, then resuspended in approximately 2 ml of 10 mM ammonium bicarbonate, 2.0% SDS, pH = 7.7.

Both SDS and Coomassie blue were removed from AP by the method of

Henderson et al. (1979). Briefly, twenty volumes of cold (-20 C) ATA (90 vol acetone: 5 vol triethylamine: 5 vol acetic acid) were added, and after 1 to 2 h at -20 C this mixture was centrifuged at 10,000 xg for 30 min (-20 C). The pellet was rinsed twice in cold ATA, twice in cold acetone, then suspended in approximately 0.5 ml of cold (4 C) sterile double-distilled water. A-protein was lyophilized, then dissolved in cold (4 C) sterile 15 mM Tris buffer, pH = 6.8. Protein concentrations of these samples were determined (Bradford 1976), and its purity analyzed by SDS-PAGE.

Lipopolysaccharide Purification. Lipopolysaccharide was purified from *A. salmonicida* strains AS-1R, AS-1S, 3.101-2 and 3.95 by the method of Westphal and Jann (1965), with only one modification. Lyophilized cells (2.0 g) were washed three times in 3 vol cold (-20 C) acetone, or until supernatant was clear (Sutherland 1978) prior to LPS extraction. The remainder of the LPS extraction procedure is fully described elsewhere (Westphal and Jann 1965), and only briefly mentioned here.

Acetone washed bacteria were suspended in a mixture of hot (65 to 70 C) phenol and water for 10 to 15 min. After cooling, slow centrifugation (3000 xg) resulted in an upper aqueous phase containing LPS, LPS fragments, and nucleic acids, a lower organic phase containing hydrophobic materials, and an interphase containing insoluble materials. The aqueous phase was recovered and LPS further purified by differential centrifugation and selective precipitation for separation from LPS fragments and nucleic acids. The purified LPS was lyophilized, weighed, and reconstituted in sterile double distilled

water to 5 mg/ml (wt/vol), and analyzed for purity by SDS-PAGE.

Fraction 4 Purification. Fraction 4 was purified from extracellular products (ECP) as described by Cipriano et al. (1981), Cipriano (1982c), and Cipriano et al. (1983). Aeromonas salmonicida strains AS-1R, AS-1S, 3.101-2 and 3.95 were grown in BHIB for 96 to 120 h. After centrifugation at 4000 xg for 30 min at 4 °C (the conditions for all F4 centrifugations) the spent culture media (supernatant) was decanted into a sterile 4.0 L flask, and slowly brought to 80% SAS by addition of crystalline ammonium sulfate. This mixture was slowly stirred at 4 °C for 4 to 16 h, centrifuged, and the resulting pellet dissolved in cold sterile double distilled water and dialyzed in three changes (12.0 L) of phosphate buffer (0.01 M K<sub>2</sub>HPO<sub>4</sub>, pH = 8.0) over 18 to 24 h at 4 °C. The retentate was saved and sodium acetate was added to 5.0% (wt/vol), followed by the addition of 5 vol cold 95% ethanol. After 30 to 60 min the mixture was centrifuged. the pellet dissolved in cold sterile double distilled water, and dialyzed in phosphate buffer as before. The retentate volume was adjusted to 30 ml with sterile phosphate buffer, then stored at -20 °C for later fractionation by anion exchange chromatography.

Chromatography was performed at 4 °C in a 2 x 40 cm column (C16/40, Pharmacia, Inc.) packed with 20 g DEAE Sephadex A-25. Media was precycled as recommended by Cooper (1977), then equilibrated in phosphate buffer. Equilibration was reached when both pH and conductivity of column effluent equalled that of the phosphate buffer. Column samples (20 ml) were applied and eluted by a four step

discontinuous NaCl gradient. Fraction 1 eluted with phosphate buffer, F2 with 0.3 M NaCl, F3 with 0.6 M NaCl, and F4 with 1.2 M NaCl. Approximately 300 ml of each eluting solution was used, with samples being collected for 2 to 2.5 h. Column flow rate was approximately 3.0 ml/min.

Elution profiles were recorded with an ISCO Type 6 spectrophotometer with a 280/310 nm filter and an ISCO UA-5 absorbance monitor (Instrument Specialty Company). Eluted samples were collected into clean tubes (6 ml/tube) using an ISCO fraction collector. Tubes corresponding to sample peaks were pooled. Fraction 4 was concentrated from approximately 100 ml to 10 ml by ultrafiltration at 4 C through a PM-10 membrane (Amicon, Corp.) at 50 psi. Samples were then dialyzed in phosphate buffer, assayed for protein content, and stored at -80 C. Purity was analyzed by SDS-PAGE.

#### Production of Antisera

Rabbits. Hyperimmune sera to formalin-killed (FK) strains of A. salmonicida and purified antigens from these bacteria were prepared in New Zealand white rabbits (weighing approximately 2.0 kg). All antisera were prepared in duplicate animals and analyzed separately to minimize the possibility of random poor immune performance. The injection schedule for antiserum production was as follows :

Day 1 : Preimmune blood collected. Injection of 1.0 ml antigen mixed in 1.0 ml Freund's complete adjuvant, FCA (Difco Laboratories) subcutaneously (s.c.) into four

dorsal sites.

Day 28 : Booster injection 1, 1/4 of the original dose of antigen was given by s.c. injection, without adjuvant. Ten ml of blood was collected to monitor antibody response.

Day 35 : Booster injection 2, 1/2 of the original dose was injected s.c. without adjuvant.

Day 42 : Booster injection 3, an amount of antigen equal to the original dose was injected s.c. without adjuvant.

Day 49 : Ten ml of blood was collected to monitor the immune response.

Day 56 : Rabbits were euthanized and exsanguinated by cardiac puncture.

Rabbits immunized with purified antigens (AP, LPS and F4) received a total of 137.5 ug as follows : Day 1, 50 ug; day 28, 12.5 ug; day 35, 25 ug; day 42, 50 ug. Formalin-killed cells were optically adjusted to OD<sub>525</sub> = 1.0 (approximately  $1.5 \times 10^9$  cfu/ml) prior to use, so that each rabbit received an approximate total of  $4.1 \times 10^8$  cfu during the immunization period.

Two rabbits were immunized with live A. salmonicida strain 3.101-2. Cells were suspended in sterile 0.85% saline and adjusted so that each injection consisted of approximately  $1.0 \times 10^5$  cfu. No adjuvant was used, but otherwise the above injection schedule was followed. No antibody response could be demonstrated by microtiter agglutination

from sera collected on day 49, so rabbits were further injected with an additional  $2.8 \times 10^5$  cfu live bacteria mixed in Freund's incomplete adjuvant, FIA, (Difco Laboratories). Sera collected 28 d after this injection lacked detectable antibodies by either agglutination or counterimmunoelectrophoresis (CIE) immunoassay methods. Therefore, these antisera were discarded.

Blood Collection and Sera Processing. Rabbits were anaesthetized by s.c. injection of 0.35 cc sublimaze inapsine (Innovar-Vet, Pitman-Moore, Inc.). After 10 to 20 min blood was collected from the central ear artery into sterile 10 ml glass tubes using a 20 gauge, 1.5 in. needle. Clotting occurred during a 2 to 3 h incubation at room temperature (22 to 25 °C), and further clot retraction proceeded at 4 °C for 12 to 16 h. Clots were centrifuged at 500 xg for 5 min (4 °C) and sera collected into sterile tubes. When necessary, sera were further clarified by a second centrifugation. Sera were heated to 56 °C for 30 min to inactivate complement proteins, passed through a 0.45 µm membrane (Gelman Sciences Inc.) and then stored at -20 °C. Thimerosal (0.01%) was added to a small aliquot of antisera used for immunoassays which were stored at 4 °C.

Fish. Rainbow trout, Oncorhynchus mykiss, averaging 500 g were injected with bacterial antigens mixed in FCA following the same schedule and doses as that used for rabbits. Initially 2.0 ml (equal parts of antigen and FCA) was injected i.p. followed by a series of three i.m. booster injections without adjuvant. Fish were

anaesthetized with 50 ppm MS-222 prior to handling for injection or bleeding. Each fish received a total of 137.5 ug of purified antigen. Formalin-killed cells were optically calibrated and injected as follows :

	<u>Amount</u>	<u>Route</u>	<u>Dose</u>
Day 1	2.0 ml	i.p.	$1.50 \times 10^9$ cfu
Day 28,35,42	0.25 ml	i.m.	$3.75 \times 10^8$ cfu

To produce antisera against undenatured bacterial antigens, live bacteria were used to subclinically infect fish. The injection schedule for avirulent live strains of *A. salmonicida* was :

	<u>Amount</u>	<u>Route</u>	<u>Dose</u>
Day 1,28,35,42	0.1 cc	i.m.	$1.0 \times 10^5$ cfu
Day 58	1.0 cc	i.p.	$6.7 \times 10^5$ cfu

The first four injections were live cells suspended in 0.85% sterile saline. Blood collected on day 49 lacked detectable agglutinating antibodies, therefore, fish were further injected with live cells in FIA on day 58. Blood was first collected 28 d after this injection, and weekly or bimonthly thereafter, until a sufficient amount of antisera had been collected.

The same time schedule was used to produce antisera against live virulent strain AS-1R, but the dosages were lower. The first four injections consisted of 50 cfu/fish, and the last injection of bacteria

in FIA consisted of  $2.3 \times 10^5$  cfu/fish.

Four to eight fish per treatment were used to produce antisera to the various bacterial antigens. Antisera from each group were pooled prior to passive immunity testing and immunoassays.

Collection and Processing of Fish Blood. Due to the smaller size of fish compared to rabbits, it was necessary to collect blood at weekly intervals to accumulate sufficient antisera for tests. Approximately 3.0 ml of blood was collected by caudal venipuncture using 20 gauge needles. Blood was allowed to clot at room temperature for 1 to 2 h, and sera was then harvested as described for rabbits. Sera collected from individual fish was pooled, heated to 45 C for 30 min, then stored at -20 C. A small aliquot for immunoassays was stored at 4 C with 0.01% thimerosal.

#### Serum Absorption

For immunoassays and passive immunization tests selected antisera were absorbed with FK or live bacterial cells. The procedure of McCarthy and Johnson (1982) was used with some modifications. Antibodies directed against cell wall components were removed by absorbing antisera with boiled *A. salmonicida* strain AS-1R or 3.101-2, as described by McCarthy et al. (1983). Bacteria were grown as previously described, washed twice in sterile 5 mM 3-(N-morpholino) proanesulfonic acid, (MOPS) buffer, (Sigma Chemical Company), pH = 7.0, and resuspended to the original culture volume in MOPS buffer. This suspension was stirred and boiled for 15 min, centrifuged once at

10,000 xg for 15 min (4 C), and boiled cells washed once in cold 0.85% saline. Cells were collected by centrifugation at and analyzed for the presence of AP by SDS-PAGE. Antisera was absorbed by mixing 1 part cells with 2 parts sera, incubating 1 h at 20 C (fish sera) or 37 C (rabbit sera) with gentle mixing, then continued incubation for 1 h at 4 C without mixing. Cells were removed by centrifugation at 10,000 xg for 15 min at 4 C. This process was repeated twice, with the final centrifugation at 20,000 xg, due to increasing softness of the bacterial pellet. McCarthy et al. (1983) reported that resulting antisera lacked antibodies directed against LPS O-antigens and other outer membrane antigens, while retaining antibodies against an additional antigen present on AP<sup>+</sup> strains (presumably anti-AP antibodies).

Antisera were also absorbed with FK or live *A. salmonicida* strain AS-1S or 3.101-3 (AP<sup>-</sup> strains) by the same method, but without boiling and using 0.85% sterile saline rather than MOPS buffer for washing cells. Selected antisera were absorbed with live *A. salmonicida* strain AS-1R or 3.101-2 to demonstrate removal of antibodies directed against AP.

#### Agglutination Titration

The level of agglutinating antibody in sera to *A. salmonicida* strain AS-1S was determined by the method of Paterson and Fryer (1974b) with minor modifications. Initial dilutions of 1:10 were made in the first well of a 96 well V-bottom microtiter plate (Costar) by adding 10  $\mu$ l sera to 90  $\mu$ l of 0.85% sterile saline. Subsequent two-fold dilutions

were made by transferring 50  $\mu$ l of this solution into 50  $\mu$ l of saline in the next well, using a Titertek digital pipette (Flow Labs).

Following serum dilution, 50  $\mu$ l of FK A. salmonicida (washed three times in sterile saline) containing 0.01% thimerosal and adjusted to OD<sub>520</sub> = 0.9 was added to each well. Plates were sealed with plastic cover sheets, gently mixed, and incubated for 2 h at 37 C, followed by 12 to 20 h at 4 C. Plates were read using a test reading mirror. Test wells where the bacterial cell button was less than or equal to 1/2 the size of the button in negative control wells (cells only, no antisera) were scored positive. Serum agglutination titers were recorded as the reciprocal of the highest dilution with positive agglutination.

#### Immunodiffusion

Gel immunodiffusion analyses were conducted on 25 x 75 mm microscope slides. Slides were precoated with 1.6 ml (0.064 ml/cm<sup>2</sup>) of 0.5% agarose (Type 5, Sigma) containing 0.05% sodium azide, and dried at room temperature for 24 to 48 h. Agarose in barbital buffer (0.05 M Barbital buffer, Sigma Chemical Co.; 0.04% calcium lactate-5·H<sub>2</sub>O; 0.05% sodium azide; in distilled water, pH=8.6) was prepared as described by Anhalt et al. (1978). Agarose was dissolved by autoclaving 10 min, then cooled to 60 C and 3.5 ml/slides (0.19 ml/cm<sup>2</sup>) added to precoated slides. Fish antisera was examined in 0.5% agarose, while 0.75% agarose was used for rabbit antisera. Agarose was solidified 5 to 10 min at room temperature, then 15 min or longer at 4 C. Slides were stored at 4 C in a humid box and were used within 4 d.

Agarose was punched to form a hexagonal pattern of wells having

inner diameters of 2.5 mm, between well spacing of 3.0 mm, and a capacity of 10  $\mu$ l. Antigens and antisera (10  $\mu$ l) were loaded into wells and slides incubated in a sealed humid box at 20 C. Readings were taken at 24 and 48 h, then slides were either dried and stained (see below), or further incubated for up to 14 d at 4 C.

To eliminate precipitin lines caused by C-reactive protein (CRP), slides were soaked 30 min in 0.1 M EDTA (disodium salt) in 0.85% saline, pH = 8.0 (Baldo and Fletcher 1973). Slides were then washed 15 min in 0.85% saline and blotted dry with Whatman No. 1 filter paper for 10 min. This was repeated three times. Slides were finally soaked for 30 min in distilled water and dried at room temperature for 24 to 48 h. Dried slides were stained 10 min in 0.5% Coomassie blue R-250 (Bio Rad Laboratories) in a solution of 43% ethanol and 10% glacial acetic acid, destained by three 10 min rinses in the solution without dye, dried at room temperature, then read and/or photographed.

#### Counterimmunolectrophoresis (CIE)

Counterimmunolectrophoresis was used to examine antisera for the presence of precipitating antibodies to selected bacterial antigens. This method has been reported to be 10 to 100 times more sensitive than immunodiffusion, detecting as little as 0.1 ng of pneumococcal polysaccharide antigen (Anhalt et al. 1978). Other advantages include its ability to detect antigens in dilute solutions, and the rapidity of the procedure.

The method of Anhalt et al. (1978) was used without modification. Briefly, slides were prepared as for immunodiffusion using 0.75%

agarose. Agarose was punched to create paired wells spaced 3.0 mm apart and having inner diameters of 3.0 mm. Each set of paired wells was separated from adjacent pairs by 5.0 mm, with each microscope slide containing six pairs of wells for six individual tests. Anodal side wells were filled with 10  $\mu$ l of undiluted antisera, while cathodal side wells received 10  $\mu$ l of antigen. A constant voltage of 8 V/cm was applied for 1 h. Gels were then removed to a humid chamber at 4°C for 15 to 60 min and then observed for precipitin lines using oblique underlighting and a 10  $\times$  hand lens. A second reading was taken at 24 h, and in some cases at 7 d. Gels were then washed, stained, dried and photographed as described for immunodiffusion.

#### Antigens for Immunodiffusion and CIE

Antigens used in CIE tests were suspended in distilled water, dilute buffer, or 0.85% saline. When these suspensions were tested by immunodiffusion, precipitin lines were often either lacking or difficult to interpret due to poor diffusion of the antigen (especially LPS) into the gel matrix. Consequently the method of Hofstra and Dankert (1980), using detergent to counteract the hydrophobicity of certain proteins and LPS, was used. Antigens for immunodiffusion were suspended in phosphate-buffered saline, PBS pH = 7.2, containing 0.2% SDS and 0.05% sodium azide, unless otherwise indicated. The following antigen preparations were used in immunodiffusion and CIE tests :

1. Washed, sonicated bacterial cells (SC). Cells were suspended to OD<sub>520</sub> = 0.25.

2. Boiled cell extract (BE). Washed cells of AP<sup>+</sup> strains AS-1R or 3.101-2 were boiled for 15 min. After centrifugation the supernatant was lyophilized, weighed and resuspended in distilled water or PBS with 0.2% SDS.

3. A-protein enriched fraction (APEF). This consisted of the 35 to 50% SAS fraction from which purified AP was derived. This solution contained many bacterial proteins, LPS, and a disproportionately large amount of AP, as determined by SDS-PAGE analysis.

4. Purified AP.

5. Purified LPS.

6. Purified F4.

Each antigen was tested at multiple concentrations to minimize the possibility of missing precipitating antibodies due to antibody or antigen excess reactions.

Recovery of Agglutinating Antibodies from Passively  
Immunized Fish

The presence of rabbit and rainbow trout anti-*A. salmonicida* antibodies in the blood of passively immunized coho (8.4g) and chinook (6.3g) salmon fingerlings was determined over a 14 to 21 d period by microagglutination titration. Fish held at 15 C (coho) or 18 C (chinook) received i.p. injections of 0.1 cc antisera. Those injected with rabbit antisera received 128 to 256 agglutinating units (Udey 1977 defined an agglutinating unit as the reciprocal agglutination titer

multiplied by the sample volume), while fish injected with trout antisera received 4096 agglutinating units. At timed intervals after injection, five fish from each treatment were removed, anaesthetized, and blood collected from the caudal vein into microhematocrit capillary tubes, 60  $\mu$ l blood/fish. After 1 to 2 h at room temperature, the blood was centrifuged for 5 min in a microhematocrit centrifuge, and sera collected, pooled and stored at -20 C. Sera were later thawed, heated to 45 C for 30 min, and titrated for the presence of agglutinating antibodies. All tests were done in replicate. Antibody longevity was measured in terms of half life, calculated by linear regression.

#### Passive Immunization Test 1

Nine month old coho salmon (8.4 g) were anaesthetized (50 ppm MS-222), injected i.p. with 0.1 cc rabbit antisera prepared against different FK A. salmonicida strains or their purified antigens, or rabbit antisera against AP<sup>+</sup> FK cells absorbed with the AP<sup>-</sup> mutant strain of A. salmonicida, and held at 15 C in aerated 15.5 L tanks with approximately 0.5 L/min water flow. Control fish were injected with rabbit preimmune sera or 0.85% sterile saline. Tests were run in replicate with 30 fish per group (Amend 1981).

After initial immersion challenge (as determined by LD<sub>50</sub> tests) proved inconsistent, fish were challenged by i.m. injection. The LD<sub>50</sub> tests indicated that fish injected i.p. with 0.1 cc sterile saline and subsequently challenged by i.m. injection of 0.1 cc A. salmonicida strain AS-1R had an LD<sub>50</sub> of 8.5 cfu/fish. Passively immunized coho salmon were anaesthetized and injected i.m. with 8 cfu/fish 4 d after

receiving antisera injections. Mortalities were monitored for 28 d, with data analyzed at the end of 10 and 28 d. Specific mortality (that due to A. salmonicida) was confirmed by reisolation of A. salmonicida from kidneys of dead fish. Other deaths were recorded as non-specific mortality. Identification of the isolates obtained was determined by cultural characteristics, pigment production, oxidase reaction, and agglutination by specific antisera.

Results were evaluated using the criteria outlined by Amend (1981), however, due to limited space and number of fish, challenges were conducted at only one level rather than two as recommended by Amend (1981). Briefly, he recommended conducting tests in duplicate with 25 to 30 fish per treatment, with all dead fish being necropsied. Deaths of undetermined nature and of non-specific causes are excluded from the test, however, non-specific mortality should not exceed 10%. Reproducibility between replicate groups is measured by percent variance between specific mortality, and should not exceed 20%. Pooling of data between replicates is allowable if either of the above two situations occurs. Relative percent survival (RPS) values greater than or equal to 60 have been correlated with efficacious vaccines (Amend 1981).

After 28 d, selected fish groups having statistically significant protection from mortality caused by A. salmonicida were further tested for the presence of latent infections. Tests were conducted following injection of one replicate group with 1.0 mg/fish Kenalog 40 (E. R. Squibb and Sons, Inc.). The second replicate group was not injected and served as a control over a 14 d observation period. Specific

mortality was determined by reisolation of A. salmonicida. After 49 d, the second replicate was also injected with Kenalog 40 to determine the carrier status of these fish.

#### Passive Immunization Test 2

Three and one half month old chinook salmon (12.4 g) were passively immunized by: i.p. injection with antisera prepared in rabbits and rainbow trout against FK cells, live cells, purified antigens of A. salmonicida; FCA; and antisera against AP<sup>+</sup> FK cells absorbed with the AP<sup>-</sup> mutant of A. salmonicida. Test conditions were as previously described, except that water temperature was 18 C. Controls consisted of fish injected with preimmune sera from trout and rabbits, or 0.85% sterile saline.

In this test, passively immunized fish were challenged, after 24 h, by immersion (for 20 min) in static tank water to which NaCl (0.90% wt/vol) and A. salmonicida strain AS-1R ( $2.45 \times 10^5$  cfu/ml) was added. After 20 min, water flow was resumed. Two groups of saline injected fish were non-challenged controls. Mortalities were monitored for 14 d and specific mortality was confirmed by reisolation of A. salmonicida from kidneys of dead fish.

After 14 d, those groups exhibiting statistically significant (chi square analysis using a 2 x 2 contingency table; Sokal and Rohlf, 1969) protection from mortality by A. salmonicida were injected with Kenalog 40 to determine the infection level of survivors. Mortalities were monitored for an additional 14 d, with all dead fish being necropsied for cause of death. Unchallenged control fish, injected with Kenalog

40, served as controls.

Detection of Carriers among Passively Immunized Fish.

Passively immunized fish surviving challenge were injected with an anti-inflammatory drug, triamcinolone acetonide, (Kenalog 40, Squibb) to determine if *A. salmonicida* was present as a latent disease (Bullock and Stuckey 1975). Anaesthetized fish were injected i.m. with 1.0 mg (0.1 ml) Kenalog 40. In passive immunization 1 an additional stressor was added by increasing water temperature 5 C, from 15 C to 20 C; in passive immunization test 2 no temperature stress was applied, and water temperature was maintained at 18 C. Fish were monitored for 14 d and specific mortality was determined by recovery of *A. salmonicida* from kidneys of dead fish.

## Results

### Virulence Testing

Strain AS-1R was highly virulent to Mad River juvenile chinook salmon by i.m. injection, having an LD<sub>50</sub> value of 8.5 cfu/fish. Strains AS-1S, 3.101-2 and 3.95 were considered avirulent by i.m. injection, having LD<sub>50</sub> values of  $1.7 \times 10^6$ ,  $1.1 \times 10^7$  and  $1.7 \times 10^8$  cfu/fish, respectively.

Moccasin Creek juvenile chinook salmon immersed in up to  $3.5 \times 10^8$  cfu/ml of strain 3.101-2 experienced no mortality. In contrast, fish immersed in strain AS-1R had an LD<sub>50</sub> of  $2.6 \times 10^2$  cfu/ml. Fish injected i.m. with  $5.25 \times 10^8$  cfu of strain 3.101-2 experienced 65% mortality compared to 90% mortality in fish receiving only 42 cfu of strain AS-1R. Virulence of strain AS-1R was determined in salmon from three sources by three different challenge methods, as summarized in Table 7.

### In Vivo Survival Tests

The results of in vivo survival of *A. salmonicida* strains AS-1R, AS-1S and 3.101-2 in chinook salmon demonstrate that only strain AS-1R is virulent (Table 8). Three groups of 20 fish were injected with either 0.9, 2.1, and  $1.4 \times 10^4$  cfu/fish of strain AS-1R, AS-1S and 3.101-2, respectively. All AS-1R injected fish died within 96 h, however, none of the AS-1S or 3.101-2 injected fish died during the 15 d study.

*Aeromonas salmonicida* was recovered from tissues of fish injected with strains AS-1S and 3.101-2, but only at the first sample period.

*Aeromonas salmonicida* was recovered from tissues of fish injected with

Table 7. Virulence of Aeromonas salmonicida strain AS-1R by injection, immersion, and immersion with 0.9% salt in juvenile salmon.<sup>1,2</sup>

Species	Source	Challenge Method	LD <sub>50</sub>
Chinook	MR	injection	8.5 cfu/fish
		immersion	NT
		immersion/salt	NT
Chinook	MC	injection	<42 cfu/fish
		immersion	$2.6 \times 10^2$ cfu/fish
		immersion/salt	NT
Chinook	IG	injection	32.4 cfu/fish
		immersion	$2.9 \times 10^6$ cfu/ml
		immersion/salt	$2.4 \times 10^5$ cfu/ml
Coho	IG	injection	9.9 cfu/fish
		immersion	$1.5 \times 10^5$ cfu/ml
		immersion/salt	NT

<sup>1</sup> Abbreviations are : Mad River hatchery (MR); Moccassin Creek hatchery (MC); Iron Gate hatchery (IG); not tested (NT).

<sup>2</sup> Water temperature for all tests was 15 C. Mad River chinook salmon were 12 mo old (25.4 g), and received no pre-challenge stressors. Moccassin Creek chinook salmon were 5 mo old (8.7 g), and were tested as above. Iron Gate chinook salmon were 3 mo old (10.8 g) when tested. Fish received i.p. injections of sterile saline 24 h prior to challenge, and water temperature was raised 3 C (from 15 to 18 C) at the time of challenge. Iron Gate coho salmon were 6 mo old (8.4 g) when challenged. Fish received i.p. saline injections 24 h prior to challenge, however, water temperature was not changed.

**Table 8.** Survival of *Aeromonas salmonicida* strains AS-1R, AS-1S and 3.101-2 in tissues of juvenile chinook salmon following intramuscular injections of the bacteria.<sup>1</sup>

Tissue	Strain	AS-1R			AS-1S			3.101-2		
		24	48	72	96	120	24	48	72	96
Blood	-	+	+	+	NA	-	-	-	-	-
Heart	-	-	+	+	NA	-	-	-	-	-
Liver	-	-	+	+	NA	-	-	+	-	-
Spleen	+	+	+	+	NA	-	-	-	-	-
Kidney	+	+	+	+	NA	+	-	+	-	-

<sup>1</sup> *A. salmonicida* was isolated from fish tissues. (+): not isolated, (-): or data not available (NA) because fish had died from infection. Amounts of bacteria injected were 0.9, 2.1 and  $1.4 \times 10^4$  cfu/fish for *A. salmonicida* strains AS-1R, AS-1S and 3.101-2, respectively.

strain AS-1R at all sample periods.

#### Antigen Purification

A-protein. A-protein was extracted by ALKOH from AP<sup>+</sup> A. salmonicida strains AS-1R and 3.101-2. The majority of AP precipitated at the 35 to 50% SAS fraction (Figure 1). The protein was further purified in preparative gels. An average of 12.2 mg of protein from the 35 to 50% SAS fraction was loaded onto each preparative gel, and 632 ug of purified AP was recovered, for an average yield of 5.2%. The majority of AP banded at approximately 49.5 kdal ( $49,463 \pm 523$  daltons) in silver nitrate and Coomassie blue stained 10% polyacrylamide gels (Figure 2). Some samples had minor additional bands slightly above and/or below the major AP band seen at 49.5 kdal (Figure 2). A-prtein from strain AS-1R and 3.101-2 appeared identical in gels of whole cells (Figure 3), SAS fractions (Figure 1), and after further purification (Figure 2). A-protein was observed only in autoaggregating A. salmonicida strains, AS-1R and 3.101-2 (Figure 3).

Lipopolysaccharide. Lipopolysaccharide was purified from lyophilized acetone-washed cells of A. salmonicida strains AS-1R, AS-1S, 3.101-2 and 3.95. An average of 49.2 mg of purified LPS was obtained from 2.0 g of lyophilized cells, for an average yield of 2.5%. Purified LPS from each strain appeared identical in silver stained 10% polyacrylamide gels (Figure 4). The O-polysaccharide chains were observed as distinct multiple bands in the 54.1 to 59.3 kdal region, and as a broad single band at 14.0 kdal (compared to protein molecular

Figure 1. Bacterial cell products from *Aeromonas salmonicida* strains AS-1R (A) and 3.101-2 (B) were extracted by alkaline hydrolysis, precipitated in 80% saturated ammonium sulfate (SAS), redissolved in distilled water, then re-precipitated at 0-35, 35-50, and 50-80 SAS intervals. These samples were separated by electrophoresis in 10% polyacrylamide gels at 24 mA for 6 h 15 min. Samples and loading for strain AS-1R (A) were : lane 2, 0-80 SAS, 0.22 mg; lane 3, 0-35 SAS (insoluble portion), 0.14 mg; lane 4, 0-35 SAS (soluble portion), 0.28 mg; lane 5, 35-50 SAS, 0.28 mg; lane 6, 50-80 SAS, 0.27 mg; and for strain 3.101-2 (B) were : lane 7, 0-80 SAS, 0.15 mg; lane 8, 0-35 SAS (insoluble portion), 0.15 mg; lane 9, 0-35 SAS (soluble portion), 0.28 mg; lane 10, 35-50 SAS, 0.26 mg; lane 11, 50-80 SAS, 0.27 mg; A-protein existed as a broad band (arrow) in the 35-50 SAS interval. Molecular weight ( $\times 1000$ ) on left is based on protein standards (lane 1).

Figure 2. A-protein (AP) extracted from preparative gels was analyzed following electrophoresis in 10% polyacrylamide gels for 6 h at 15 mA, and staining with Coomassie blue. Lane 1, washed cells of strain AS-1R; lane 3, AP, AS-1R; lane 5, AP, 3.101-2; lanes 2, 4 and 6, sample buffer. Molecular weight ( $\times 1000$ ) on right is based on protein standards (lane 7).

1

A

B

94  
68  
43  
30  
14.3

1 2 3 4 5 6 7 8 9 10 11



2

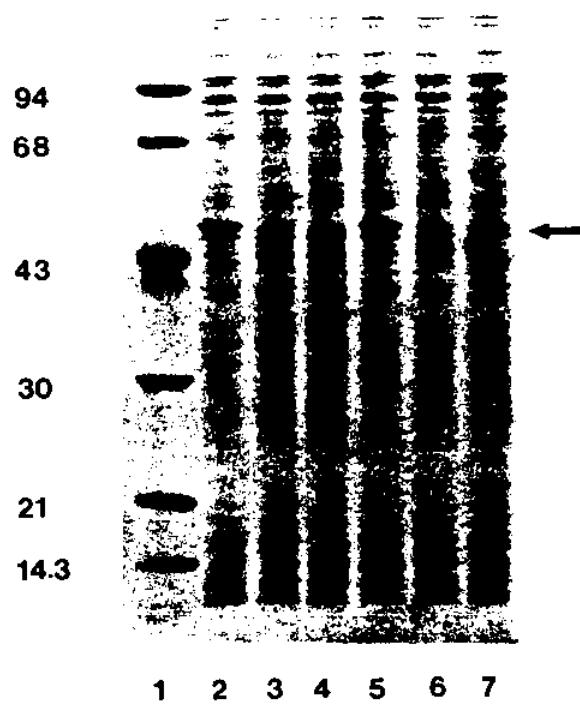
200  
130  
94  
68  
43  
30  
14.3

1 2 3 4 5 6 7

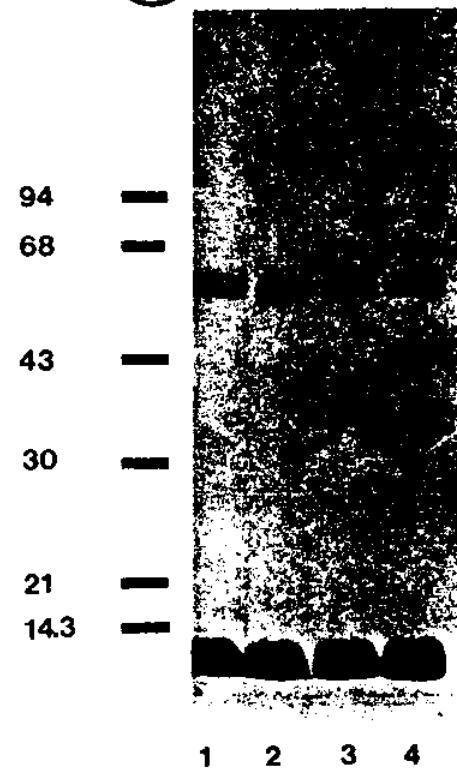
Figure 3. Polypeptides from six strains of Aeromonas salmonicida were examined for the presence of A-protein (AP). Cells were washed three times in sterile 0.85% saline, pelleted by centrifugation (10,000 xg, 15 min, 4 C), and resuspended to 0.625% (wet weight/vol) in sample buffer. After electrophoresis, the proteins were stained with Coomassie blue R-250. Lane 2, AS-1R; lane 3, AS-1S; lane 4, 3.101-1; lane 5, 3.101-2; lane 6, 3.101-3; lane 7, 3.95. Arrow indicates the level of AP, lanes 2 and 5. Molecular weight (x 1000) on left is based on protein standards (lane 1).

Figure 4. Purified lipopolysaccharide (LPS) from four strains of Aeromonas salmonicida was analyzed in a 10% polyacrylamide gel electrophoresed for 16 h at 40 V and stained with silver. Each well contained 1.0 ug LPS derived from A. salmonicida strain AS-1R (lane 1), AS-1S (lane 2), 3.101-2 (lane 3) or 3.95 (lane 4). Molecular weight (x 1000) indicated by horizontal bars is based on protein standards.

(3)



(4)



weight standards) by SDS-PAGE.

Fraction 4. Extracellular products from each of four *A. salmonicida* strains fractionated by anion exchange chromatography had similar UV absorption profiles (Figure 5), consisting of five distinct absorption peaks. The total amount of protein collected in each fraction varied widely between the four *A. salmonicida* strains (Table 9), with strain AS-1S producing more F4 than the other strains. Lipopolysaccharide and numerous proteins were seen by SDS-PAGE in Fractions 1, 2 and 3 (data not shown).

Fraction 1 eluted in 0.1 M K<sub>2</sub>HPO<sub>4</sub>, pH = 8.0, as a single peak. It was visually opalescent. Large amounts of LPS (55 to 60 kdal) and approximately 11 protein bands between 10 and 55 kdal were observed in silver-stained polyacrylamide gels. Average protein yield was 6390 ug.

Fraction 2 eluted in 0.3 M NaCl as a bimodal peak. Lipopolysaccharide and six proteins (10 to 45 kdal) were seen in silver-stained polyacrylamide gels. The proteins were not unique to this fraction, being present also in F1. Average protein yields were 8514 and 4768 ug for the first and second peaks, respectively.

Fraction 3 eluted in 0.6 M NaCl as a single peak, was brown in color, and contained LPS and six to eight proteins. Average protein yield was 6255 ug.

Fraction 4 eluted in 1.2 M NaCl as a single peak. Average protein concentration at peak UV absorbance was 66 (50 to 75) ug/ml, yielding a total protein average of 1477 ug of F4 per column sample (Table 9). The major component of F4 was LPS, as determined by comparison of F4 to

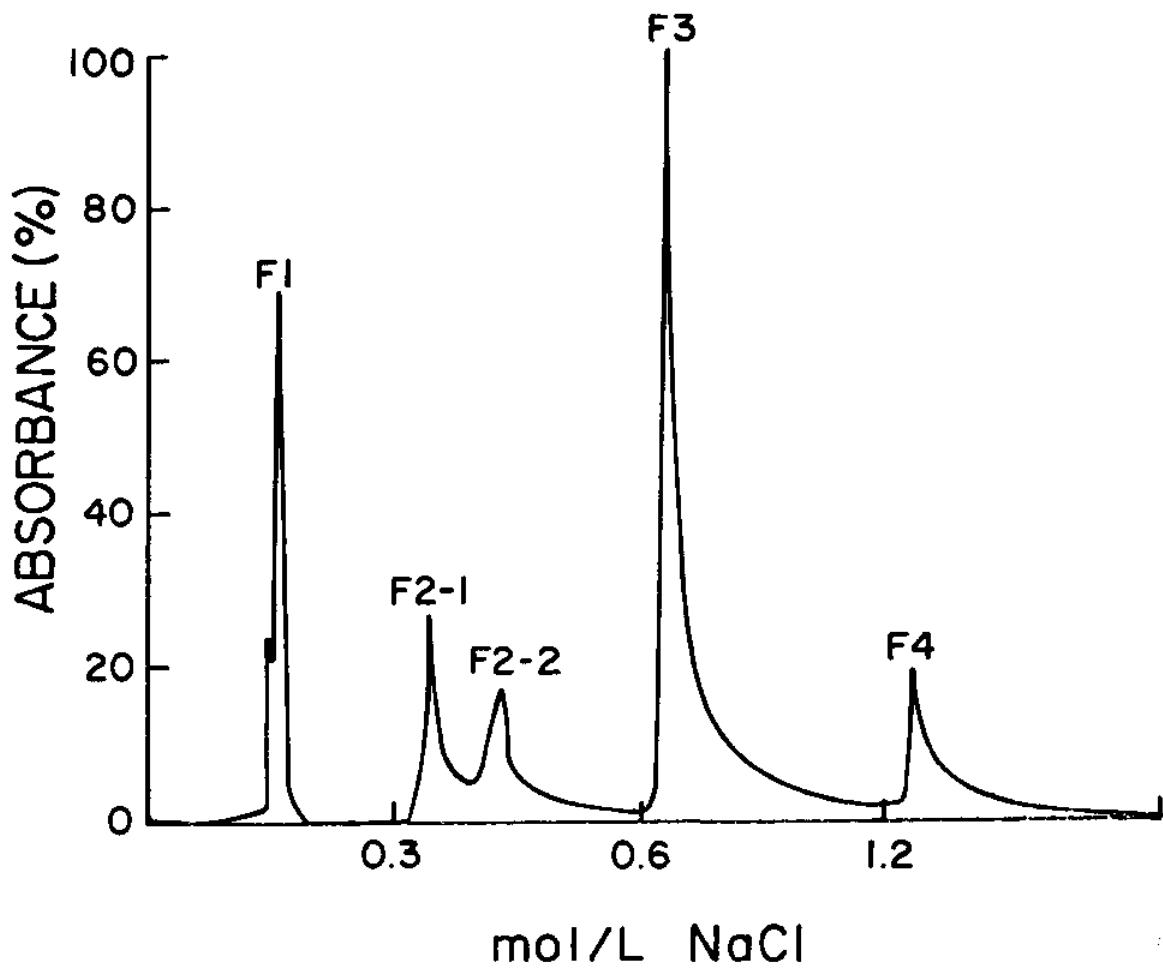


Figure 5. Elution profile of ammonium sulfate concentrated extracellular products from a 96 h broth culture of Aeromonas salmonicida strain 3.101-2. Fractions eluted from DEAE sephadex A-25 in a discontinuous NaCl gradient from 0.0 to 1.2 mol/L. Ultraviolet absorbance was measured with a 280/310 nm filter.

Table 9. Total protein yields of extracellular product fractions from four *Aeromonas salmonicida* strains following anion exchange chromatography.<sup>1</sup>

Column Fraction	Strain				
	AS-1R	AS-1S	3.101-2	3.95	AVE
F1	5487	8460	5282	6330	6390
F2-1	8496	10225	5615	3719	8514
F2-2	5782	8580	1986	2725	4768
F3	7021	7980	4661	5356	6255
F4	1692	2084	775	1238	1447

<sup>1</sup> Protein yield was recorded in ug. Average, (AVE).

purified LPS in 10% polyacrylamide gels (Figure 6) and later in serological tests. Fraction 4 of *A. salmonicida* strains AS-1R, AS-1S, 3.101-2 and 3.95 appeared identical in polyacrylamide gels (Figure 6), consisting primarily of LPS and a low molecular weight (18.6 kdal) protein.

#### Comparison of Purified Antigens

Purified LPS, F4 and AP were compared in silver-stained 10% polyacrylamide gels (Figure 7), and by immunodiffusion (see antisera characterization). Fraction 4 existed as: 1) four to five distinct golden-brown bands in the 54.1 to 59.3 kdal molecular weight range, and 2) a wide dark staining band of 14.0 kdal. These bands corresponded exactly with those seen in purified LPS. Additional bands present in F4 but not in LPS were an 18.6 kdal protein, and several lightly staining bands of low molecular weight (Figure 6). A-protein was distinct from either F4 or LPS, banding at 49.5 kdal (Figure 7). A-protein multimers of approximately 100 kdal were sometimes observed.

#### Antisera Characterization

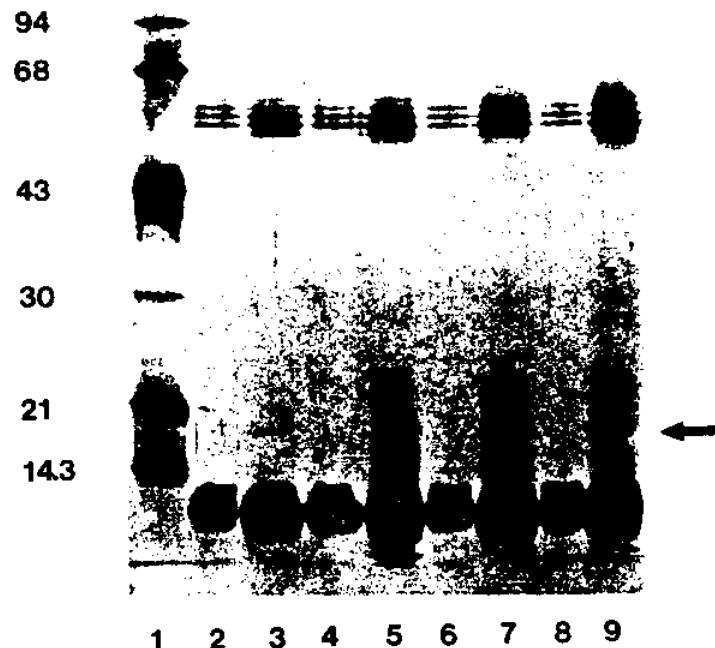
Rabbits. All rabbits immunized with FK *A. salmonicida* cells or F4 mixed in FCA had high agglutination titers (320 to 5120), however, rabbits immunized with live bacteria in FIA or purified AP or LPS had low (0 to 80) agglutination titers (Table 10).

Precipitating antibodies against six *A. salmonicida* antigen solutions were detected by CIE (Table 10). Purified antigens (AP, LPS and F4) always developed a single precipitin line with antisera, in

Figure 6. Lipopolysaccharide (LPS) and Fraction 4 (F4) antigens from four strains of Aeromonas salmonicida were electrophoresed and compared in 10% polyacrylamide gels. Samples and amounts are: lane 2, LPS, AS-1R, 0.50 ug; lane 3, F4, AS-1R, 1.25 ug; lane 4, LPS, AS-1S, 0.50 ug; lane 5, F4, AS-1S, 2.00 ug; lane 6, LPS, 3.101-2, 0.50 ug; lane 7, F4, 3.101-2, 1.25 ug; lane 8, LPS, 3.95, 0.75 ug; lane 9, F4, 3.95, 1.25 ug. Samples were electrophoresed for 4 h 10 min at 15 mA and then stained with silver. Some antigens were present in F4 (arrow) but absent from LPS. Molecular weight ( $\times 1000$ ) on left is based on protein standards (lane 1).

Figure 7. A-protein (AP), Fraction 4 (F4) and Lipopolysaccharide (LPS) antigens from Aeromonas salmonicida strain 3.101-2 were compared within the same gel. Samples and loading are: lane 1, AP, 2.88 ug; lane 2, F4, 0.625 ug; lane 3, LPS, 1.00 ug. Samples were electrophoresed in a 3% stacking and 10% separating polyacrylamide gel for 6 h 15 min at 12 mA, and silver stained. Molecular weight ( $\times 1000$ ) on right is based on protein standards (lane 4).

(6)



(7)

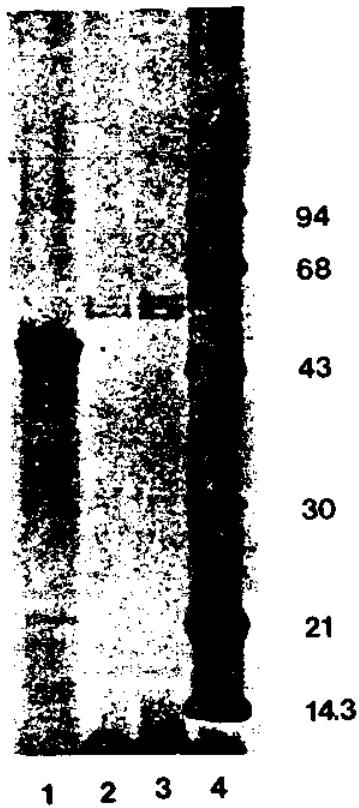


Table 10. Characterization of rabbit antisera prepared against whole cells and purified antigens of six strains of *Aeromonas salmonicida* by agglutination titers and counterimmuno-electrophoresis (CIE) precipitin reactions.

Antigen <sup>2</sup>	Rabbit Number	Titer <sup>3</sup>	CIE PRECIPITINS <sup>1</sup>					
			SC	BE	APEF	AP	LPS	F4
AS-1R:FK	1	5120	2	1	3	1	1	1
	2	640	0	1	2	1	1	1
AS-1S:FK	1	5120	1	1	1	0	1	1
	2	5120	3	1	3	NT	1	1
3.101-1:FK	1	640	1	1	1	0	1	1
	2	1280	2	2	1	NT	1	1
3.101-2:FK	1	2560	1	1	2	1	1	1
	2	5120	3	1	2	1	1	1
3.101-3:FK	1	320	1	1	1	0	1	1
	2	320	1	1	1	NT	1	1
3.95:FK	1	2560	2	1	1	0	1	1
	2	1280	2	1	2	NT	1	1
3.101-2:live	1	10	0	0	0	NT	0	0
	2	0	0	0	0	NT	0	0
AP:AS-1R	1	40	1	1	1	0	1	1
	2	40	0	1	1	NT	1	1
AP:3.101-2	1	30	0	2	2	0	1	1
	2	died						
LPS:AS-1R	1	10	0	0	0	0	0	0
	2	80	0	0	0	NT	0 <sup>4</sup>	0
F4:AS-1R	1	640	0	1	1	0	1	1
	2	1280	1	1	1	NT	1	1
F4:AS-1S	1	5120	1	1	1	0	1	1
	2	640	1	1	1	NT	1	1
F4:3.101-2	1	1280	1	1	1	0	1	1
	2	640	1	1	1	NT	1	1
F4:3.95	1	1280	1	1	1	0	1	1
	2	960	1	1	2	NT	1	1

Table 10 (cont.)

<sup>1</sup> Antigens and their optimal concentrations used for CIE were : SC, sonicated cells ( $OD_{520} = 0.25$ ); BE, boiled extract of *A. salmonicida* strain AS-1R or 3.101-2 cells, (150  $\mu g/ml$ ); APEF, A-protein enriched fraction from strains AS-1R or 3.101-2, (125  $\mu g/ml$ ); AP, A-protein, (125  $\mu g/ml$ ); LPS, lipopolysaccharide, (250  $\mu g/ml$ ); F4, fraction 4, (25  $\mu g/ml$ ). Precipitin lines recorded represent the number of lines observed in stained slides. Some antigens were not tested (NT) due to limited supplies.

<sup>2</sup> Antigens used to immunize rabbits were : live bacteria; formalin-killed (FK) whole cells; A-protein (AP); lipopolysaccharide (LPS); and fraction 4 (F4). Live *A. salmonicida* strain 3.101-2 was mixed in Freund's Incomplete Adjuvant while other antigens were mixed in Freund's Complete Adjuvant.

<sup>3</sup> Agglutination titer was recorded as the reciprocal of the highest serum dilution where the cell pellet was less than or equal to 1/2 the size of the pellet in the negative control well. Values recorded were the average of two readings.

<sup>4</sup> A precipitin line was observed against purified lipopolysaccharide by immunodiffusion.

contrast to multiple bands (up to three) with complex antigen mixtures (SC, BE and APEF). Antisera against purified LPS and live *A. salmonicida* strain 3.101-2 lacked precipitating antibodies by CIE, however, antibodies were detected by immunodiffusion in one of the rabbits immunized with LPS. All other antisera contained antibodies against both LPS and F4.

Antibodies against AP were detected by two methods 1) by precipitation with purified AP after CIE (Figure 8A), and 2) by precipitation of absorbed antisera with APEF or purified AP after CIE (Figures 8B and 8E). Rabbits immunized with FK AP. *A. salmonicida* strain AS-1R or 3.101-2 or AP from strain 3.101-2 developed specific anti-AP antibodies (Tables 10 and 11, Figures 8B and 8E). Similar antibodies could not be demonstrated in rabbits immunized with F4, LPS, or AP-cells of *A. salmonicida* (Tables 10 and 11, Figures 8C and 8D).

All antisera absorbed with strain AS-1S lacked agglutinating antibodies, as demonstrated by a fall in titer to zero (Table 11). Absorption also removed precipitating antibodies against LPS and F4 (Figures 8B to 8D, 9A).

Absorption of antisera possessing anti-AP antibodies with boiled or FK AP+ *A. salmonicida* strains AS-1R or 3.101-2 removed all anti-AP antibodies when tested by CIE and immunodiffusion tests (data not shown).

Although many concentrations of antigens were used in CIE reactions, optimal results were usually obtained using SC adjusted to OD<sub>520</sub> = 0.25, BE at 150 ug/ml, APEF at 250 ug/ml, AP at 125 ug/ml, LPS at 250 ug/ml, and F4 at 25 ug/ml.

Figure 8. Characteristics of rabbit antisera prepared against whole cells and purified antigens of Aeromonas salmonicida were demonstrated by counterimmunoelectrophoresis (CIE). Precipitin lines were stained 10 min with Coomassie blue.

A. Rabbit antisera against formalin-killed (FK) strain AS-1R formed a single distinct precipitin line near the antibody (anodal, left) well when reacted against purified A-protein (AP) from strain 3.101-2. Odd (left) numbered wells contained antisera. Even (right) numbered wells contained dilutions of AP: well 2, 480 ug/ml; well 6, 240 ug/ml; well 10, 120 ug/ml; well 4, 60 ug/ml; well 8, 30 ug/ml; well 12, 15 ug/ml.

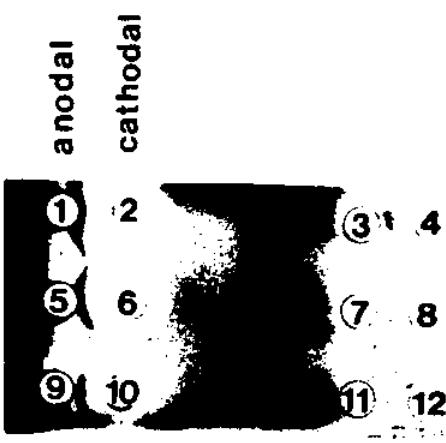
B. Rabbit antisera against FK strain AS-1R formed two precipitin lines (arrowhead and arrow, left) when reacted against A-protein enriched fraction (APEF) from strain AS-1R. Only one of these precipitin lines was removed by absorbing antisera with AP- strain AS-1S (arrowhead, right). Wells 1, 5 and 9, rabbit anti-AS-1R; wells 3, 7 and 11, absorbed AS-1R antisera; wells 2 and 4, APEF, AS-1R, 125 ug/ml; wells 6 and 8, LPS, AS-1R, 250 ug/ml; wells 10 and 12, F4, AS-1R, 25 ug/ml.

C. Rabbit antisera against AP- strain AS-1S formed nearly identical precipitin lines against tested antigens as did antisera against AP+ strain AS-1R (Figure 8B). However, the line nearest well 1 (arrowhead) was much lighter, and was removed by absorbing antisera with strain AS-1S (wells 3, 4). Wells 1, 5 and 9 contained rabbit anti-AS-1S; wells 3, 7 and 11 contained absorbed antisera; other wells were as listed in

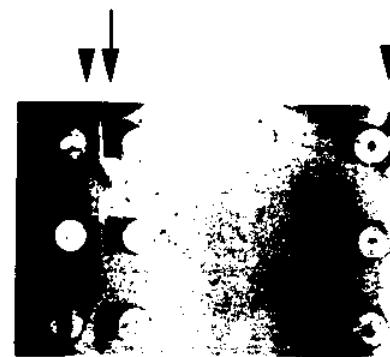
Figure 8B.

D. Rabbit antisera against F4 antigen lacked the extra precipitin line observed with rabbit anti-AS-1R (Figure 8B, arrowhead). All precipitin lines were removed by absorbtion of sera with strain AS-1S. Wells 1, 5 and 9, anti-F4, as-1R; wells 3, 7 and 11, anti-F4, AS-1R, absorbed; other wells are as in Figure 8B.

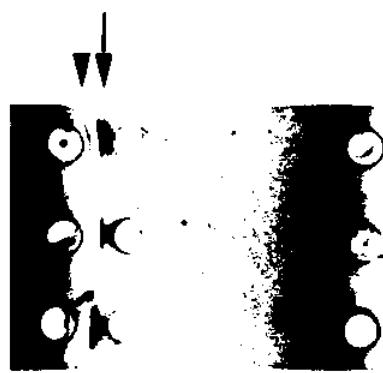
E. Rabbit antisera against strain AS-1R reacted identically against APEF from either strain AS-1R or 3.101-2, producing two precipitin lines (arrowhead and arrow, left). Only one of these lines was removed by absorbing antisera with AP- strain AS-1S (arrowhead, right). Wells 1 and 5, rabbit anti-AS-1R; wells 3 and 7, absorbed antisera; wells 2 and 4, APEF, AS-1R, 125 ug/ml; wells 6 and 8, APEF, 3.101-2, 125 ug/ml.



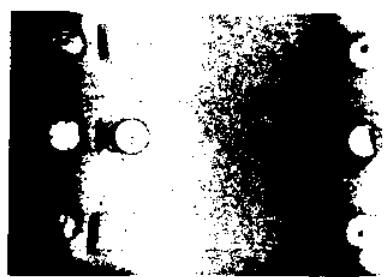
A



B



C



D



E

Table 11. Characterization by agglutination titer and counterimmuno-electrophoresis (CIE) reactions of rabbit antisera prepared against whole cells and purified antigens of four strains of Aeromonas salmonicida which were absorbed with A. salmonicida strain AS-1S.<sup>1</sup>

Antigen	Rabbit Number	Absorbed Titer <sup>2</sup>	CIE Precipitins		
			APEF	LPS	F4
AS-1R:FK	1	0	1	0	0
AS-1S:FK	2	0	0	0	0
3.101-2:FK	2	0	1	0	0
3.95:FK	2	0	0	0	0
AP:AS-1R	1	0	0	0	0
AP:3.101-2	1	0	1	0	0
F4:AS-1R	1	0	0	0	0
F4:AS-1S	1	0	0	0	0
F4:3.101-2	2	0	0	0	0
F4:3.95	2	0	0	0	0

<sup>1</sup> Antigens and their optimal concentrations used for CIE were : APEF, A-protein enriched fraction from strains AS-1R or 3.101-2, (125 ug/ml); LPS, lipopolysaccharide, (250 ug/ml); F4, fraction 4, (25 ug/ml). Precipitin lines recorded represent the number of lines observed in stained slides. Antigens used to immunize rabbits were : formalin-killed (FK) whole cells; A-protein (AP); and fraction 4 (F4).

<sup>2</sup> Antisera was absorbed three times with FK A. salmonicida strain AS-1S.

Figure 9. Characteristics of rabbit antisera prepared against whole cells and purified antigens of Aeromonas salmonicida demonstrated by double immunodiffusion (ID). Precipitin lines were visualized after staining 10 min with Coomassie blue.

A. Immunodiffusion reactions of rabbit antisera against strain AS-1R (left) and absorbed antisera (right) against sonicated AP+ cells, LPS, and F4. Reactions of identity were observed between F4 and LPS (wells 4, 5), LPS derived from two different A. salmonicida strains (wells 3, 4) and LPS and sonicated cells (wells 2, 3). Additional antigens not related to LPS were present in sonicated cell preparations, as indicated by a spur (well 3) and an extra distinct precipitin line (wells 1,2). Antibodies against one of these extra antigens were not removed from antisera by absorption with AS-1S (AP-) cells (wells 7, 8). Additionally, the extra antigen was serologically identical between strain AS-1R and 3.101-2, as evidenced by a reaction of identity (wells 7, 8). Well A, rabbit anti-AS-1R; well B, Anti-AS-1R, absorbed; wells 1 and 7, sonicated AS-1R cells; wells 2 and 8, sonicated 3.101-2 cells; wells 3 and 9, strain 3.101-2 LPS, 50 ug/ml; wells 4 and 10, strain AS-1R LPS, 50 ug/ml; wells 5 and 11, strain AS-1R F4, 50 ug/ml; wells 6 and 12, strain 3.101-2 F4, 50 ug/ml.

B. A-proteins purified from strain AS-1R and 3.101-2 were recognized as being identical by immunodiffusion. Well A, rabbit anti-AS-1R; well 1, AP, 3.101-2, 125 ug/ml; well 2, AP, AS-1R, 245 ug/ml; well 3, AP, 3.101-2, 455 ug/ml; well 4, AP, 3.101-2, 1560 ug/ml; well 5, AP, AS-1R, 640 ug/ml; well 6, AP, AS-1R, 675 ug/ml.

C. The effect of LPS concentration on precipitin line pattern was observed (this and the following two figures). High LPS concentrations (2.5 mg/ml) resulted in compact precipitin lines and incomplete reactions of identity between LPS antigens derived from four strains of A. salmonicida. Peripheral wells contain 10 ul of LPS at 2.5 mg/ml. The samples and sources are: well A, rabbit anti-3.101-2; well 1, Phosphate buffered saline / sodium dodecyl sulfate (PBS/SDS); well 2, AS-1R; well 3, 3.101-2; well 4, 3.95; well 5, AS-1R; well 6, AS-1S.

D. Multiple precipitin lines and reactions of identity were observed when LPS was used at a concentration of 500 ug/ml. Samples and locations are as in Figure 9C.

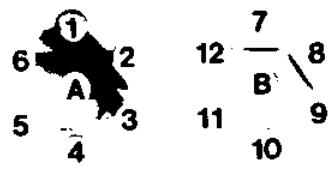
E. Precipitin lines against LPS were poorly defined when used at a concentration of 50 ug/ml. Samples and locations are as in Figure 9C.

F. Rabbit antisera against F4 reacted strongly with purified LPS. Reactions of identity were observed between LPS from the four A. salmonicida strains. Well A, rabbit anti-F4, AS-1R; well 1, PBS/SDS; well 2, LPS, AS-1R, 500 ug/ml; well 3, LPS, 3.101-2, 500 ug/ml; well 4, LPS, 3.95, 500 ug/ml; well 5, LPS, AS-1R, 500 ug/ml; well 6, LPS AS-1S, 500 ug/ml.

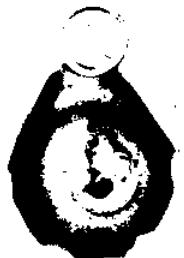
G. Lipopolysaccharide and F4 reacted against rabbit anti-AS-1R antisera developed distinct reaction of identity precipitin lines. Well A, rabbit anti-AS-1R; well 1, PBS/SDS; well 2, LPS, AS-1R, 100 ug/ml; well 3, F4, AS-1R, 50 ug/ml; well 4, LPS, 3.95, 100 ug/ml; well 5, F4, 3.95, 50 ug/ml; well 6, LPS, AS-1R, 100 ug/ml.

H. Lipopolysaccharide and F4 suspended in distilled water had broad precipitin lines and reactions of identity when reacted against rabbit

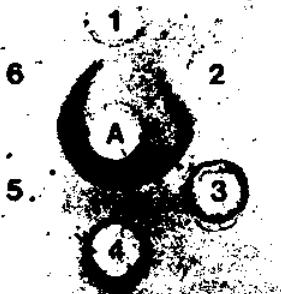
anti-AS-1R. Purified AP precipitated in approximately the same region, however, the narrowness of the precipitin line and the presence of a spur suggest a separate identity. Fraction 4 antigens had an additional poorly diffusing antigen also seen in LPS samples at higher loading (see Figure 9D), but not seen with AP. Well A, rabbit anti-AS-1R; well 1, AP, 3.101-2, 480 ug/ml; well 2, F4, 3.101-2, 50 ug/ml; well 3, LPS, 3.101-2, 50 ug/ml; well 4, AP, 3.101-2, 480 ug/ml; well 5, F4, 3.95, 50 ug/ml; well 6, LPS, 3.95, 50 ug/ml.



**A**



**C**



**B**



**D**



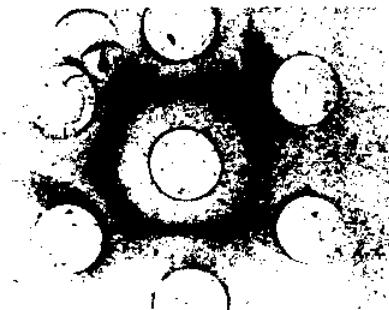
**E**



**F**



**G**



**H**

The serological relationships between different antigens were studied by double immunodiffusion. Purified LPS from *A. salmonicida* strains AS-1R, AS-1S, 3.101-2 and 3.95 was identical (Figures 9C to 9E), however, the precipitin line pattern observed was variable with antigen concentration and individual antisera. When low concentrations of LPS were suspended in distilled water or buffer without detergent, precipitin lines were absent, diffuse or present at the periphery of the well (Figure 9H). Enhanced diffusion of LPS into the gel matrix was achieved by the addition of 0.2% SDS to the buffer (Figures 9C to 9G).

Fraction 4 antigens from *A. salmonicida* strains AS-1R, AS-1S, 3.101-2 and 3.95 were identical. Reactions of identity were also seen between LPS and F4 from the same or heterologous *A. salmonicida* strains (Figure 9G). These results, and those from SDS-PAGE analysis, indicate that the major antigenic component of F4 is LPS. However, the presence of two distinct precipitin lines by CIE in rabbit anti-F4, 3.95 reacted against APEF (Table 10) also suggest that other antigens exist in F4. This observation was confirmed by SDS-PAGE (Figure 6).

Purified AP was serologically distinct from either F4 or LPS (Figure 9H). A-protein from either strain AS-1R or 3.101-2 was antigenically identical, as determined using absorbed antisera against APEF (Figures 8E, 9A) and reactions of non-absorbed antisera with purified AP from different sources (Figure 9B).

Rainbow Trout. Trout immunized with FK *A. salmonicida* cells, purified LPS or F4 mixed in FCA developed high agglutination titers (5120 to

81920). In contrast, low titers (0 to 1280) were measured in fish immunized with live bacteria, purified AP or FCA (Table 12).

Trout immunized with virulent and avirulent strains of live *A. salmonicida* lacked precipitating antibodies by CIE, and developed only low levels of agglutinating antibodies (0 to 640). Those infected with virulent strain, AS-1R, produced a notable amount of C-reactive protein, which precipitated in CIE tests as a comet shaped arc around the antibody (anodal) well (data not shown). This line was first observed 15 to 30 min after completion of CIE tests, and was removed by rinsing for 30 min in 0.1 M EDTA in 0.85% saline, pH = 8.0.

Trout antisera against FK cells or AP reacted with complex antigen mixtures (SC, BE, APEF) developed multiple (up to two) precipitin lines after CIE (Table 12, Figure 10A). Antisera against LPS or F4 developed single precipitin lines against these antigens. No precipitin lines were observed against AP by either CIE or immunodiffusion. Antibodies against LPS and F4 existed in all antisera developed against FK cells, against LPS, and F4 antigens. No antibodies were detected in fish injected with only FCA. All absorbed antisera lacked agglutinating antibodies to whole cells and precipitating antibodies to APEF, F4 and LPS.

Immunodiffusion tests demonstrated homogeneity among LPS purified from four strains of *A. salmonicida*, and as seen in rabbits, the precipitin line pattern observed was related to LPS concentration (Figures 10B to 10D). Fraction 4 antigens from four *A. salmonicida* strains were also recognized as being serologically identical (Figure 9E). Homogeneity between F4 and LPS was also seen (Figure 9F).

Table 12. Characterization of rainbow trout antisera prepared against whole cells and purified antigens of six strains of *Aeromonas salmonicida* strains by agglutination titers and counterimmunoelectrophoresis (CIE) precipitin reactions.<sup>1,2</sup>

Antigen	Titer	CIE Precipitins					
		SC	BE	APEF	AP	LPS	F4
AS-1R:FK	30720	1	2	1	0	1	1
AS-1S:FK	81920	1	2	1	0	1	1
3.101-1:FK	20480	1	1	1	NT	1	1
3.101-2:FK	40960	2	2	2	0	1	1
3.101-3:FK	10240	2	1	1	NT	1	1
3.95:FK	10240	1	2	1	0	1	1
AS-1R:live <sup>3</sup>	40	0	0	0	0	0	0
AS-1S:live	640	0	0	0	NT	0	0
3.101-1:live	320	0	0	0	NT	0	0
3.101-2:live	80	0	0	0	0	0	0
3.101-3:live	40	0	0	0	NT	0	0
3.95:live	0	0	0	0	NT	0	0
AP:AS-1R	1280	0	0	2	0	1	1
AP:3.101-2	80	0	0	0	0	1	1
LPS:AS-1R	5120	0	0	1	0	1	1
F4:AS-1R	20480	0	1	1	0	1	1
F4:3.95	10240	0	1	1	NT	1	1
FCA	0	0	0	0	NT	0	0

<sup>1</sup> Antigens and their optimal concentrations used for CIE were : SC, sonicated cells ( $OD_{520} = 0.25$ ); BE, boiled extract of *A. salmonicida* strain AS-1R or 3.101-2 cells, (150  $\mu g/ml$ ); APEF, A-protein enriched fraction from strains AS-1R or 3.101-2, (125  $\mu g/ml$ ); AP, A-protein, (125  $\mu g/ml$ ); LPS, lipopolysaccharide, (250  $\mu g/ml$ ); F4, fraction 4,

Table 12. (cont.)

(25 ug/ml). Precipitin lines recorded represent the number of lines observed in stained slides. Some antigens were not tested (NT) due to limited supplies. Antigens used to immunize rabbits were : live bacteria; formalin-killed (FK) whole cells; A-protein (AP); lipopolysaccharide (LPS); and fraction 4 (F4). Live *A. salmonicida* strain 3.101-2 was mixed in Freund's Incomplete Adjuvant while other antigens were mixed in Freund's Complete Adjuvant. Agglutination titer was recorded as the reciprocal of the highest serum dilution where the cell pellet was less than or equal to 1/2 the size of the pellet in the negative control well. Values recorded were the average of two readings.

<sup>2</sup> Antisera from four to eight fish were pooled prior to testing. Agglutination titers are the average of two replicates.

<sup>3</sup> An EDTA soluble precipitin line was observed prior to staining in all CIE reactions using antisera against live AS-1R.

Figure 10. Characteristics of rainbow trout antisera prepared against whole cells and purified antigens of Aeromonas salmonicida demonstrated by counterimmunoelectrophoresis (CIE) and double immunodiffusion (ID). Precipitin lines were stained 10 min with Coomassie blue.

A. Trout antisera against strain 3.101-2 formed single precipitin lines against five different A. salmonicida antigenic preparations. No reaction was observed against A-protein (AP). Left (odd) wells, antisera; well 2, sonicated cells (SC), AS-1R, 5% (wt/vol); well 4, boiled cell extract (BE), AS-1R, 500 ug/ml; well 6, A-protein enriched fraction (APEF), AS-1R, 125 ug/ml; well 8, lipopolysaccharide (LPS), AS-1R, 250 ug/ml; well 10, AP, AS-1R, 40 ug/ml; well 12, F4, 3.95, 50 ug/ml.

B. Trout antisera reacted identically with LPS from four A. salmonicida strains. As seen with rabbit antisera, LPS concentration affected the precipitin line pattern observed (Figures 10B to 10D). Outer wells contain 10 ul of LPS at 2.5 ug/ml. The samples and sources are: well A, trout anti-AS-1R; well 1, AS-1R; well 2, AS-1S; well 3, 3.101-2; well 4, 3.95; well 5, AS-1S; well 6, 3.101-2.

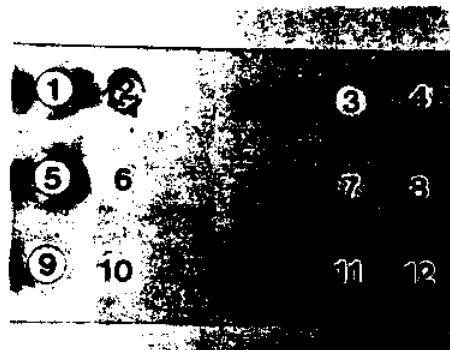
C. Trout antisera against strain AS-1R formed reactions of identity with LPS. A single precipitin line was observed. Outer wells contained 10 ul of LPS at 625 ug/ml. Samples and locations are as listed in Figure 10B.

D. When trout antisera to strain AS-1R was reacted against dilute LPS, a diffuse single precipitin line was observed. Outer wells contained 10 ul of LPS at 78 ug/ml. Samples and locations are listed

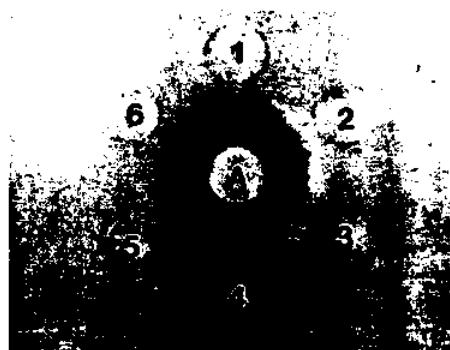
in Figure 10B.

E. Fraction 4 antigens from each of four *A. salmonicida* strains were recognized as equal by trout antisera. Well A, trout anti-AS-1R; well 1, F4, AS-1R, 60 ug/ml; well 2, F4, AS-1S, 95 ug/ml; well 3, F4, 3.101-2, 75 ug/ml; well 4, F4, 3.95, 75 ug/ml; well 5, F4, AS-1S, 95 ug/ml; well 6, F4, 3.95, 75 ug/ml.

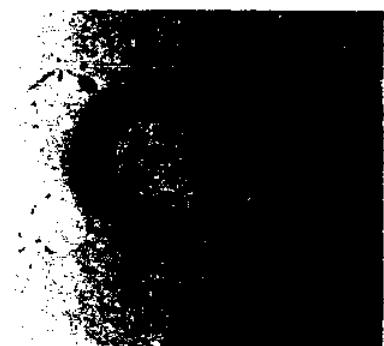
F. Fraction 4 and LPS reacted identically with trout antisera. Well A, trout anti-AS-1S; wells 1, 3 and 5, F4, 3.95, 165 ug/ml; well 2, LPS, AS-1R, 100 ug/ml; well 4, LPS, 3.95, 100 ug/ml; well 6, LPS, 3.101-2, 100 ug/ml.



A



B



C



D



E



F

Detection of Agglutinating Antibodies from Passively  
Immunized Fish

Agglutinating antibodies to A. salmonicida were detected in the blood of passively immunized coho and chinook salmon fingerlings at the first sample period (24 h), and until the end of the monitoring period (Tables 13 and 14). Initially, titers were approximately equal to the number of agglutinating units injected, and remained at these levels for up to 9 d before declining in chinook salmon fingerlings. The half life of rabbit anti-A. salmonicida antibodies in coho salmon at 15 C was 152.2 h (6.6 d). Rabbit antibodies in chinook salmon at 18 C had a half life of 248.6 h (10.4 d), and rainbow trout antibodies in chinook salmon at 13 C had a half life of 387.3 h (16.1 d).

Passive Immunization Test 1

Protection, or lack of protection, from mortality caused by A. salmonicida was evaluated using RPS values and chi square probabilities. Arbitrary values were adopted creating three groups: 1) protected, 2) marginally or equivocally protected, and 3) non-protected. Protected groups had high RPS values ( $\geq 60$ ) and/or low chi square probabilities ( $\leq 0.005$ ). Equivocally protected groups had RPS values between 30 and 60 and/or chi square probabilities between 0.01 and 0.10. Non-protected groups had low RPS values ( $< 30$ ) and/or high probabilities ( $> 0.10$ ).

Data to day 10. Saline-injected coho salmon challenged by i.m. injection with A. salmonicida strain AS-1R experienced an average of 51.7% specific mortality during the first 10 d. Fish receiving rabbit

Table 13. Reciprocal agglutination titers from sera of coho salmon fingerlings passively immunized with rabbit Aeromonas salmonicida antisera.<sup>1</sup>

Time (days) <sup>2</sup>	anti AS-1R		anti 3.95	
	Titer	AVE	Titer	AVE
0	0/0	0	0/0	0
1	320/160	240	20/80	50
2	80/80	80	40/40	40
4	40/80	60	10/40	25
6	10/40	25	10/20	15
10	10/20	15	10/20	15
14	10/20	15	0/0	0

<sup>1</sup> Each fish was injected i.p. with 0.1 cc rabbit antisera against formalin-killed A. salmonicida strain AS-1R (256 agglutinating units) or strain 3.95 (128 agglutinating units).

<sup>2</sup> Agglutinating titers recorded on day 0 were from blood collected prior to passive immunization.

Table 14. Reciprocal agglutination titers from sera of chinook salmon fingerlings passively immunized with rabbit and rainbow trout Aeromonas salmonicida antisera.<sup>1</sup>

Day <sup>2</sup>	Trout anti-AS-1S						Rabbit anti-AS-1S					
	Rep1	ave	Rep2	ave	AVE	Rep1	ave	Rep2	ave	AVE		
0	0	0	0	0	0	0	0	0	0	0	0	0
1	5120		5120			160		320				
	5120	5120	10240	7680	6400	160	160	320	320	240		
2	5120		2560			320		320				
	5120	5120	2560	2560	3840	320	320	160	240	280		
4	5120		5120			160		320				
	5120	5120	5120	5120	5120	160	160	160	240	200		
6	5120		2560			160		160				
	5120	5120	2560	2560	3840	160	160	160	160	160		
9	5120		5120			80		80				
	5120	5120	5120	5120	5120	80	80	80	80	80		
12	5120		2560			80		80				
	5120	5120	2560	2560	3840	160	120	80	80	100		
16	2560		2560			40		20				
	2560	2560	2560	2560	2560	40	40	20	20	30		
23	2560		320			10		20				
	2560	2560	320	320	1440	10	10	20	20	15		

<sup>1</sup> Fish injected i.p. with trout antisera against formalin-killed (FK) *A. salmonicida* strain AS-1S received 4096 agglutinating units; those injected with rabbit antisera against FK strain AS-1S received 256 agglutinating units.

<sup>2</sup> Agglutination titers recorded for day 0 are from blood collected prior to passive immunization.

preimmune sera had significantly ( $\chi^2 = 29.4$ ,  $p = 0.005$ ) greater mortality during the same period, averaging 98.3% (Table 15). This mortality level was much greater than desired making evaluation of results difficult. Experimental groups receiving different antisera were compared to controls injected with preimmune sera for statistical analysis. Due to the very high mortality rate in control fish, some vaccinated fish groups having statistically significant ( $p \leq 0.005$ ) protection from mortality by chi square analysis had relatively low RPS values (Table 15).

Significant protection was observed among all groups immunized with FK A. salmonicida cells, and absorbed antisera. Equivocal protection among groups receiving antisera against purified AP, LPS and F4 from strain 3.95 was observed. No protection was afforded by antisera to F4 from strain AS-1R.

Formalin-killed A. salmonicida strains AS-1R, AS-1S and 3.101-2 provided better protection than strain 3.95. Average mortality among the four strains was 21.9% compared to 98.3% in controls. Excessive variation between replicates (>20%) in AS-1R, FK tests necessitated pooling of data for statistical analysis (Amend 1981). Pooled data gave RPS and chi square values of 77.9 and 69.5 ( $p = 0.005$ ), respectively.

Antisera against FK AP<sup>+</sup> cells absorbed with AP<sup>-</sup> mutant A. salmonicida strains were also protective, having an average RPS of 61.0. Excessive non-specific mortality was observed in each of the absorbed sera treatments. This was attributed to the use of live A. salmonicida AP<sup>-</sup> strains for sera absorption, and failure to filter

Table 15. Survival of coho salmon fingerlings passively immunized with rabbit antisera against Aeromonas Salmonicida antigens 10 d after injection challenge with A. salmonicida strain AS-1R.<sup>1,2</sup>

Antigen	Rep	N	Number Percent	N'	Number Percent	$\chi^2$	prob	RP	RPS	Var	SM
Saline	1	30	0	0.0	30	20	66.7				30.0
	2	30	0	0.0	30	11	36.7				
Preimmune	1	30	0	0.0	30	30	100.0				3.4
	2	30	1	3.3	29	28	96.6				
AS-1R:FK	1	30	0	0.0	30	3	10.0	45.6	0.005	10.0	90.0
	2	30	0	0.0	30	10	33.3	22.9	0.005	2.9	65.5
AS-1S:FK	1	30	0	0.0	30	5	16.7	39.5	0.005	6.0	83.3
	2	30	1	3.3	29	4	13.3	36.9	0.005	7.0	85.7
3.101-2:FK	1	30	0	0.0	30	4	13.3	42.4	0.005	7.5	86.7
	2	30	0	0.0	30	0	0.0	51.1	0.005	inf.	100.0
3.95:FK	1	30	0	0.0	30	13	43.3	21.0	0.005	2.3	56.7
	2	30	1	3.3	29	13	44.8	16.3	0.005	2.2	53.6
AP:AS-1R	1	30	1	3.3	29	24	82.8	3.5	0.100	1.2	17.2
	2	30	1	3.3	29	18	62.1	9.5	0.005	1.6	35.7
AP:3.101-2	1	30	0	0.0	30	16	53.3	15.8	0.005	1.9	46.7
	2	30	3	10.0	27	22	81.5	1.9		1.2	15.6
LPS:AS-1R	1	30	1	3.3	29	23	79.3	5.0	0.050	1.3	20.7
	2	30	0	0.0	30	16	53.3	12.5	0.005	1.8	44.8

Table 15. (cont.)

Antigen	Rep	N	NSM Number Percent	N'	SM Number Percent	$\chi^2$	Prob	RP	RPS	Var
F4:AS-1R	1	30	1	3.3	29	27	93.1	0.5	1.1	6.9
	2	30	1	3.3	29	26	99.7	2.3	1.1	7.1
F4:3.95	1	30	1	3.3	29	21	72.4	7.5	0.010	1.4
	2	30	3	10.0	27	16	59.3	11.7	0.005	1.6
AS-1R:Abs	1	30	0	0.0	30	6	20.0	40.9	0.005	5.0
	2	30	4	13.3	26	7	26.9	25.5	0.005	3.6
3.101-2:Abs	1	30	10	33.3	20	9	45.0	18.1	0.005	2.2
	2	30	5	16.7	25	12	9.0	39.2	0.005	12.1

<sup>1</sup> Anaesthetized fish were injected i.m. with 8 cfu (equal to a LD<sub>46.5</sub>) of *A. salmonicida* strain AS-1R four days after being passively immunized. Data was analyzed after a 10 d monitoring period.

<sup>2</sup> Abbreviations used in this table are : N, number of fish; N', N minus mortalities from non-specific causes; NSM, non-specific mortality; SM, specific mortality;  $\chi^2$ , chi square; Prob, probability; RP, relative potency (% specific control mortality / % specific vaccinee mortality); RPS, relative percent survival ( $1 - [1/ RP] \times 100$ ); Var, variation between percent mortality in replicate groups one and two; Abs, absorbed antisera with *A. salmonicida* strain AS-1S or 3.101-3; FK, formalin-killed.

absorbed sera prior to injecting fish. Avirulent, AP<sup>-</sup> strains of *A. salmonicida* were reisolated from kidneys of dead fish during the first 7 d of the test. Variation in mortality between replicate groups passively immunized with 3.101-2, FK, absorbed antisera was excessive (37.0%). Pooled data had RPS and chi square values of 75.2 and 59.4 ( $p = 0.005$ ), respectively. Because the effects of live AP<sup>-</sup> *A. salmonicida* in the absorbed antisera were unknown, and excessive between replicate variation existed, these tests should have been repeated. However, due to a lack of additional fish this was not possible.

Antisera against purified antigens provided equivocal protection, as indicated by lower RPS values. In each of the AP groups (AS-1R and 3.101-2 derived) only one of the replicates had significant ( $p = 0.005$ ) protection, while the second replicate had poor ( $p \geq 0.100$ ) protection. Pooled data (due to excessive variation) from fish injected with AP. AS-1R had RPS and chi square values of 26.3 and 13.9 ( $p = 0.005$ ), respectively, while those injected with AP. 3.101-2 had RPS and chi square values of 32.1 and 18.3 ( $p = 0.005$ ), respectively.

Fish injected with rabbit anti-LPS were protected at approximately the same level as those receiving AP antisera. Excessive variation between replicates existed, and pooled data had RPS and chi square values of 32.8 and 18.8 ( $p = 0.005$ ), respectively.

Fish injected with antisera from F4, AS-1R were not protected from mortality caused by *A. salmonicida*. However, antisera from F4, 3.95 provided equivocal protection, comparable to AP and LPS antisera.

Data to day 28. Mortalities were monitored for an additional 18 d,

making a total of 28 d (Table 16). Results observed during the initial 10 d period were essentially unchanged. The excessive variation between replicates was diminished for certain groups (AP, AS-1R, and LPS, AS-1R). Fish immunized with antisera against FK cells experienced an average mortality of 25.7% (compared to 100.0% in controls), and had an average RPS of 74.3. Excessive variation between replicates remained in groups injected with AS-1R, FK antisera, and pooled data had RPS and chi square values of 76.7 and 70.5 ( $p = 0.005$ ), respectively.

Fish injected with absorbed antisera had average RPS values of 64.9. Pooled data from fish injected with strain 3.101-2, FK, absorbed antisera had RPS and chi square values of 68.9 and 54.8 ( $p = 0.005$ ), respectively.

Rabbit anti-AP injected fish had average RPS values of 21.8. Excessive variation remained between replicate groups injected with AP, 3.101-2, and pooled data had average RPS and chi square values of 29.8 and 18.1 ( $p = 0.005$ ), respectively. Fish receiving anti-LPS sera had average RPS values of 23.7. Fraction 4 injected fish had an average RPS of 13.3.

#### Detection of Latent Infection among Surviving Coho Salmon

Coho salmon passively immunized with antisera against FK strains of A. salmonicida and surviving 28 d post challenge, were injected i.m. with 1.0 mg Kenalog 40 and monitored for an additional 14 d. The second replicate group of fish was not injected, serving as control groups. Carriers existed in all groups (Table 17), with the higher

Table 16. Survival of coho salmon fingerlings passively immunized with rabbit antisera against *Aeromonas* *Salmonicida* antigens 28 d after injection challenge with *A. salmonicida* strain AS-1R.<sup>1,2</sup>

Antigen	Rep	N	Number	Percent	N'	Number	Percent	$\chi^2$	prob	RP		RPS	Var
										SM	RP		
Saline	1	30	0	0.0	30	24	80.0					26.7	
	2	30	0	0.0	30	16	53.3						
Preimmune	1	30	0	0.0	30	30	100.0					0.0	
	2	30	1	3.3	29	29	100.0						
AS-1R:FK	1	30	0	0.0	30	3	10.0	45.5	0.005	10.0	90.0		
	2	30	0	0.0	30	11	36.7	24.1	0.005	2.7	63.3	26.7	
AS-1S:FK	1	30	0	0.0	30	7	23.3	34.3	0.005	4.3	76.7		
	2	30	1	3.3	29	4	13.8	40.5	0.005	7.2	86.2	9.5	
3.101-2:FK	1	30	0	0.0	30	5	16.7	39.5	0.005	6.0	83.3		
	2	30	0	0.0	30	0	0.0	55.1	0.005	inf.	100.0	16.7	
3.95:FK	1	30	0	0.0	30	16	53.3	15.8	0.005	1.9	46.7		
	2	30	1	3.3	29	15	51.7	15.9	0.005	1.9	48.3	1.6	
AP:AS-1R	1	30	1	3.3	29	26	89.7	1.4		1.1	10.3		
	2	30	1	3.3	29	21	72.4	7.1	0.010	1.4	27.6	17.3	
AP:3.101-2	1	30	0	0.0	30	17	56.7	14.1	0.005	1.8	43.3		
	2	30	3	10.0	27	23	85.2	2.8	0.100	1.2	14.8	28.5	
LPS:AS-1R	1	30	1	3.3	29	23	79.3	5.0	0.050	1.3	20.7		
	2	30	0	0.0	30	22	73.3	7.0	0.010	1.4	26.7	6.0	

Table 16. (cont.)

Antigen	Rep	NSM		SM		$\chi^2$	prob	RP	RPS	Var
		N	Number Percent	N'	Number Percent					
F4:AS-1R	1	30	1	3.3	29	27	93.1	0.6	1.1	6.9
	2	30	1	3.3	29	26	89.7	1.4	1.1	10.3
F4:3.95	1	30	1	3.3	29	25	86.2	2.4	1.2	13.8
	2	30	3	10.0	27	21	77.8	5.1	0.025	1.3
AS-1R:Abs	1	30	0	0.0	30	9	30.0	29.3	0.005	3.3
	2	30	4	13.3	26	11	42.3	20.1	0.005	2.4
3.101-2:Abs	1	30	10	33.3	20	12	60.0	11.5	0.005	1.7
	2	30	5	16.7	25	2	8.0	43.1	0.005	12.5

<sup>1</sup> Experimental conditions were as listed in Table 14, except fish were monitored an additional 18 d (a total of 28 d).

<sup>2</sup> Abbreviations used in this table are : N, number of fish; N', N minus mortalities from non-specific causes; NSM, non-specific mortality; SM, specific mortality;  $\chi^2$ , chi square; prob, probability; RP, relative potency (% specific control mortality / % specific vaccinee mortality); RPS, relative percent survival ( $1 - [1/ RP] \times 100$ ); Var, variation between percent mortality in replicate groups one and two; Abs, absorbed antisera with *A. salmonicida* strain AS-1S or 3.101-3; FK, formalin-killed.

Table 17. Detection of latent infection with *Aeromonas salmonicida* in coho salmon passively immunized with rabbit antisera prepared against formalin-killed cells.<sup>1</sup>

Antisera	Treatment	N	NSM	N'	SM	Carrier Rate
Saline	Kenalog	14	1	13	13	100.0
	None	6	0	6	4	66.7
AS-1R	Kenalog	19	0	19	16	84.2
	None	27	0	27	0	0.0
AS-1S	Kenalog	25	0	25	1	4.0
	None	23	0	23	1	4.3
3.101-2	Kenalog	30	2	28	1	3.6
	None	25	0	25	0	0.0
3.95	Kenalog	14	0	14	11	78.6
	None	14	0	14	0	0.0

<sup>1</sup> Abbreviations used in this table are : N, number of fish; N', N minus mortalities from non-specific causes; NSM, non-specific mortality; SM, specific mortality.

percentages (84.2 and 78.6%) in fish injected with AS-1R and 3.95 antisera. Fish injected with A. salmonicida strain AS-1S or 3.101-2 antisera had low carrier rates, 4.0 and 3.6%, respectively. Specific mortality among control fish was low (0.0 to 4.3%) during this period.

Controls (above) were injected with Kenalog 40 56 d after challenge. Carriers were found only among fish immunized with FK AS-1S antisera, with 84.2% (16/19) dying from furunculosis. No carriers were detected in fish immunized with FK AS-1R, (0/24), FK 3.101-2, (0/22), or FK 3.95, (0/13) antisera.

#### Passive Immunization Test 2

Saline-injected chinook salmon challenged by immersion in  $2.45 \times 10^5$  cfu/ml A. salmonicida strain AS-1R experienced an average mortality of 65.0% (60.0 to 70.0%). This level of mortality did not differ significantly from that observed in control fish injected with either rabbit (66.1%) or fish (71.7%) preimmune sera. The level of mortality among control fish was within the 60 to 80% level recommended by Amend (1981) for best evaluation of treatments.

Chinook Salmon Injected with Rabbit Antisera. Significant protection was observed among chinook salmon immunized with rabbit antisera against A. salmonicida strains AS-1R, AS-1S, 3.101-2 and 3.101-3, and absorbed 3.101-2 antisera. Equivocal protection was observed among fish receiving antisera against strain 3.101-1, absorbed AS-1R antisera, and antisera against F4, 3.95. No protection was afforded by antisera against strain 3.95 or purified AP, LPS, or F4, AS-1R (Table

18).

Rabbit antisera against AP<sup>+</sup> A. salmonicida strains provided better protection than AP<sup>-</sup> antisera. Relative percent survival values averaging 89.9 and 97.5 for strains AS-1R and 3.101-2, respectively indicated excellent protection. In comparison, antisera against AP<sup>-</sup> strains were protective for only two of four strains tested. Lower RPS values were observed (62.1 and 62.2 for strains AS-1S and 3.101-3, respectively). Strain 3.101-1 provided equivocal protection, having an RPS of 46.2, while strain 3.95 was not protective.

Absorbed antisera of AP<sup>+</sup> strains AS-1R and 3.101-2 provided marginal to good protection. Excessive variation existed in AS-1R absorbed antisera, but pooled data had  $\chi^2 = 14.0$  ( $p = 0.005$ ) and an RPS of 53.8. Better protection was observed with 3.101-2 absorbed antisera, having an average RPS of 87.4.

Among the purified antigens, equivocal protection was provided by antisera against F4, 3.95. Other purified antigens (AP, LPS, F4, AS-1R) were not protective.

Chinook Salmon Injected with Rainbow Trout Antisera. Lower RPS values were observed among salmon immunized with trout antisera, compared to those receiving rabbit antisera. None of the passive immunization treatments provided good protection, using the guidelines previously described. Marginal or equivocal protection was observed among fish receiving antisera against FK cells of strain AS-1R and 3.101-2, absorbed antisera of strains AS-1R and 3.101-2, and antisera against F4, 3.95. All other treatments failed to protect salmon against

Table 18. Survival of chinook salmon fingerlings passively immunized with rabbit antisera against *Aeromonas salmonicida* antigens after immersion challenge with *A. salmonicida* strain AS-1R.<sup>1,2</sup>

Antigen	Rep	NSM		N <sup>†</sup>	Number Percent	SM	$\chi^2$	prob	RP	RPS	Var
		N	Number Percent								
Saline	1	30	0	0.0	30	21	70.0				
	2	30	0	0.0	30	18	60.0				
Pre immune	1	30	0	0.0	20	66.7					1.2
	2	30	1	3.3	29	19	65.5				
AS-1R:FK	1	30	0	0.0	30	0	0.0	27.1	0.005	inf.	100.0
	2	30	0	0.0	40	4	13.3	14.8	0.005	4.9	79.7
AS-1S:FK	1	30	0	0.0	30	7	23.3	9.7	0.005	2.9	65.0
	2	30	0	0.0	30	0	26.7	7.4	0.010	2.5	59.2
3.101-1:FK	1	30	0	0.0	30	4	13.3	15.6	0.005	5.0	80.1
	2	30	0	0.0	30	17	56.7	0.2		1.2	13.8
3.101-2:FK	1	30	0	0.0	30	0	0.0	27.1	0.005	inf.	100.0
	2	30	0	0.0	30	1	3.3	22.9	0.005	19.9	95.0
3.101-3:FK	1	30	0	0.0	30	0	0	26.7	6.1	0.025	2.5
	2	30	0	0.0	30	7	23.3	8.9	0.005	2.8	64.4
3.95:FK	1	30	0	0.0	30	22	73.3	-0.1		0.3	-9.9
	2	30	1	3.3	29	20	69.0	0.0		1.0	-5.3
AP:AS-1R	1	30	0	0.0	30	24	80.0	-0.8		0.8	-20.5
	1	30	0	0.0	30	26	86.7	-2.5		0.8	-31.6

Table 18. (cont.)

Antigen	Rep	N	Number Percent	NSM		$\chi^2$	prob	RP	RPS	Var
				N'	Percent					
AP:3.101-2	1	30	0	0.0	30	23	76.7	-0.3	0.9	-14.9
	2	30	0	0.0	30	20	66.7	0.0	1.0	-2.0
LPS:AS-1R	1	30	0	0.0	30	24	80.0	-0.8	0.8	-20.5
	2	30	0	0.0	30	21	70.0	0.0	0.9	-6.4
F4:AS-1R	1	30	0	0.0	30	19	63.3	0.0	1.1	4.8
	2	30	0	0.0	30	23	76.7	-0.4	0.9	-17.6
F4:3.95	1	30	0	0.0	30	14	46.7	1.7	1.4	30.1
	2	30	0	0.0	30	8	26.7	7.4	0.010	2.5
AS-1R:Abs	1	30	0	0.0	30	5	16.7	13.4	0.005	4.0
	2	30	0	0.0	30	13	43.3	2.1	1.5	33.8
3.101-2:Abs	1	30	0	0.0	30	3	10.0	18.0	0.005	6.7
	2	30	0	0.0	30	2	6.7	19.9	0.005	9.8
										3.3

<sup>1</sup> Abbreviations used in this table are: N, number of fish; N', N minus mortalities from non-specific causes; NSM, non-specific mortality; SM, specific mortality;  $\chi^2$ , chi square; prob, probability; RP, relative potency (% specific mortality / % specific vaccinee mortality); RPS, relative percent survival ( $1 - [1/ RP] \times 100$ ); Var, variation between percent mortality in replicate groups one and two; Abs, absorbed antisera with *A. salmonicida* strain AS-1S or 3.101-3; FK, formalin-killed.

<sup>2</sup> Fish were challenged by addition of sodium chloride to the tank (0.9%, wt/vol), followed by addition of *A. salmonicida* strain AS-1R to a final concentration of  $2.45 \times 10^5$  cfu/ml for 20 min.

<sup>3</sup> Pooled data from replicate groups 1 and 2 had  $\chi^2 = 10.4$  ( $p = 0.005$ ) and RPS = 46.2.

Table 18 (cont.)

\* Pooled data from replicate groups 1 and 2 had  $\chi^2 = 9.3$  ( $p = 0.005$ ) and RPS = 43.6.

† Pooled data from replicate groups 1 and 2 had  $\chi^2 = 14.0$  ( $p = 0.005$ ) and RPS = 53.8.

mortality caused by A. salmonicida (Table 19).

Detection of Latent Infection Among Surviving Chinook Salmon. Control groups and fish having significant protection were injected with 1.0 mg Kenalog 40 at the end of the fourteenth day, and mortalities monitored an additional 14 d. Carrier rates were near 100.0% (Table 20) in all groups, demonstrating a high level of infection in all surviving fish.

Table 19. Survival of chinook salmon fingerlings passively immunized with rainbow trout antisera against *Aeromonas salmonicida* antigens after immersion challenge with *A. salmonicida* strain AS-1R.<sup>1,2</sup>

Antigen	Rep	N	NSM		N <sup>1</sup>	SM		$\chi^2$	prob	RP	RPS	Var
			Number	Percent		Number	Percent					
Saline	1	30	0	0.0	30	21	70.0					10.0
	2	30	0	0.0	30	18	60.0					
Preimmune	1	30	0	0.0	30	22	73.3					3.3
	2	30	0	0.0	30	21	70.0					
FCA	1	30	0	0.0	30	28	93.3	-3.0	0.100	0.8	-27.3	
	2	30	0	0.0	30	23	76.7	-0.1	0.9	0.9	-9.6	16.6
AS-1R:FK	1	30	0	0.0	30	13	43.3	4.4	0.050	1.7	40.9	
	2	30	0	0.0	30	8	26.7	9.6	0.005	2.6	61.9	16.7
AS-1S:FK	1	30	0	0.0	30	26	86.7	-0.9	0.9	0.9	-18.3	
	2	30	0	0.0	30	28	93.3	-4.0	0.050	0.8	-33.3	6.6
3.101-1:FK	1	30	0	0.0	30	23	76.7	0.0		1.0	-4.6	
	2	30	0	0.0	30	17	56.7	0.6		1.2	19.0	20.0 <sup>3</sup>
3.101-2:FK	1	30	0	0.0	30	8	26.7	11.3	0.005	2.8	63.6	
	2	30	0	0.0	30	10	33.3	6.7	0.010	2.1	52.4	6.6
3.101-3:FK	1	30	0	0.0	30	21	70.0	0.0		1.1	4.5	
	2	30	0	0.0	30	19	63.3	0.1		1.1	9.6	6.7
3.95:FK	1	30	0	0.0	30	23	76.7	0.0		1.0	-4.6	
	2	30	0	0.0	30	25	83.3	-0.8	0.8	-19.0	6.6	

Table 19. (cont.)

Antigen	Rep	N	Number	Percent	N'	Number	Percent	$\chi^2$	Prob	RP	RPS	Var
<b>AS-1R:live</b>	1	30	0	0.0	30	20	66.7	0.1	1.1	9.0		
	2	30	0	0.0	30	26	86.7	-1.6	0.8	-23.9	20.0*	
<b>AS-1S:live</b>	1	30	0	0.0	30	24	80.0	-0.1	0.9	-9.1		
	2	30	1	3.3	29	24	82.8	-0.7	0.9	-18.3	2.8	
<b>3.101-2:live</b>	1	30	0	0.0	30	19	63.3	0.3	1.2	13.6		
	2	30	1	3.3	29	24	82.8	-0.7	0.9	-18.3	19.5	
<b>AP:AS-1R</b>	1	30	0	0.0	30	17	56.7	1.2	1.3	22.6		
	2	30	1	3.3	29	20	69.0	0.0	1.0	1.4	12.3	
<b>AP:3.101-2</b>	1	30	0	0.0	30	20	66.7	0.1	1.1	9.0		
	2	30	0	0.0	30	25	83.3	-0.8	0.8	-19.0	16.6	
<b>LPS:AS-1R</b>	1	30	0	0.0	30	22	73.3	0.0	1.0	0.0		
	2	30	0	0.0	30	20	66.7	0.0	1.1	4.7	6.6	
<b>F4:AS-1R</b>	1	30	0	0.0	30	18	60.0	0.7	1.2	18.1		
	2	30	0	0.0	30	21	70.0	0.0	1.0	0.0	10.0	
<b>F4:3.95</b>	1	30	0	0.0	30	16	53.3	1.8	1.4	27.3		
	2	30	1	3.3	29	11	37.9	4.8	0.050	1.9	45.9	15.4
<b>AS-1R:Abs</b>	1	30	0	0.0	30	13	43.3	4.4	0.050	1.7	40.9	
	2	30	0	0.0	30	13	43.3	3.3	0.100	1.6	38.1	0.0

Table 19. (cont.)

Antigen	Rep	NSM		N'	SM		$\chi^2$	prob	RP	RPS	Var
		N	Number		Percent						
3.101-2:Abs	1	25	0	0.0	25	16	64.0	0.2	1.2	12.7	
	2	25	0	0.0	25	7	28.0	7.9	0.005	2.5	60.0

<sup>1</sup> Fish were challenged by addition of sodium chloride to the tank (0.9%, wt/vol), followed by addition of A. *salmonicida* strain AS-1R to a final concentration of  $2.45 \times 10^5$  cfu/ml for 20 min.

<sup>2</sup> Abbreviations used in this table are: N, number of fish; N', N minus mortalities from non-specific causes; NSM, non-specific mortality; SM, specific mortality;  $\chi^2$ , chi square; prob, probability; RP, relative potency (% specific mortality / % specific vaccine mortality); RPS, relative percent survival [ $(1 - [1/RP] \times 100)$ ]; Var, variation between percent mortality in replicate groups one and two; Abs, absorbed antisera with A. *salmonicida* strain AS-1S or 3.101-3; FK, formalin-killed.

<sup>3</sup> Pooled data from replicate groups 1 and 2 had  $\chi^2 = 0.2$ .

<sup>4</sup> Pooled data from replicate groups 1 and 2 had  $\chi^2 = 0.2$ .

<sup>5</sup> Pooled data from replicate groups 1 and 2 had  $\chi^2 = 6.5$  ( $p = 0.025$ ) and RPS = 35.8.

Table 20. Detection of latent infection with *Aeromonas salmonicida* in chinook salmon passively immunized with rabbit or rainbow trout antisera.<sup>1,2</sup>

Antisera	Rep	N	NSM	N'	SM	Carrier Rate
Saline	1	9	0	9	9	100.0
	2	12	0	12	12	100.0
Rabbit preimmune	1	8	0	8	8	100.0
	2	9	0	9	7	77.8
Rabbit AS-1R:Fk	1	28	0	28	28	100.0
	2	24	0	24	24	100.0
Rabbit AS-1S:Fk	1	23	0	23	23	100.0
	2	22	0	22	22	100.0
Rabbit 3.101-1:Fk	1	24	0	24	24	100.0
	2	12	0	12	11	91.7
Rabbit 3.101-2:Fk	1	30	0	30	30	100.0
	2	29	0	29	28	96.6
Rabbit 3.101-3:Fk	1	22	0	22	22	100.0
	2	21	0	21	21	100.0
Rabbit F4:3.95	1	14	0	14	14	100.0
	2	19	0	19	19	100.0
Rabbit AS-1R:Abs	1	24	0	24	24	100.0
	2	17	0	17	17	100.0
Rabbit 3.101-2:Abs	1	27	0	27	27	100.0
	2	25	0	25	25	100.0
Trout preimmune	1	6	0	6	5	83.3
	2	8	0	8	8	100.0
Trout AS-1R:Fk	1	16	0	16	16	100.0
	2	19	0	19	17	89.5
Trout 3.101-2:Fk	1	22	0	22	20	90.1
	2	18	0	18	18	100.0
Trout F4:3.95	1	14	0	14	14	100.0
	2	16	0	16	16	100.0

Table 20. (cont.)

Antigen	Rep	N	NSM	N <sup>1</sup>	SM	Carrier Rate
Trout AS-1R:Abs	1	17	0	17	17	100.0
	2	14	0	14	14	100.0
Trout 3.101-2:Abs	1	9	0	9	8	88.9
	2	18	0	18	18	100.0
Saline Unchallenged	1	29	17 <sup>2</sup>	12	0	0.0
	2	28	0	28	0	0.0

<sup>1</sup> Passively immunized fish surviving 14 d after immersion challenge were injected with 1.0 mg of Kenalog 40, and mortalities monitored for an additional 14 d.

<sup>2</sup> Fish were challenged by addition of sodium chloride to the tank 0.9%, wt/vol), followed by addition of *A. salmonicida* strain AS-1R to a final concentration of  $2.45 \times 10^3$  cfu/ml for 20 min.

<sup>3</sup> Cause of death was attributed to infection with *Cystic* and/or *A. hydrophilia*.

## Discussion

### Virulence of Strains

Aeromonas salmonicida strains AS-1S, 3.101-2 and 3.95 were avirulent by i.m. injection into juvenile chinook salmon, having LD<sub>50</sub> values greater than 10<sup>6</sup> cfu/fish. Strain AS-1R was virulent, having an LD<sub>50</sub> of 8.5 to 32.4 cfu/fish by i.m. injection. Others (Udey and Fryer 1978, Michel 1979, Ishiguro et al. 1981, Cipriano et al. 1981, Sakai 1985a, Olivier et al. 1985a) have reported similar LD<sub>50</sub> values by injection for virulent and avirulent strains of A. salmonicida.

Strain AS-1R was also virulent by immersion challenge to chinook salmon, having LD<sub>50</sub> values ranging from 2.6 × 10<sup>2</sup> to 2.9 × 10<sup>6</sup> cfu/ml. Similar values have been reported by Cipriano (1982a) in brook trout (2.0 × 10<sup>5</sup> cfu/ml), and by McCarthy (1983) in five species of Pacific salmon (1.0 to 7.0 × 10<sup>4</sup> cfu/ml). Differences in lethal doses have been observed by Michel (1979) in rainbow trout. In this study, chinook salmon from Moccasin Creek hatchery were relatively susceptible to mortality caused by A. salmonicida, with 2.6 × 10<sup>2</sup> cfu/ml killing 50% of the fish. Chinook salmon from Iron Gate hatchery, however, were more resistant, having an LD<sub>50</sub> of 2.9 × 10<sup>6</sup> cfu/ml. Michel (1979) suggested that these differences may be due to 1) differences in the virulence of bacterial cultures used for challenges, 2) differences in sizes, strains and resistance of fish used, and 3) variations in experimental conditions.

Because strain 3.101-2 lacked virulence but possessed AP, it was of interest to determine its ability to survive within fish tissues. Munn et al. (1982) demonstrated that AP+ strains were resistant to serum

lysis in the presence of antibody and complement. It therefore seemed possible that strain 3.101-2 may be capable of surviving within the host without causing either pathology or mortality. Results from this study indicate that strain 3.101-2 neither replicates nor survives within host tissues, and is truly an avirulent strain (Table 8). A similar lack of virulence was shown with strain AS-1S (AP<sup>-</sup>, avirulent). In contrast, strain AS-1R (AP<sup>+</sup>, virulent) survived, replicated, and killed fish within 96 h of injection. Similarly, Munn and Trust (1984) also found that AP<sup>+</sup> virulent and AP<sup>-</sup> avirulent strains of *A. salmonicida* could be separated by their ability to survive and replicate within fish tissues. Avirulent AP<sup>+</sup> strains, such as 3.101-2, are candidates for live vaccines since they present native antigens (AP, LPS) on their surfaces which may elicit better protective immune responses than inactivated bacterins. Some work in this area has been completed with strain 3.101-2 (Cox and Hedrick 1987), and further research is under consideration.

#### Antisera Against Whole Cells

Rabbits injected with formalin-killed (FK) AP<sup>+</sup> *A. salmonicida* strains (AS-1R, 3.101-2) in FCA developed average agglutination titers of 3360 (640 to 5120), and contained precipitating antibodies against AP, F4 and LPS antigens. Similar agglutination titers (562 to 1280) have been observed by Udey (1977), McCarthy et al. (1983) and Olivier et al. (1985a). Agglutinating antibodies against AP in rabbit antisera against AP<sup>+</sup> cells have been reported by McCarthy et al (1983) and Udey (1977).

Coho and chinook salmon passively immunized with rabbit antisera against AP<sup>+</sup> cells were significantly protected ( $p = 0.005$ ), having RPS values of 84.2 (63.3 to 100.0) and 93.7 (79.7 to 100.0) in coho and chinook salmon, respectively. Passive immunization tests using rabbit antisera in coho (Olivier et al. 1985a) and sockeye (McCarthy et al. 1983) salmon showed comparable results. Previous active immunization studies using FK AP<sup>+</sup> cells injected in FCA (Fryer et al. 1976, McCarthy et al. 1983, Olivier et al. 1985a) have also shown protection. However, investigators using AP<sup>+</sup> strains injected without adjuvant have not observed protection (Michel 1979, Cipriano 1982b, Michel 1985, Newman and Majnarich 1985).

Rabbits injected with FK AP<sup>+</sup> *A. salmonicida* cells in FCA developed average agglutination titers of 2133 (320 to 5120). Antisera lacked precipitating antibodies against AP, but contained antibodies against LPS and F4. Similarly, Udey (1977) and McCarthy et al. (1983) observed titers of 1280 and 1024, respectively, and a lack of antibody against AP in rabbit antisera against AP<sup>-</sup> cells.

Coho and chinook salmon passively immunized with rabbit antisera against AP<sup>-</sup> strains were inconsistently protected from mortality. Antisera to strains AS-1S and 3.101-3 were protective, however, RPS values were lower than for AP<sup>+</sup> strain antisera. Average RPS values for these strains in chinook salmon were 62.2 and 93.7 for AP<sup>-</sup> and AP<sup>+</sup> strains, respectively. Antisera against strain 3.95 provided protection to coho salmon, but failed to protect chinook salmon. Strain 3.101-1 provided equivocal protection. Similar inconsistencies on the ability of rabbit antisera against AP<sup>-</sup> strains to confer

protection were seen by Olivier et al. (1985a) who observed protection in coho salmon and McCarthy et al. (1983) who observed no protection in sockeye salmon. These studies demonstrate that rabbits produce protective antibodies to antigens present on some AP<sup>-</sup> strains, however, the level of protection is lower and more inconsistent than that observed for AP<sup>+</sup> strains. Therefore, it is advantageous to use AP<sup>+</sup> strains for protective immunity.

Rainbow trout injected with FK AP<sup>+</sup> *A. salmonicida* cells in FCA developed high agglutinating antibody titers (35,840). Precipitating antibodies against LPS and F4, but not AP, were detected by CIE. These titers are somewhat higher than those reported by others. Fryer et al. (1976) observed agglutination titers of 1024 to 2048 in coho salmon, Olivier et al. (1985a) 2560 to 10,240 in coho salmon, Spence et al. (1965) titers exceeded 320 in rainbow trout, Udey (1977) 2560 to 20,480 in coho salmon. The lack of detectable antibodies against AP in fish antisera to FK cells has been reported by others (Udey 1977), however, Hastings (pers. comm.) has observed anti-AP antibodies in fish antisera by western blotting. The failure to detect antibodies to AP in this study may be due to a lower sensitivity of the CIE test compared to western blotting.

Chinook salmon passively immunized with trout antisera against AP<sup>+</sup> cells were marginally protected, having RPS values of 54.7 (40.9 to 63.6). Similarly, Spence et al. (1965) observed protection of coho salmon immunized with rainbow trout antisera, and Udey (1977) and Olivier et al. (1985a) passively protected juvenile coho salmon by immunization with subadult coho salmon antisera against an AP<sup>+</sup> strain.

Rainbow trout injected with FK AP<sup>+</sup> A. salmonicida cells in FCA developed agglutinating antibody titers of 30,720 (10,240 to 81,920), and had precipitating antibodies against LPS and F4 antigens. Similar titers (2560 to 10,240) have been reported for fish injected with AP<sup>-</sup> cells by Krantz et al. (1963), Krantz et al. (1964a), Udey (1977) and Olivier et al. (1985a). No protection was observed using this antisera in passive immunization tests with chinook salmon. Udey (1977), McCarthy et al. (1983) and Olivier et al. (1985a) also observed a lack of protection by fish antisera against AP<sup>+</sup> strains. Apparently fish are unable to respond to the protective antigen on some AP<sup>-</sup> strains which is recognized by rabbits.

Rabbits injected s.c. with live A. salmonicida strain 3.101-2 developed no agglutinating or precipitating antibodies, therefore passive protection tests using this antisera were not conducted. Olivier et al. (1985a), however, observed titers of 640 in rabbit antisera against a live frozen AP<sup>+</sup> strain of A. salmonicida. A difference in the route of administration (Olivier et al. 1985a injected i.v., whereas in this study antigens were injected s.c.) of the antigen may have accounted for the difference in humoral immune response. The i.v. route was purposefully avoided in this study due to previous mortality among rabbits injected with bacterial cells by this route. Olivier et al. (1985a) observed that antisera against live AP<sup>+</sup> cells of A. salmonicida passively protected juvenile coho salmon from furunculosis.

Rainbow trout injected with live AP<sup>+</sup> and AP<sup>-</sup> strains of A. salmonicida developed low levels of agglutinating antibodies. These

ranged from 40 to 80 (60) for AP<sup>+</sup> strains to 0 to 640 (250) for AP<sup>-</sup> strains. Michel (1982) observed similar titers (40 to 80) in rainbow trout injected repeatedly with sublethal doses of virulent *A. salmonicida*, and Cipriano (1982b) measured titers averaging 512 in rainbow trout injected with either AP<sup>+</sup> or AP<sup>-</sup> strains. No precipitating antibodies were observed in any of the antisera in this study, however, an EDTA soluble precipitin (presumed to be C-reactive protein) was observed in antisera against virulent strain AS-1R. The humoral immune response to live antigens administered without FCA was poor compared to that observed using FK cells in FCA. Additionally, none of these antisera were protective in passive immunization tests. Krantz et al. (1964b) and Paterson (1972) also observed a poor humoral immune response using antigens without adjuvant. Cipriano (1982b), in contrast to results in this study, observed protection among brook trout passively immunized with rainbow trout antisera against live AP<sup>+</sup> or AP<sup>-</sup> strains of *A. salmonicida* without adjuvant.

#### Absorbed Antisera

Fish passively immunized with antisera against FK AP<sup>+</sup> cells absorbed against their mutant AP<sup>-</sup> derivatives were protected from mortality due to *A. salmonicida*. Relative percent survival values exceeding 60 (average was 67.8) indicated excellent protection. McCarthy et al. (1983) and Olivier et al. (1985a) also observed protection in sockeye and coho salmon passively immunized with absorbed rabbit antisera.

Absorbed rabbit antisera lacked agglutinating antibodies against AP<sup>-</sup> cells and precipitating antibodies against LPS and F4, but

possessed precipitating antibodies against AP. Similarly, Trust et al. (1982) were able to remove all LPS but no AP antibodies from rabbit antisera by this absorption method.

Absorbed rainbow trout antisera lacked detectable precipitating or agglutinating antibodies against AP or any of the tested A. salmonicida antigens. However, some protection (average RPS was 37.7) was observed. Others have been unable to detect antibodies against AP in fish antisera (Udey 1977), and Dorson (1987) observed that fish respond to only about 10% of the antigens normally immunogenic to mammals. Hastings (pers. comm.), however, has detected antibodies against AP in rainbow trout antisera by western blotting methods. Because antisera lacked complement activity (due to heating) or antibodies to LPS or other cell associated antigens (due to absorption) the protection conferred by absorbed trout antisera is believed to be due to (non-detected) antibodies against AP.

The results of this study indicate that AP is a protective antigen of A. salmonicida, in agreement with McCarthy et al. (1983) and Olivier et al. (1985a). However, an appropriate adjuvant, such as FCA, is required to elicit a protective humoral immune response. An adjuvanting effect of LPS (also present on the surface of AP<sup>+</sup> cells) has been described (Johnson et al. 1956), however, LPS alone appears to be inefficient in eliciting amounts of anti-AP antibody sufficient for protection. Although cellular immunity is an important factor in protection against furunculosis (Olivier et al. 1985b), results from this study demonstrate that antibody plus "unprimed" host immune cells are sufficient for protection. Also, virulent A. salmonicida strains

provided better protection from mortality due to furunculosis than avirulent strains (Michel 1982, Paterson 1981, Olivier et al. 1985a, Udey and Fryer 1978, McCarthy et al. 1983). This protection was associated with the presence of an additional antigen, AP, on virulent strains (Udey 1977, McCarthy et al. 1983, Olivier et al. 1985a).

#### Purified Antigens

A-protein. Purified AP from *A. salmonicida* strains AS-1R and 3.101-2 appeared identical by SDS-PAGE, having molecular weights of  $49.5 \pm 0.5$  kdal, as previously shown by Trust et al. 1980 (50 kdal), Kay et al. 1981 (48.9 + 0.5 kdal), Evenberg and Lugtenberg 1982 (54 kdal), Phipps et al. 1983 (50.1  $\pm$  0.6 kdal), Kay et al. 1984 (48 to 50 kdal), Johnson et al. 1985 (49 kdal) and Ward et al. 1985 (51 kdal).

Contaminants were observed in purified AP at approximately 55 and 42 kdal. These were believed to be LPS (55 kdal) and an outer membrane protein (42 kdal). Others have also noticed contamination of purified AP with outer membrane proteins between 40 and 50 kdal (Kay et al. 1981, Kay et al. 1984, Evenberg et al. 1982).

Lipopolysaccharide contamination of purified AP has previously been detected in rabbit immune sera (Trust et al. 1982, Phipps et al. 1983). Using solid phase ELISA (enzyme linked immunosorbant assay), Trust et al. (1982) and Munn et al. (1982) detected antibodies against LPS and AP in rabbit antisera against purified AP. Phipps et al. (1983) were unable to detect LPS contamination in purified AP using gas liquid chromatography, or Schiff or silver staining of polyacrylamide gels. However, rabbits injected with purified AP developed antibodies against

LPS. The authors speculated that LPS may have been adsorbed onto AP during the purification procedure.

In my study, both rabbit and rainbow trout injected with purified AP developed antibodies to LPS, detected by CIE. These antibodies corresponded to LPS contamination observed in silver stained SDS-PAGE of purified AP (Figure 3).

Immunological homogeneity of AP from various *A. salmonicida* strains has been demonstrated by cross reactivity of rabbit antisera against purified AP from one isolate with AP from various other *A. salmonicida* isolates (Evenberg et al. 1982, Kay et al. 1984), using the methods of gel radiimmunoassay, ELISA, western blotting, and indirect fluorescent antibody technique (IFAT). Homogeneity was also seen between AP from *A. salmonicida* strains AS-1R and 3.101-2 in this study using rabbit antisera against FK AP+ cells reacted against purified AP (Figure 9B), absorbed antisera against FK AP+ cells reacted against sonicated cells (Figure 9A), and absorbed antisera against purified AP, 3.101-2 reacted by CIE against APEF, AS-1R (Table 11).

Antisera against purified AP was cross reactive with native AP on live *A. salmonicida* cells by IFAT (Kay et al. 1984) and by western blotting (Phipps et al. 1983). Such cross reactivity indicates retention of native configuration of antigenic epitopes on purified AP, and supports the suitability of using purified AP for immunization studies.

Precipitating antibodies against AP were detected by CIE in antisera from rabbits injected with purified AP from *A. salmonicida* strain 3.101-2, but not in antisera from rabbits injected with AP from strain

AS-1R. Precipitin lines in stained CIE gels indicated (non-quantitatively) small amounts of antibody against AP, but large amounts of antibody against LPS and F4. Rabbits injected with purified AP from strain AS-1R had distinct precipitin lines against LPS and F4, but lacked any reaction with AP. These observations suggested a relatively larger amount of antibody against LPS/F4 than against AP. Antisera against AP developed agglutination titers of 30 to 40 against AP<sup>-</sup> *A. salmonicida* cells. The immunogenicity of purified AP in rabbits has been reported by Evenberg et al. (1982), Munn et al. (1982), Trust et al. (1982), Phipps et al. (1983), and Kay et al. (1984). Antibodies against AP have been detected and/or titrated by gel radicimmunoassay, ELISA, IFAT, western blotting, and agglutination with AP<sup>+</sup> *A. salmonicida* cells. Trust et al. (1982) and Munn et al. (1982) prepared rabbit antisera against purified AP and found 10 to 100 times more antibody against AP than LPS using an ELISA titration. The same investigators reported agglutination titers of 40 and 32, respectively, in antisera from rabbits injected with purified AP.

Rainbow trout injected with purified AP developed higher agglutination titers (1280) than observed in rabbits (35). Precipitating antibodies against LPS and F4 were observed, however, no antibodies against AP were detected. No reports were found in the literature, for comparison purposes, on fish injected with purified AP.

In this study, fish passively immunized with either rabbit or trout antisera against purified AP were not protected from challenge with virulent *A. salmonicida*. These tests appear to be the first attempt to use purified AP in either active or passive immunization tests. Others

(Trust et al. 1982, Munn et al. 1982) have observed good antibody responses to purified AP in rabbits, but have not conducted passive immunization tests. The lack of protection observed in this study was probably due to a low level of antibodies against AP in either fish or rabbit sera. A higher level of antibodies to AP, and passive protection to fish from furunculosis, was observed in absorbed antisera from rabbits injected with AP+ FK cells. Reasons for the poor humoral immune response to purified AP are unknown, however, purified AP may be less immunogenic than cell associated AP, possibly due to structural changes occurring during the purification process. Also, cell associated AP may have better immunogenicity because of close association with other cell bound antigens (LPS, outer membrane proteins). Additionally, no antibodies against AP were detected in rabbits or fish injected with live AP+ cells in FIA. Thus it appears that a suitable adjuvant (FCA) is required to elicit a measurable response to AP.

Lipopolysaccharide. The total yield of LPS purified from lyophilized *A. salmonicida* cells was 2.5%, within the range (2.0 to 4.5%) reported for this method by Westphal and Jann (1965) and Williams and Chase (1968) for gram-negative bacteria. Characteristics of *A. salmonicida* LPS by SDS-PAGE were in agreement with Chart et al. (1984), who reported a high molecular weight O-polysaccharide-core-oligosaccharide-lipid A fraction in multiple bands between 45 and 65 kdal, and a fast migrating low molecular weight core oligosaccharide-lipid A fraction of 10 kdal. They observed resolution of the high molecular weight

fraction into several distinct, yet closely associated light brown bands in silver stained gels. Similar bands were observed in this study for *A. salmonicida* strains AS-1R, AS-1S, 3.101-2 and 3.95. Results from this study indicated a molecular weight of 54.1 to 59.3 kdal and 14.0 kdal for the high and low molecular weight fractions, respectively.

Chart et al. (1984) observed O-polysaccharide side chains to be highly immunogenic in rabbits (antibody titers were not reported). Rabbit antisera against purified *A. salmonicida* LPS was cross reactive with heterologous *A. salmonicida* LPS tested by immunoblotting and IFAT. Similarly, LPS from four *A. salmonicida* strains in this study were immunologically identical in immunodiffusion tests.

In contrast to the highly immunogenic nature of LPS reported in mammals (Elin and Wolff 1974) and rabbits (Chart et al. 1984), a poor immune response was observed in this study. Rabbit antisera against purified LPS had a poor agglutinating antibody titer (10 to 80) and no precipitating antibodies were detectable by CIE. Fish passively immunized with this antisera were not protected.

Trout immunized with *A. salmonicida* LPS developed agglutinating antibody titers of 5120, and precipitating antibodies against LPS and F4 were observed by CIE. Paterson (1972) also observed agglutinating antibody titers of 960 to 2560 following injection of Al(OH)<sub>3</sub> precipitated *A. salmonicida* LPS into coho salmon. He observed precipitating antibodies (by immunodiffusion) against LPS only if antisera agglutinating titer equalled or exceeded 1280. Ingraham and Alexander (1980) also observed high agglutinating antibody titers

(averaging 19,500) by indirect hemagglutination in brown trout injected with Salmonella typhimurium LPS mixed in FCA. Olivier et al. (1985a) observed high agglutinating antibody titers in coho and sockeye salmon injected with LPS purified from a virulent A. salmonicida strain. Challenged fish, however, were not protected from furunculosis. Similarly, passively immunized salmon in this study were not protected by antisera against purified A. salmonicida LPS.

Fraction 4. The UV absorbance profile of eluted fractions from concentrated ECP obtained from 4 to 5 d old broth cultures of A. salmonicida was similar to that described by Cipriano et al. (1981) and Cipriano (1983b). The elution of F2 as two separate peaks (F2-1 and F2-2), however, differed from Cipriano et al. (1981). Protein yields for each fraction were within the ranges listed by Cipriano et al. (1981), who observed a protein concentration of 59 ug/ml (35 to 75 ug/ml) for F4. In comparison, 66 ug/ml (50 to 75 ug/ml) of F4 were obtained in this study.

Lipopolysaccharide was the major component of F4, with multiple bands observed between 54.1 to 59.3 kdal, and a single band at 14.0 kdal. An 18.6 kdal protein was also observed. Newman (pers. comm.) has also observed LPS and a protein of about 20 kdal in F4 preparations. Faintly staining bands between 20 and 50 kdal observed in some F4-samples probably represented proteins present in very low amounts. In contrast, Cipriano (1982c) described F4 as a glycoprotein with a 66 kdal protein subunit and a carbohydrate moiety of approximately 55 kdal. It is likely that the carbohydrate fraction

observed by Cipriano (1982c) was the LPS O-side chain (54.1 to 59.3 kdal) observed in this study. The 66 kdal protein was not observed in this study, or by Newman (pers. comm.). Cipriano (1982c) included bovine plasma albumin (MW = 66 kdal) as a molecular weight standard in his polyacrylamide gels, and it is possible that it may have contaminated F4 sample wells.

Williams and Chase (1968) noted that only the first component eluting from a column can be obtained in pure form. Following eluting components are always contaminated by preceding components. Cipriano et al. (1983) recognized F1 as being composed primarily of endotoxin (LPS), therefore, F2, F3 and F4 would also contain LPS in smaller and smaller amounts, according to Williams and Chase (1968). In this study LPS was observed in each fraction (data not shown), with the largest amounts present in F1. Also, Newman (pers. comm.) found F1 to be composed mostly of LPS, F2 was about 80% LPS with some protein, F3 contained less LPS and several proteins, and F4 contained the least LPS and only one major protein. Others, (Overbeeke et al. 1980, Hcfstra and Dankert 1980) have also observed LPS to contaminate highly purified bacterial proteins.

Rabbit or trout antisera against F4 developed high agglutinating antibody titers, and precipitated LPS and F4, but not AP when tested by CIE. By immunodiffusion, anti-F4 sera recognized F4 and LPS as identical, but failed to react with purified AP. In contrast, rabbit antisera against AP<sup>+</sup> strain AS-1R precipitated AP, F4 and LPS, demonstrating AP to be distinct from LPS/F4. These results differ from those of Cipriano (1982c) who found F4 to be immunologically distinct

from either AP or LPS. The differences observed may be attributed to the use of 0.2% SDS in this study to counteract the hydrophobicity and enhance the diffusion of purified LPS into agarose gels. Cipriano (1982c), however, suspended F4 and LPS in phosphate buffer prior to immunodiffusion tests. If F4 is a degraded form of LPS, it may diffuse into agarose more rapidly than the purified, hydrophobic LPS molecule, and thus may form a precipitin line with antibody at a different location than LPS-antibody precipitin lines. These differences would be recognized as immunological reactions of non-identity, however, in fact they are due to different migration rates of antigenic molecules into the agarose gel matrix.

Coho and chinook salmon passively immunized with rabbit or trout antisera against F4 antigens either lacked protection or were marginally and inconsistently protected (depending on the strain of F4). Rabbit anti-F4, AS-1R provided low (0.0 to 8.6) RPS values and was not protective. However, rabbit anti-F4, 3.95 provided higher RPS values (18.0 to 44.7) and offered marginal protection. Similar results were observed with chinook salmon passively immunized with trout antisera. Trout anti-F4 from strain AS-1R provided low (0.0 to 18.1) RPS values and no protection, while trout anti-F4, 3.95 provided higher RPS (27.3 to 45.9) values and marginal protection.

Because rabbit or trout antisera against washed FK 3.95 cells was not protective, the presence of a non-cell associated protective antigen in broth culture supernatants from strain 3.95 seems likely. Although Cipriano did not conduct passive immunization tests using anti-F4 sera, results from active immunization tests using F4 derived

from virulent, avirulent and atypical strains of A. salmonicida indicated excellent protection (Cipriano 1982c). In this study a low level of protection by passive immunization indicated the production of low levels of protective antibodies to F4. Since antibodies to LPS were not protective, the protective antibodies must have been against other antigens in F4. Purified extracellular proteases from A. salmonicida have provided protection in active immunization tests (Sheih 1984) and were probably the protective antigens in F4. Cipriano (1982c) may have stimulated a low humoral response to proteases combined with activation of the cellular immune system, which together gave excellent protection.

Recovery of Agglutinating Antibodies from Passively  
Immunized Fish

Groberg et al. (1979) observed a rapid uptake of immunoglobulin from the peritoneal cavity of i.p. injected coho salmon into blood, with maximum levels existing 8 h post injection. Groberg et al. (1979) and Voss et al. (1980) studied the catabolism of radiolabelled coho salmon immunoglobulin in coho salmon. They observed half lives of approximately 49 h at 12 C. Faster catabolic rates were observed at higher temperatures, and the relationship was expressed by a temperature coefficient equal to 1.5 h/deg. Results in this study indicate half lives (as measured by recovery of serum agglutinating antibodies) of 152 h for rabbit antisera in coho salmon at 15 C, 249 h for rabbit antisera in chinook salmon at 18 C, and 387 h for rainbow trout antisera in chinook salmon at 18 C. The slower catabolism

observed in this study may be associated with 1) a lack of existing enzyme systems to catabolize heterospecific antibodies, or 2) the use of whole serum rather than purified antibody. Further differences in the half lives observed by Groberg et al. (1979) and Voss et al. (1980) and this study may be associated with differences in detection methods. The prolonged presence of circulating antibody in passively immunized fish supports the suitability of using passive immunization/challenge procedures to measure the protective abilities of antibodies.

#### Detection of Latent Infection

Chinook salmon passively immunized with rabbit antisera against AP<sup>+</sup> *A. salmonicida* strains and immunosuppressed 14 d after challenge had a 99.2% infection (carrier) rate. Coho salmon similarly treated had infection rates of 41.3% at 42 d and 0.0% at 56 d. Fish passively immunized with rabbit antisera against AP<sup>-</sup> strains had corresponding infection rates of 99.0% at 14 d, 43.9% at 42 d and 42.1% at 56 d. From these data it appears that, in addition to protecting from mortality, antibodies against AP may aid fish in the slow elimination of *A. salmonicida* from host tissues.

In contrast, Michel (1979) observed a 65% carrier rate in surviving rainbow trout vaccines (AP<sup>+</sup> strain) 90 d following challenge. Inconsistencies in the establishment or detection of carrier states were observed by Michel (1985). He observed carriers in both vaccinated/non-protected and control groups of rainbow trout immunosuppressed 60 d after immersion challenge. He found three of nine groups tested had *A. salmonicida* carriers, with no difference

observed between vaccine and control groups.

The mechanisms for the establishment and elimination of the carrier state are unknown, and further work in this area is needed.

"Privileged" sites (e.g. host tissues with little or no operative defense mechanisms) may be colonized by A. salmonicida to create carrier fish. McArdle et al. (1986) have observed A. salmonicida microcolonies on or between secondary gill lamellae of carrier fish.

The bacteria are enclosed by a thin membrane continuous with the basement membrane of the secondary lamellae, and this may represent a privileged site. Another such privileged site may exist within fish phagocytic cells. Invasive A. salmonicida cells resist lysis by host antibody and complement (Munn et al. 1982) and the opsonized bacteria are phagocytized by fish leukocytes (Sakai 1985b). Munn and Trust (1984) have suggested that A. salmonicida cells may survive within leukocytes as facultative intracellular parasites, however, mechanisms allowing A. salmonicida to resist phagolysosomal enzymes have yet to be described.

Vaccines which protect against mortality must not lead to the establishment of carrier states. Release of latently infected hatchery reared salmonid fishes into waters containing native fishes susceptible to furunculosis could have devastating effects. Additionally, stock transfers of carrier fish between fish farms would widely disseminate A. salmonicida.

## Summary and Conclusions

1. Rabbit antisera containing antibodies against AP consistently protected passively-immunized coho and chinook salmon from mortality due to furunculosis.
2. Rabbit antisera against AP- *A. salmonicida* cells contained antibodies against LPS but lacked antibodies against AP. These antisera provided inconsistent and poorer protection than corresponding antisera against AP+ cells.
3. Rainbow trout antisera against AP+ *A. salmonicida* cells, and absorbed antisera, lacked detectable antibodies against AP, however, these antisera were marginally protective.
4. Rainbow trout antisera against AP- *A. salmonicida* cells were not protective.
5. A-protein is a protective antigen of *A. salmonicida*.
6. A-protein was purified by a novel procedure. It was extracted from bacterial cells by alkaline hydrolysis (ALKOH), selectively precipitated with ammonium sulfate, and purified by preparative gel electrophoresis.
7. The molecular weight of AP was  $49.5 \pm 0.5$  kdal.

8. The AP of strains AS-1R and 3.101-2 were identical by SDS-PAGE analysis.

9. Immunological homogeneity of AP was observed by immunodiffusion and counterimmunoelectrophoresis (CIE).

10. Purified AP was poorly immunogenic in rabbits and rainbow trout. Juvenile salmon passively immunized with antisera against purified AP were not protected. The reasons for the poor immunogenicity of purified AP are unknown, however, the possibility exists of a structural change during purification, resulting in lowered antigenicity.

11. Lipopolysaccharide (LPS) purified from four A. salmonicida strains by the Westphal-Jann procedure was identical by SDS-PAGE, consisting of a high molecular weight O-polysaccharide-core-oligosaccharide-lipid A component in distinct multiple bands (at light loading) between 54.1 and 59.3 kdal, and a low molecular weight core-oligosaccharide-lipid A fraction at 14.0 kdal.

12. Immunological homogeneity of LPS from four strains of A. salmonicida was demonstrated by immunodiffusion and CIE.

13. Purified LPS was poorly immunogenic in rabbits, but strongly immunogenic in rainbow trout.

14. Antisera from rabbits or trout against LPS were not protective in passive immunity tests.

15. Lipopolysaccharide is not a protective antigen of A. salmonicida.

16. Fraction 4 obtained by the methods of Cipriano et al. (1981) was composed primarily of LPS, but also contained a major protein of 18.6 kdal.

17. Fraction 4 from four A. salmonicida strains was identical by SDS-PAGE analysis. Immunological homogeneity was observed by immunodiffusion and CIE. Cross reactivity was also observed between F4 and purified LPS.

18. Fraction 4 was highly immunogenic in both rabbits and rainbow trout.

19. Passive protection experiments with antisera against F4 gave variable results. Antisera against F4, AS-1R failed to passively protect juvenile salmon. Antisera against F4, 3.95 provided an inconsistent, low level of protection.

20. The protective abilities of F4, 3.95 are presumably due to a non-cell associated antigen.

21. Carrier fish having latent infections with A. salmonicida existed

among surviving passively-immunized salmon receiving AP+ or AP- antisera. At 14 d post-immunization the carrier rate was near 100% among all treatments tested. However, at 56 d post immunization only fish immunized with AP- antisera harbored latent infections. This suggests that antibodies against AP may aid in the slow elimination of A. salmonicida from host tissues.

22. Passively immunized salmon had measurable levels of agglutinating antibodies 10 d (rabbit antisera) to 23 d (trout antisera) after injection. Antibody half lives were 200.5 h and 387.0 h for rabbit and trout antisera, respectively.

23. An unusual AP+ avirulent strain of A. salmonicida was isolated, and its avirulence confirmed by standard LD<sub>50</sub> testing and in vivo tissue survival tests. Its potential use as a live vaccine for furunculosis warrants further attention.

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Appendix A. Biochemical characteristics of Aeromonas salmonicida  
strains grown at room temperature (22 to 25 C) for  
48 h on API-20E test strips.

Test	Strain					
	AS-1R	AS-1S	3.101-1	3.101-2	3.101-3	3.95
ONPG	-	-	-	-	-	-
ADH	+	+	d	+	+	+
LDC	-	-	-	-	-	+
ODC	-	-	-	-	-	+
CIT	-	-	-	-	-	-
H <sub>2</sub> S	-	-	-	-	-	-
URE	-	-	-	-	-	-
TDA	-	-	-	-	-	-
IND	+	-	-	-	-	-
VP	-	-	-	-	-	-
MOT	-	-	-	-	-	-
GEL	+	+	+	+	+	+
GLU	+	+	+	+	+	+
MAN	+	+	+	+	+	+
INO	-	-	-	-	-	-
SOR	-	-	-	-	-	-
RHA	+	-	-	-	-	-
SAC	+	-	-	-	-	-
MEL	+	-	-	-	-	-
AMY	+	-	-	-	-	-
ARA	-	-	-	-	-	-
OXI	+	+	+	+	+	+
NIT REC	+	+	+	+	+	+
NO <sub>2</sub> GAS	-	-	-	-	-	-

+: positive reaction.

-: negative reaction.

d: variable reaction.

Appendix B. Sensitivities of Aeromonas salmonicida strains to selected antibiotic drugs.

	<u>Strain</u>									
	<u>E. coli</u> <sup>1</sup>		AS-1R		AS-1S		3.101-2		3.95	
Drug <sup>2</sup>	ZI <sup>3</sup>	RX <sup>4</sup>	ZI	RX	ZI	RX	ZI	RX	ZI	RX
CHL	25	S	26	S	30	S	30	S	30	S
ERY	6	R	10	R	12	R	12	R	10	R
GEN	22	S	22	S	18	S	19	S	20	S
MYX	21	S	25	S	24	S	25	S	22	S
NIT	21	S	22	S	21	S	22	S	23	S
NOV 30	10	R	18	I	16	R	18	I	16	R
NOV 5	6	R	15	R	11	R	12	R	12	R
PEN	6	R	16	I	14	I	16	I	12	I
SULF X	24	S	6	R	6	R	6	R	6	R
SULF D	25	S	12	R	6	R	6	R	12	R
TET 30	24	S	17	S	17	S	13	R	12	R
TET 5	18	I	6	R	6	R	6	R	6	R
VIB	21	S	6	R	6	R	6	R	6	R

<sup>1</sup> *Escherichia coli* reference strain 25922 from the American Type Culture Collection (ATCC) was used for control reactions.

<sup>2</sup> Antibiotic drugs and amount used were : CHL, chloramphenicol, 30 ug; ERY, erythromycin, 15 ug; GEN, gentamycin, 10 ug; MYX, myxin, 10 ug; NIT, nitrofurazone, 300 ug; NOV 30, novobiocin, 30 ug; NOV 5, novobiocin, 5 ug; PEN, penicillin G, 10 units; SULF X, sulfasoxazole, 300 ug; SULF D, sulfadiazine, 1000 ug; TET 30, tetracycline, 30 ug; TET 5, tetracycline, 5 ug; VIB, vibriostat, 150 ug.

<sup>3</sup> ZI : zone of inhibition measured in mm.

<sup>4</sup> RX : reaction (R = resistant, S = sensitive, I = intermediate).