Bacterial and Viral Diseases of Fish

Molecular Studies

Jorge H. Crosa, Editor

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To Erling J. Ordal, teacher
and pioneer in fish microbiology

Preface

The aquaculture industry has in the past few years increased very significantly on a world-wide basis, although it has long been an established component of the Pacific Northwest economy.

The pen rearing of Pacific salmon (Onchorhynchus sp.) demonstrated not only the potential of this industry but also the impact of disease. In most cases disease may be the result of interactions between the fish and infectious agents, although physical and perhaps chemical factors may also play a role on the precipitation of a diseased condition. Hence the relationship between a pathogen, the host fish and the environment is very complex and many parameters may have a strong influence on its final outcome and the resultant fish loss.

Losses from some bacterial disease situations could be eliminated (or reduced) by the use of antibiotics but because of the risk inherent in antibiotic therapy, considerable efforts have been channeled towards obtaining effective vaccines which in the case of diseases of a viral etiology may be one of the few approaches available.

Several laboratories are intensively working towards an increased understanding of the molecular mechanisms of pathogenesis of bacterial and viral diseases of fish. Knowledge of the molecular causes of viral and bacterial virulence will hopefully lead to the development of efficacious vaccines.

It is the intention of this book to put together in one collection current research carried out on some of the most important bacterial and viral diseases of salmonids. The first paper gives a detailed account of those diseases which are considered to be major sources of mortality among populations of cultured salmonids. This paper also deals with the methods used for the prevention of the spread of these diseases. The focus turns next to the analysis of those bacterial and viral pathogens of fish in which the molecular aspects of the systems are fully documented. Thus, the three following papers analyze the causative

agents of vibriosis: Vibrio anguillarum and V. ordalii and furunculosis: Aeromonas salmonicida.

The molecular study of two viral systems, infectious hematopoietic necrosis (IHN) and infectious pancreatic necrosis (IPN) viruses is the subject of the last two papers in this collection.

Although brief, I hope that this book will give the reader an appreciation of the techniques used and the general status of the knowledge in this fascinating field.

I wish to express my gratitude to Dr. William R. Davis for his continuing encouragement and support and to Dr. Stanley Murphy for believing in the feasibility of a molecular approach to the study of fish disease. I am also grateful to the Washington Sea Grant Communications staff for the excellent job done in putting together this collection.

J.H. Crosa
Portland, October 1982

Bacterial and Viral Diseases of Cultured Salmonids in the Pacific Northwest

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Introduction

This report reviews the important bacterial and viral diseases encountered among populations of cultured salmonids in the Pacific Northwest. Methods utilized for preventing the spread of these diseases are described and factors influencing the host-parasite relationship are discussed.

The control of infectious diseases of fish remains the single most important factor in the success of aquaculture. In the past two decades, the construction of new state and federal fish hatcheries and the development of private aquaculture have intensified the rearing of salmonid fish. There is an increased realization of the necessity to avoid infectious agents and to maintain healthy stocks of fish to enhance production. Of particular concern are those diseases which do not respond to chemotherapeutic substances and are classed as nontreatable diseases. Examples are the viruses, drug resistant bacteria and certain of the deep tissue protozoans.

Bacterial Diseases

A variety of bacteria produce infectious diseases of fish. These bacteria and the diseases associated with them are listed in Table 1. Among stocks of salmon in the Pacific Northwest there are 10 of these bacterial diseases of major concern because of the economic loss they cause among commercially important fish. Included for each disease discussed is a description of the causative agent, number of serotypes, host range, control methods, and selected features of the host-parasite relationship.

Furunculosis

Aeromonas salmonicida, the causative agent of furunculosis, is the most intensively studied bacterial pathogen of fish. It is ubiquitous among populations of cultured salmonids and has been

Table 1. Bacterial pathogens of fish and the diseases associated with them.

Bacterium	Disease
Aeromonas salmonicida	Furunculosis
Aeromonas hydrophila	Motile aeromonas septicemia
Yersinia ruckeri	Enteric redmouth
Vibrio anguillarum	Vibriosis
Vibrio ordalii	Vibriosis
Flexibacter columnaris	Columnaris
Cytophaga psychrophila	Low temperature disease
Cytophaga or *Flavobacterium*	Bacterial gill disease
Renibacterium salmoninarum	Bacterial kidney disease
Lactobacillus sp.	Pseudokidney disease
Edwardsiella tarda	Edwardsiellosis
Pseudomonas sp.	Bacterial hemorrhagic septicemia
Pasteurella sp.	Pasteurellosis
Cytophaga and others	Fin rot
Sporocytophaga sp.	Salt water columnaris
Mycobacterium sp.	Mycobacteriosis
Norcardia asteroides	Nocardiosis
Streptomyces sp.	Streptomycosis
Streptococcus sp.	Streptococcosis
Eubacterium tarentellus	Eubacterial meningitis

responsible for severe mortality. The disease may occur in an acute or chronic form depending upon water temperature, host resistance, number of bacteria and virulence of the strain. In the acute form, losses may be very severe without the development of typical symptoms (Snieszko and Bullock 1975).

The bacterium is a short, nonmotile, nonsporulating, nonencapsulated Gram negative rod and is an oxidase positive facultative anaerobe. The G+C content of the DNA is 58%. Most strains appear antigenically similar. Significant differences exist, however, in the virulence of strains. Differences in highly virulent strains have been shown to be associated with the presence of an additional layer (A layer) on the cell surface (Udey and Fryer 1978; Munn et al. 1982).

Aeromonas salmonicida can be grown on a wide variety of bacteriological media forming small, white, round, raised, convex colonies. Most strains produce a brown, water soluble pigment after about 48 hr incubation at 18°C. Three subspecies are commonly recognized based on the production of pigment, gas and fermentation reactions (Paterson et al. 1980).

All salmonid species are susceptible to infection with A. salmonicida and epizootics have been observed in both fresh and saltwater. The bacterium is considered an obligate pathogen of freshwater fish, but may be found free-living for short periods of time. Carrier fish are the main reservoir of A. salmonicida and transmission occurs primarily via water. Infected fish may exhibit either chronic or acute disease depending on host resistance, strain virulence and environmental factors such as temperature. The gross pathology is similar to that of other Gram negative septicemias in fish. Areas of focal necrosis, abscesses, hemorrhaging of fins and internal organs and a generalized bacteremia are the most common signs of the disease process. In addition, hemorrhage of the gills and bluish areas under the skin are sometimes observed (Wood 1974).

Epizootics of A. salmonicida are commonly controlled by feeding chemotherapeutic substances such as sulfa drugs or oxytetracycline. Of particular concern in recent years, is the emergence of drug resistant strains of A. salmonicida which can not be controlled with either of these antimicrobial compounds. A potentiated sulfonamide (RO5-0037) has been tested against A. salmonicida with very encouraging results (Bullock et al. 1974). The criteria for FDA approval have nearly been completed and the drug may be available to fish culturists in the near future. Effective vaccines against A. salmonicida have proven difficult to develop but currently research is being devoted to this problem (Paterson 1981).

Motile aeromonas septicemia

Aeromonas hydrophila is another Gram negative bacterial fish pathogen which produces a hemorrhagic septicemia and severe losses under certain conditions. Most strains do not seem to be highly

invasive but, when the environment becomes unfavorable for the host, A. hydrophila is capable of producing mortality.

Like most other members of the genus, A. hydrophila is a motile, nonsporeforming, nonencapsulated rod. It is facultatively anaerobic, oxidase positive, fermentative and usually nonpigmented. The G+C content ranges from 57-63% and many serotypes exist. The organism grows well at 37°C and produces white, circular, convex colonies on agar media.

All cultured species of freshwater fish seem susceptible to A. hydrophila and the bacterium is widespread geographically. Under conditions of stress or at elevated water temperatures (Groberg et al. 1978), the disease process resembles other Gram negative septicemias of fish. Control of motile aeromonas septicemias is by administration of oxytetracycline or sulfa compounds in the feed. Due to the large numbers of serotypes of A. hydrophila, little hope for an effective vaccine may be expected.

Enteric redmouth

Yersinia ruckeri was formerly referred to as the Hagerman redmouth or enteric redmouth bacterium (Ewing et al. 1978). Originally, this organism was thought to occur principally in stocks of rainbow trout in the Hagerman Valley of Idaho (Ross et al. 1966). With improvements in diagnostic methodology, Y. ruckeri is now known to occur among populations of many salmonid species throughout most of the United States.

The bacterium is a short, oxidase negative, Gram negative rod, motile by peritrichous flagella with a G+C content of 48%. It grows well on agar media forming colonies which are circular, creamy, entire and convex. Motility is present at 22°C but absent at 37°C (O'Leary et al. 1979). At least three serotypes are now known (Stevenson and Daly 1982).

The disease caused by Y. ruckeri is commonly known as enteric redmouth or ERM and is characterized by a bacteremia with inflammation and hemorrhage of the jaw and palate. Additional signs resemble other Gram negative septicemias of fish. While a chronic form of the disease accompanied by low mortality is common, epizootics may occur if environmental conditions become unfavorable for the fish. The disease can often be controlled with oxytetracycline and sulfa drugs although many drug-resistant strains of Y. ruckeri exist. Efficacious vaccines have been developed against Y. ruckeri (Tebbit et al. 1981).

Vibriosis

Vibrio anguillarum and Vibrio ordalii, the causative agents of vibriosis in salmonids, produce serious losses in fish reared in saltwater along the Pacific Coast. These bacteria are extremely infectious, and produce a bacterial hemorrhagic septicemia with somewhat different pathology (Ransom 1978). The advent of salmonid mariculture was dependent upon development of methods for

the control of this disease. Fish transported from fresh to saltwater would often develop signs of vibriosis within four to five days with catastrophic losses occurring within the following seven to ten days.

Like other members of the Vibrionaceae, these bacteria are short, curved, nonencapsulated, nonsporeforming, Gram negative rods, motile by polar flagella. Both are facultative anaerobes, oxidase positive and have G+C contents of 43-46%. Vibrio anguillarum grows rapidly producing circular, convex, cream-colored colonies. Vibrio ordalii is slower growing on primary isolation, requiring 48 hr at 18°C to form similar colonies. The most important serotype of V. anguillarum is serotype I. Vibrio ordalii is a new species formerly classified as V. anguillarum serotype II (Schiewe et al. 1981.Other serotypes of V. anguillarum are known to be pathogenic for fish (Aoki et al. 1981, Strout et al. 1978).

These bacteria are found in salt or brackish water and some strains are isolated from fish in freshwater. All species of salmonids are susceptible. Vibriosis is a typical Gram negative septicemia with extensive hemorrhaging and necrosis of internal organs and necrotic foci in the musculature. The disease may be but is rarely chronic. Recent studies by Crosa (1980) have identified virulence factors associated with plasmids in certain of these vibrios.

Control of vibriosis in cultured salmon was previously restricted to the use of antimicrobial compounds such as oxytetracycline. Highly effective vaccines have been developed against both V. anguillarum and V. ordalii (Amend and Johnson 1981).

Columnaris

Columnaris disease, caused by Flexibacter columnaris has long been recognized as a major disease of salmonid fish. A thorough review of the organism has been prepared by Becker and Fujihara (1978). Massive losses have been attributed to F. columnaris among populations of juvenile salmonids in hatcheries and among adult fish returning to spawn.

Because of improved environmental conditions in certain hatcheries and in holding facilities for adult salmon, losses due to columnaris have been reduced. However, F. columnaris is a ubiquitous pathogen in the Northwest and will become a problem whenever appropriate environmental conditions occur.

Flexibacter columnaris is a slender Gram negative rod which exhibits gliding motility on solid surfaces. The cells in vitro and in vivo tend to lie in parallel masses or columns, giving the organism its name. The bacterium is aerobic and requires a dilute, simple medium for growth (Cytophaga agar, Anacker and Ordal 1959). Colonies are dry, rhizoid and yellowish after 3 days incubation at 20°C. Four serological groups are recognized and significant differences in virulence have been shown among selected strains.

Fish affected with columnaris disease exhibit lesions of the gills, skin and musculature. The gills are most commonly involved where yellowish lesions, severe necrosis and erosion of the gill filaments are typical signs. Bacteria in columnar masses can be observed in wet mounts made from the leading edges of the lesions. The organism becomes systemic and the disease process is greatly accelerated at higher water temperatures (Holt et al. 1975).

Oxytetracycline has been used to control outbreaks of columnaris in hatcheries, and bath treatments with the herbicide, Diquat, are also recommended (Wood 1974). Reduction of water temperature is the single most effective method of prevention of columnaris among salmonids. No effective vaccines against this disease are currently available.

The largest natural fish kill ever recorded in the State of Oregon occurred in Klamath Lake, Klamath County, in 1971 and was caused by F. columnaris. The mortality was composed of nonfood fish (rough fish) and the resident biologists estimated thirteen to fourteen million fish were killed during the epizootic (Rohovec and Fryer 1979).

Low-temperature disease

Cytophaga psychrophila is the causative agent of low-temperature disease or bacterial cold-water disease. It is particularly common among cultured coho salmon fry in the coastal streams of western Oregon and Washington when water temperatures are in the range of 5 to 10°C. The disease can usually be controlled by the use of oxytetracycline substances provided the fish have been transferred to ponds and are taking food. Cold-water disease is particularly severe among young fry in hatchery troughs where treatment is difficult. Annual epizootics of cold-water disease occur among cultured coho salmon at hatcheries where the bacterium is endemic.

Cytophaga psychrophila is a long, thin, Gram negative rod which exhibits gliding motility. It does not form microcysts nor fruiting bodies and also requires a simple dilute media (Anacker and Ordal 1959) for growth. Colonies are moist, spreading, and yellowish resembling a "fried egg" after 3-4 days incubation at 18°C. Only one serotype has been reported (Pacha 1968).

Fish infected with C. psychrophila typically show saddle-like lesions near the dorsal fin. Masses of cytophagal organisms may be found in these lesions. The fish often become dark and, in advanced cases, a bacteremia will occur. Oxytetracycline is commonly used in controlling outbreaks of cold-water disease. No vaccines are available against C. psychrophila.

Bacterial gill disease

Bacterial gill disease is caused by a long, thin, filamentous Gram

negative rod. The organism has never been appropriately classified, and it is possible that more than one agent is involved. When environmental conditions impose unreasonable stress on the host, this disease can result in excessive fish mortality. An estimated nine million fall chinook salmon died at the Bonneville Salmon Hatchery in Oregon in the spring of 1978 as a result of bacterial gill disease.

Koch's postulates, required to establish the causative agent of a disease, have never been completed for bacterial gill disease. Bacteria can be isolated from diseased fish with relative ease and grown in pure culture. However, when susceptible hosts are exposed to these isolates under experimental conditions, the typical disease process fails to occur (Wakabayashi et al. 1980).

Bacterial gill disease is reported worldwide in hatchery reared salmonids. Wet mounts of gills from fish affected with this disease show masses of flavobacterial or cytophagal-like organisms covering large areas of the gills. Gill filaments are often swollen, clubbed or fused together. Control of the disease usually involves elimination of the bacteria from the gill surface with bath treatments of germicides or disinfectants (Wood 1974).

Bacterial kidney disease

Bacterial kidney disease remains one of the most serious bacterial infections of salmonids. The causative agent, Renibacterium salmoninarum, does not respond effectively to any of the readily available chemotherapeutic substances and because of the slow, insidious nature of the disease may continue to produce deaths over long periods of time. There is increasing evidence that the infectious process continues in salmonids after they have entered salt water (Ellis et al. 1978).

Renibacterium salmoninarum is a short, nonmotile, nonsporeforming, nonacid-fast, oxidase negative, catalase positive, Gram positive rod that was formerly classified as a Corynebacterium (Sanders and Fryer 1980). The organism is slow-growing and requires a complex medium containing cysteine and usually serum. The optimal temperature for growth is 15-18°C and several weeks may be required before small, circular, convex, white or creamy yellow colonies can be observed. The G+C content is 53% and only one serotype has been identified (Fryer and Sanders 1981).

The bacterium is an obligate pathogen occurring intracellularly. Fish infected with R. salmoninarum develop a slow chronic disease characterized by grey-white necrotic abscesses in the kidney (Bullock et al. 1975). Other internal organs and the eyes may be affected in severe cases. All salmonid species tested are susceptible and the disease is present in many stocks of fish throughout the Northwest. No effective control method for the disease is known.

Pseudokidney disease

Brood trout (primarily rainbow and cutthroat) occasionally show signs of a condition referred to as pseudokidney disease. Examination of these fish often reveals the presence of a large Gram positive bacterium belonging to the genus Lactobacillus (Ross and Toth 1974) and Gram stains of this organism are occasionally confused with those of R. salmoninarum.

The Lactobacillus sp. associated with pseudokidney disease is an oxidase negative, catalase negative, facultative anaerobe with a G+C content of 34-36%. Optimal growth occurs at 25-30°C on tryptic soy agar which helps distinguish this organism from R. salmoninarum. Colonies are white, pinpoint, circular and entire.

Fish infected with this organism may show no pathological signs. Chronic infection may lead to a distended abdomen, muscular abscesses and ascites. Higher temperatures and the stress of spawning are thought to accelerate the disease process. Oxytetracycline has been used prophylactically before spawning to reduce the mortality associated with this disease.

Edwardsiellosis

Edwardsiella tarda was first reported as a fish pathogen by Meyer and Bullock (1973). The agent has since become recognized as a serious problem in cultured catfish in the southern United States. Recently, however, E. tarda has been isolated from Pacific salmon in the Northwest (Amandi et al. 1982).

The organism is a nonsporeforming, Gram negative rod which is motile by peritrichous flagella. It grows well at an optimal temperature of 37°C forming small, grey, circular, transparent, raised colonies. The bacterium is oxidase negative, produces H_2S gas and has a G+C content of 53-59%. A wide range of homeotherms and poikilotherms are hosts for E. tarda. Isolates from fish are serologically similar.

Catfish infected with E. tarda typically exhibit a chronic disease process. Mortality is often low until the fish are stressed. Then, gas-filled abscesses begin to form in the musculature, becoming necrotic and foul-smelling. In catfish, the name "emphysematous putrefactive disease" was given to this condition but the terms "edwardsiellosis" or "edwardsiella septicemia" are now more commonly used to name this disease.

Salmon have been found to be naturally infected with the bacterium at warmer water temperatures. Experimentally, the organism has been shown to be capable of causing serious mortality in salmonid fish. Edwardsiellosis may be controlled by antimicrobial compounds; however, reduction in water temperature is an important adjunct to chemotherapy.

Table 2. Viruses of fish which have been isolated and characterized.

Virus	Group
Infectious Pancreatic Necrosis Virus	Reovirus-like
Infectious Hematopoietic Necrosis Virus	Rhabdovirus
Herpesvirus salmonis	Herpesvirus
Channel Catfish Virus	Herpesvirus
NeVTA Virus	Herpesvirus
Oncorhynchus masou Virus	Herpesvirus
Lymphocystis Virus	Iridovirus
Carp Gill Necrosis Virus	Iridovirus-like
Bluegill Virus	Orthomyxovirus
Eel Virus 2	Orthomyxovirus
Eel Virus A	Rhabdovirus
Eel Virus X	Rhabdovirus
Viral Hemorrhagic Septicemia Virus	Rhabdovirus
Spring Viremia of Carp Virus	Rhabdovirus
Pike Fry Rhabdovirus	Rhabdovirus
Golden Shiner Virus	Reovirus-like
Eel Virus E	Reovirus-like
Chum Salmon Virus	Reovirus
Eel Virus 1	Ungrouped
Grunt Fin Agent	Ungrouped

Viral Diseases

At least 20 viruses have been isolated from fish (Table 2) and an additional 14 (Table 3) have been observed by means of electron

Table 3. Viruses of fish observed by electron microscopy.

Virus	Group
Erythrocytic Necrosis Virus	Iridovirus
Virus of Epitheliosum Papillosum	Herpesvirus
Turbot Herpesvirus	Herpesvirus
Pleuronectid Papilloma Virus	Ungrouped
Walleye Sarcoma Virus	Ungrouped
Walleye Epidermal Hyperplasia Virus	Ungrouped
White Sucker Epidermal Papilloma Virus	Ungrouped
Brown Bullhead Papilloma Virus	Ungrouped
Atlantic Salmon Papilloma Virus	Ungrouped
Intraerythrocytic Virus of Rainbow Trout	Ungrouped
Northern Pike Epidermal Proliferation Virus	Ungrouped
Ulcerative Dermal Necrosis Virus	Ungrouped
Esocid Lymphosarcoma Virus	Ungrouped
Atlantic Salmon Fibrosarcoma Virus	Ungrouped

microscopy (Wolf and Mann 1980). In the Pacific Northwest, four viruses have been identified among populations of salmonids. Three of these (infectious pancreatic necrosis virus, infectious hematopoietic necrosis virus, and Herpesvirus salmonis) have been isolated and characterized, while the fourth (erythrocytic necrosis virus) has yet to be isolated in cell culture. The viruses of fish have been reviewed by Pilcher and Fryer (1980) and McAllister (1979).

Infectious pancreatic necrosis

Infectious pancreatic necrosis virus (IPNV) was isolated in the Pacific Northwest in the early 1960's, but it was not observed to be a major cause of mortality among salmonids until the early 1970's. During the years 1971 through 1973, the entire brook trout program in the State of Oregon for those years was eliminated as a result of this virus. Heavy losses of rainbow trout also occurred. Infectious pancreatic necrosis virus remains

widespread throughout portions of the Columbia River Basin and in certain high mountain lakes.

Infectious pancreatic necrosis is a viral infection that characteristically causes epizootics among the fry of various species of salmonids. Brook trout are especially susceptible. Infected fry exhibit whirling, darkening, exopthalmia and petechia in the muscles and viscera. The mortality rate is usually high.

The IPN virion is nonenveloped, has icosahedral symmetry and is approximately 65 nm in diameter. The genome of the virus is composed of 2 segments of double-stranded RNA. The virus can be propagated in several fish cell lines at 20°C where it causes a cytopathic effect (CPE) composed of rounded, detached and spindle-shaped cells. At least 3 serotypes of IPNV are recognized. The disease and virus have been reviewed by Scherrer (1973), Wolf (1976), and Pilcher and Fryer (1980).

Infectious hematopoietic necrosis

Infectious hematopoietic necrosis (IHN) is a viral disease principally affecting fry of sockeye and chinook salmon and rainbow trout. Reviews of this disease have been published by Amend et al. (1973), Wolf (1976) and Pilcher and Fryer (1980). The natural range of IHNV is the Pacific Coast of North America extending from the Sacramento-San Joaquin River system in California to Alaska where the virus occurs endemically.

Infectious hematopoietic necrosis virus is a rhabdovirus (Amend and Chambers, 1970). The virion is a bullet-shaped particle measuring approximately 170 x 80 nm. It is enveloped and the genome is composed of single-stranded RNA (McAllister et al. 1974, McCain et al. 1974). The virus replicates in most of the commonly used fish cell lines producing a CPE consisting of rounded cells in "grape-like" clusters. Only one serotype has been reported, but some serological differences in strains of the virus are thought to exist (McCain et al. 1971).

Salmon fry infected with IHNV exhibit darkening, exopthalmia, abdominal swelling, petechia of musculature and fins and often trail white fecal casts. Epizootics usually occur in the spring when water temoeratures rise and fry of a susceptible size (1-2 g) are present. Losses to IHNV can exceed 80-90% in some outbreaks.

Infectious hematopoietic necrosis virus will remain a major management problem because of the high incidence of carriers among adult salmon returning to streams where the virus is endemic. No treatment is available for viral diseases of fish, but elevation of water temperature has been used at some hatcheries to reduce the severity of epizootics. This treatment has not been effective in reducing the numbers of carrier fish. Research with attenuated and killed vaccines is still in the experimental stage but shows some promise.

Viral erythrocytic necrosis

Viral erythrocytic necrosis (VEN) was first described by Laird and Bullock (1969), as a pathological condition in the erythrocytes of three fish species collected from coastal waters of eastern Canada and the northeastern USA. Eosinophilic inclusion bodies measuring up to 1 µm in diameter were observed in the cytoplasm of infected erythrocytes. The nucleus was distorted, and round clear vesicles developed, containing dense staining particles from 250 to 500 nm in diameter. A possible viral etiology was suggested, and the disease was first named piscine erythrocytic necrosis (PEN).

Additional studies of this disease in Atlantic cod have been reported by Appy et al. (1976) and Walker and Sherborne (1977). These included electron microscopic examinations of infected cells. Both groups observed cytoplasmic particles with hexagonal or pentagonal profiles resembling iridovirus virions.

Viral erythrocytic necrosis has also been reported in fish from the Pacific Coast of North America. Evelyn and Traxler (1978) observed VEN in chum and pink salmon reared in pens at Departure Bay, British Columbia. The virus has also been found in adult chinook, coho and chum salmon, steelhead trout and herring from the coastal waters of Oregon (Rohovec and Amandi 1981).

Limited progress in understanding this disease is anticipated until the infectious agent can be isolated and propagated. At the present time, it is not known if a single viral agent or a group of related viruses is responsible for this disease.

Herpesvirus disease

Wolf and Taylor (1975) and Wolf (1976) reported the occurrence of a 30 to 50% post-spawning mortality among rainbow trout broodstock. This loss had occurred annually since 1971 at the Winthrop Fish Hatchery in Washington State. Each year, an agent presumed to be a virus was isolated by W. G. Taylor from ovarian fluids of the diseased fish, but its viral nature was not confirmed until 1975. The characteristics of the virus indicate it is a member of the herpesvirus group. The agent has been named Herpesvirus salmonis (Wolf et al. 1978).

Rainbow trout fry have been used to study pathological changes caused by H. salmonis (Wolf and Smith 1981). The effect of the virus on adults has not been determined. Herpesvirus salmonis was originally isolated from ovarian fluid, indicating that a carrier state existed in some adult rainbow trout. The virus has only been observed in the USA and perhaps Japan (Sano 1976) where several similar viruses have been isolated.

The origin of the virus is unclear, but it may have been transmitted via egg shipments from Japan to the U.S. The Winthrop hatchery was disinfected and all carrier brood fish were

destroyed. Since then, H. salmonis has not been isolated from fish at the hatchery and may have been eliminated from North America.

Host Parasite Relationships and Disease Control

Disease in fish is the result of an interaction between the host, the pathogen and the environment (Wedemeyer 1970, Snieszko 1974). With this relationship in mind it is possible to consider how temperature, density, genetics and presence of the pathogen affect the disease process.

It is well established that salmonid fish are highly sensitive to changes in the quality of their environment. Experiments have been conducted in our laboratory to test the effects of water temperature or rainbow trout, and coho and chinook salmon infected with A. salmonicida and A. hydrophila (Groberg et al. 1978), bacterial kidney disease (Sanders et al. 1978), Ceratomyxa shasta (Udey et al. 1975) and F. columnaris (Holt et al. 1975). In each case an optimal range of temperature for the progress of infection was observed. With the exception of bacterial kidney disease, lower temperatures favored the host, and higher temperatures accelerated the disease process.

Rearing fish in excess of the carrying capacity of the hatchery is a major contributing factor to disease. Crowding favors the transmission of infectious agents and produces stress in the host which depresses disease resistance. Bacterial gill disease outbreaks have been observed to occur regularly at densities in excess of 0.6 Kg of fish per liter of water per minute (Wood 1974).

Selective breeding of salmonid fish has been practiced in one form or another for many years. In addition to characteristics such as ocean survival, fecundity and maximum size, it is also possible to select stocks showing resistance to a specific disease. In a study conducted in our laboratory (Zinn et al. 1977) it was found that fall chinook salmon raised from eggs of parents taken from rivers in which the protozoan C. shasta is endemic were highly resistant to challenge with the agent and suffered only 0-13% mortality. On the contrary, those fish from parents taken from rivers where the parasite was absent, suffered an 88-100% mortality. This difference is presumed to be the result of natural selection in the presence of the pathogen.

In addition to environmental and host considerations, one of the most effective methods for controlling disease results from efforts to eliminate or reduce the contact with disease agents. Several approaches have been used to achieve this goal. In the hatchery, ponds and equipment are routinely sanitized between groups of fish with beneficial results. Sanitizing agents containing chlorine or iodine are most commonly employed (Wood 1974).

The disinfection of eggs, both before shipment and upon receipt at

the hatchery, has become routine. Iodophors are the disinfectant of choice and will eliminate many disease agents which could be transported on the surface of the egg (Amend 1974). Recent evidence, however, seems to indicate that IPN virus and R. salmoninarum may not be killed by this method, perhaps because these agents are inside the egg or somehow sequestered from contact with the disinfectant.

Pasteurization of the fish processing wastes which are incorporated into salmon diets has effectively eliminated fish tuberculosis caused by Mycobacterium sp. This disease was formerly widespread in the Columbia River basin hatcheries, and its disappearance is thought to be a result of breaking the cycle of reinfection caused by the feeding of raw salmon viscera.

Hatchery water supplies have also been modified in order to reduce contact with disease agents. Wherever possible, hatcheries utilize well or spring water sources that are fish pathogen free. Other hatcheries have constructed extensive treatment facilities using high rate sand filtration followed by ultraviolet radiation, ozone or chlorination systems to render water supplies pathogen free. Hatchery designs using reduced flows, recycled water and water flowing through consecutive ponds are regarded as less desirable from a disease standpoint as they allow the number of pathogens to increase and favor disease outbreaks.

Contact with disease agents can also be reduced by policies which will eliminate or reduce the numbers of carrier fish both in a hatchery or in the wild. While such a commitment may be difficult to initiate, it has been successful where applied. Fall River in central Oregon is a short, spring-fed stream on which a trout hatchery is located. Below the hatchery is a natural obstruction (waterfall) which prevents fish passage. An epizootic in 1973 was diagnosed as IPN and further sampling revealed IPNV carrier fish in the stream above the hatchery. The decision was made to kill the surviving fish as well as all wild fish above the hatchery and to sanitize the entire facility. Following these procedures the river and hatchery were restocked with virus-free fish. Since that time, no isolation of IPN virus has occurred at the station.

Recent evidence indicates that the stocking of virus-free fish alone may reduce or eliminate the carrier population. In Canada, trout from lakes have been sampled to determine the percentage of IPNV carriers before and during stocking with IPNV-free fish. The percentage of IPNV carriers declined to very low or non-detectable levels in these lakes (Yamamoto 1978). A similar example of the dilution of virus carriers is reported from Utah which stocked IPNV carrier fish in 1968 and 1969 in Schofield reservoir. Beginning in 1970 only virus-free fish were stocked and no detection of IPNV has occurred in samples taken after 1971 (FHS Newsletter 1978).

One of the most important management techniques for controlling the spread of infectious agents involves the establishment of a

disease control policy for a given geographic area such as a watershed, system of lakes, state or country (Fryer et al. 1979). The first requirement of such a policy is an extensive sampling program to determine the geographic distribution of various disease agents within that area (Mulcahy et al. 1980). In the Pacific Northwest, certain serious fish pathogens (whirling disease, caused by Myxosoma cerebralis, and Viral Hemorrhagic Septicemia) have never been detected.

Once the geographic distribution of known fish pathogens has been determined, a rational disease-inspection and certification program can be established. The disease-free certification of stocks to be imported or moved between watersheds will prevent the introduction or further spread of those agents with a limited geographic range. Many states have recently implemented such a disease control policy. In Oregon, examinations must be conducted for the viruses: VHS, IHN and IPN; the protozoans: C. shasta and M. cerebralis; the bacteria: A. salmonicida and Y. ruckeri; and R. salmoninarum. Currently, at least 34 of the 50 states in the USA have some type of required disease inspection. The establishment of an effective disease control policy, coupled with the other management techniques dicussed, will continue to reduce the incidence and severity of fish diseases in the Pacific Northwest.

Acknowledgments

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The Genetics of Plasmid-Mediated Virulence In the Marine Fish Pathogen *Vibrio anguillarum*

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Introduction

The many demonstrations of fish disease due to Vibrio anguillarum establish this bacterium as one of the most prevalent of marine pathogens (Canestrini, 1893; Cisar and Fryer, 1969; Evelyn, 1971; Rucker et al., 1953). However, despite the voluminous literature on this vibrio as a common pathogen, the actual molecular basis for this important disease is poorly understood, although its similarities with invasive septicemic diseases in man are quite appalling.

The sequence of events is similar to mammalian inflammation except for a very slow digestion and lysis by the phagocytic cells. The fish inflammatory response takes longer to initiate and the whole process goes slower (Finn and Nielson, 1971; Ingram and Alexander, 1977). Experimental infections caused by low-virulence strains of V. anguillarum (Braaten and Hodgins, 1976) do not proceed beyond these initial stages but if the infecting V. anguillarum strain is of a high-virulence phenotype, the disease progresses with the appearance of characteristic bloody lesions in the musculature and a generalized septicemia; death ensues from hypoxia and dysfunction of various organs (Harbell et al, 1979; D. Ransom, Ph.D. Thesis, Oregon State University, Corvallis, 1978). Obviously, the high-virulence strains of V. anguillarum have developed the ability to evade the fish specific and non-specific mechanisms of defense against infection.

The many similarities of the pathogenesis of this disease as compared to invasive septicemic diseases in man, together with the relative facility with which fish can be manipulated make the fish - V. anguillarum system an attractive animal model. By using the fish model we have recently shown that many of the high-virulence strains of V. anguillarum isolated from epizootics harbor a specific plasmid class which is absent from low-virulence strains (Crosa, et al 1977). Curing experiments have confirmed a link between the presence of this specific plasmid which we called pJM1, and the ability of V. anguillarum to establish infections

(Crosa, et al, 1980), suggesting that this plasmid carries genetic determinants for an important component of the virulence repertoire of this bacterium.

In general, all bacterial virulence factors promote growth in the antagonistic environment of the host defense mechanisms. One line of defense is provided by the proteins transferrin and lactoferrin which bind iron, an essential element for bacterial growth (Bullen et al, 1978; Neilands, 1981; Weinberg, 1978). These proteins are normally unsaturated and limit the availability of iron in blood and on mucosal surfaces which, in turn, may result in enhancement of non-specific resistance to bacterial infections (Bullen et al, 1978; Neilands, 1981; Payne and Finkelstein, 1978; Weinberg, 1978). A mechanism whereby invading bacteria may selectively and successfully compete for the bound iron could become crucial in enabling them to proliferate in body fluids and tissues, and thus establish an infection.

We have recently determined that the V. anguillarum virulence plasmid specifies a very efficient iron sequestering system enabling high virulence plasmid-carrying V. anguillarum strains to survive and grow in vitro under conditions of limited iron availability in a culture medium containing high concentrations of transferrin (Crosa, 1979; 1980). Under the same conditions, low-virulence cured strains (plasmidless) are completely inhibited.

In experimental infections of fish with a mixture of both, a plasmid-carrying high-virulence strain and its cured, (plasmidless) low-virulence derivative, there was a selective advantage for the plasmid-carrying high-virulence strain. However, when iron was included in the inoculum this advantage was greatly diminished. These results demonstrated that the ability to take up iron under conditions of iron limitation is a very important virulence determinant in V. anguillarum (Crosa, 1979; 1980).

Concomitant with an efficient iron uptake by plasmid-carrying strains of V. anguillarum, new outer membrane proteins are induced. At least one of them OM2, an 86,000 dalton protein is induced only when the virulence plasmid is present (Crosa and Hodges, 1981). This outer membrane protein may play a role as a receptor for a putative plasmid-mediated siderophore.

In order to map the location of iron uptake genes and determine their association with virulence in the fish model, we initiated a mutational analysis of the plasmid-mediated iron-uptake system in V. anguillarum. In this paper, we describe the utilization of transposon-generated mutations to characterize the plasmid-mediated iron uptake system in V. anguillarum.

Analysis of Iron-uptake Deficient Mutants

A number of recent studies have demonstrated that several antibiotic resistance genes reside upon discrete sequences of DNA,

transposons, that can migrate from replicon to replicon. Transposition events occur virtually at random into a plasmid or chromosome and if the insertion event occurs within the continuity of a gene, it leads to the generation of non-leaky polar mutations (Hedges and Jacob, 1974; Heffron et al, 1975).

Recent experiments in my laboratory have shown that P1 group plasmids can be transferred from Escherichia coli K12 to V.anguillarum. RP4, one member of this incompatibility group which possesses the transposition sequence Tn1, carrying ampicillin resistance determinants (Hedges and Jacob, 1974; Heffron et al, 1975) has been especially useful as a donor of the transposition sequence Tn1 to the pJM1 plasmid in V. anguillarum (Crosa et al, 1980).

By using the Tn1 donor capacity of RP4 we were now able to generate a series of pJM1 derivatives in which the Tn1 sequence has been inserted at different sites. The methodology we used to develop these transposed derivatives is described in a previous work (Crosa et al, 1980).

Table 1 shows the properties of five of these derivatives and figure 1 shows the relative mobility of two of the derivative plasmids as compared to pJM1 in an agarose gel electrophoresis determination. All of the strains tested are now resistant to higher levels of ampicillin and as expected, the molecular weight of the virulence plasmid in most of these strains has been increased by about 3.5 megadaltons (Mdal), due to the insertion of Tn1 in the plasmid genome, although in one case 775:Tn1 (pJHC 9-8), the plasmid has actually suffered a deletion of about 8.5 Mdal, induced by the Tn1 integration (see Fig 1).

Table 1. Properties of Tn1 insertion derivatives of the V. anguillarum pJM1 plasmid.

Strain	Plasmid	Ability of plasmid-carrying bacteria to grow in the presence of 3 μM transferrin	Induction of outer membrane proteins OM2	OM 3	Virulence
775 (pJM1)	pJM1	+	+	+	1.2×10^3
775-7:Tn 1	pJHC11	+	+	+	2.5×10^3
775-8:Tn 1	pJHC12	+	+	+	4.0×10^3
775-9:Tn 1	pJHC13	+	+	+	1.5×10^3
775-6:Tn 1	pJHC91	-	+*	+	6.0×10^7
775-5:Tn 1	pJHC9-8	-	-	+	7.0×10^8

(*) Induction of OM 2 occurs in this mutant at lower levels than in the wild-type 775 (pJM1) strain

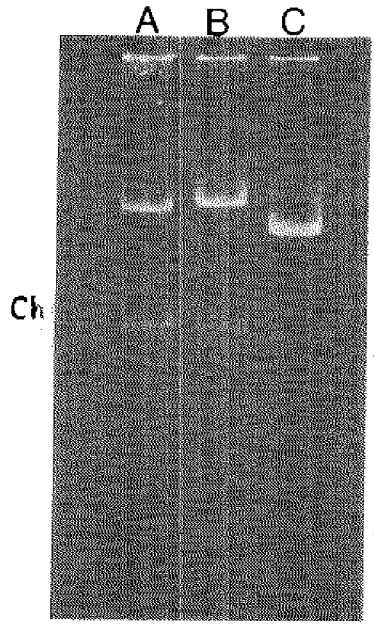

FIGURE 1: Agarose gel electrophoresis of plasmid DNA. Plasmid DNA was electrophoresed in a 0.7% agarose gel as previously described (Crosa et al, 1978). Ch band corresponds to chromosome DNA.

Lane A: pJM1
Lane B: pJHC91
Lane C: pJHC9-8

Most of the strains containing the pJM1-insertion derivatives are iron-proficient and of high virulence indicating that insertion of Tn1 took place in a plasmid region non-essential for the iron-uptake process or virulence. However, the deletion mutant 775:Tn1 (pJHC9-8) and one of the insertion derivatives, 775-6:Tn1 (pJHC91), showed an impairment in their ability to grow under conditions of iron limitation. In addition, these two derivatives showed a decreased virulence in the fish system, the LD50 (lethal dose 50: number of bacteria killing 50 percent of the fish inoculated as determined by the method of Reed and Muench, 1938) increasing by about 4 to 5 logs (Table 1). These results indicated that the iron uptake genes have been affected by insertional inactivation with Tn1 to generate the pJHC91 plasmid, as well as by the insertion-deletion process which generated pJHC9-8. Additionally our mutant data demonstrate the intimate relation between iron uptake proficiency and the high-virulence phenotype of these bacteria.

In all the known specific systems of iron transport at least two components are involved. One component is a specific siderophore (a low molecular weight compound that will bind iron) and a second is a specific membrane receptor for the iron-siderophore complex.

These two components appear to be necessary for the internalization of the iron ions by means of a specific mechanism. If the plasmid iron-uptake system in V. anguillarum acts in this fashion then our iron-uptake deficient mutants might have lesions in the production or function of either of these two components. Moreover, it seemed logical to hypothesize this type of mechanism after the finding that an iron regulated outer membrane protein, OM2, (which could be a receptor) is associated with the presence of the pJM1 plasmid. With this in mind, we decided to examine our two mutants with respect to siderophore and specific receptor usage.

Consequently, further insight into the nature of the plasmid mutations was obtained by cross-feeding experiments. Figure 2 shows that under conditions of iron limitation the iron-uptake deficient mutant 775:6:Tn1 (pJHC91) can grow only around filter discs containing supernatants from strains carrying an intact pJM1 type plasmid. Growth does not occur if the filter discs contain supernatants from either of the two iron-uptake deficient mutants or from a plasmidless strain.

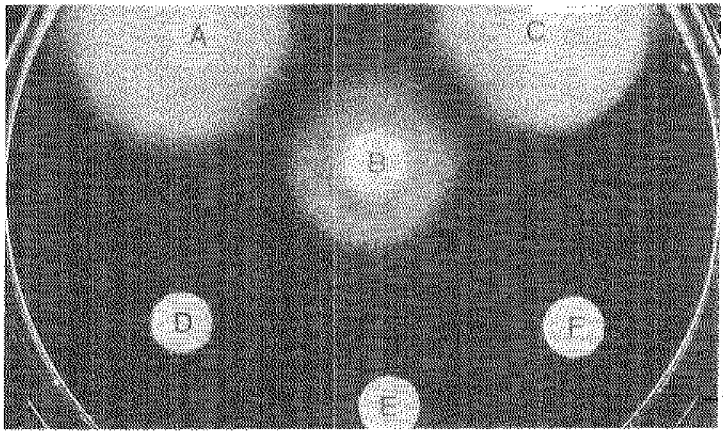

FIGURE 2: The ability of supernatants to support the growth of the 775-6:Tn1 (pJHC91) mutant in iron-depleted medium was tested by impregnating a sterile filter disc with 10 µl of supernatant from the growth of different V. anguillarum strains. Strains were grown in a minimal medium containing 10 µM EDDA (ethylenediamine-o-hydroxyphenyl acetic acid), a non-assimilable iron chelator, for 16 hrs. at 23°C. After centrifugation of the cells the supernatants were removed and filter sterilized. The discs were placed on a minimal agar plate containing 10 µM EDDA and that had been seeded with a lawn of 0.1 ml (10^8 cells) of the 775-6:Tn1 (pJHC91) strain in 4 mls of plain top agar. Supernatants tested were from the strains carrying the wild type plasmids:775 (pJM1) on disc A; 775-1:Tn1 (pJM11) on disc B; 775-8:Tn1 (pJHC12) on disc C. Supernatants from the mutants 775-5:Tn1 (pJHC 9-8) on disc D; 775-6:Tn1 (pJHC91) on disc E and the plasmidless strain 775-3 on disc F.

Figure 3 shows that this cross-feeding effect is specific for the 775-6:Tn1 (pJHC91) mutant as wild-type plasmid-carrying strains cannot cross-feed the plasmidless strain H775-3.

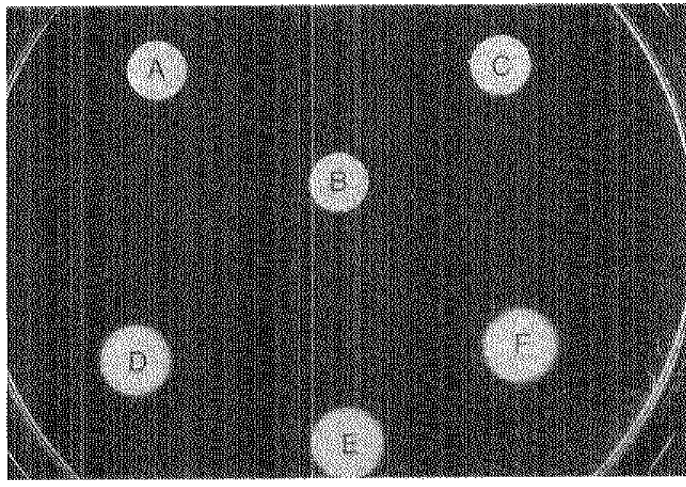

FIGURE 3. Same as Figure 2 except that the bacterial strain used for the lawn was the plasmidless derivative H775-3 and that 20 µl of bacterial supernatant per disc was used.

The very thin halos seen in this photograph around discs D,E, and F are caused by growth of the lawn bacteria utilizing the iron present at higher concentration (24 µM in the medium used to grow these iron uptake-deficient derivatives: 775-5:Tn1 (pJHC9-8) 775-6:Tn1 (pJHC91) and 775-3 (plasmidless).

The ability to utilize a diffusible siderophore produced only by wild-type plasmid-carrying strains, indicate that the plasmid-mediated outer membrane receptors in the mutant 775-6:Tn1 (pJHC91) are still functional and thus, the Tn1 insertion only affected genes involved in the synthesis of the plasmid-mediated siderophore. It can also be inferred from Fig 2 that the deletion mutant 775-5:Tn1 (pJHC9-8) could not cross-feed the insertion mutant 775-6:Tn1 (pJHC91), suggesting that the deletion process which generated pJHC9-8 also affected the plasmid genes involved in siderophore biosynthesis. An important result (shown in Fig 4) is that the deletion mutant cannot be cross-fed by wild-type plasmid carrying strains.

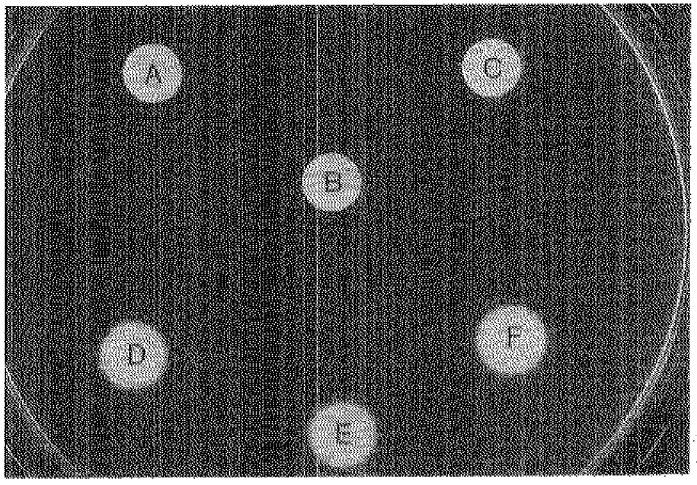

FIGURE 4. Same as Figure 3 except that the bacterial strain used for the lawn was the deletion mutant 775-5:Tn1 (pJHC9-8). The same type of thin halos are seen in D,E, and F, although these are smaller because only 10 µl of supernatant was used.

These results indicate that the 8.5 Mdal deletion affected not only the putative siderophore genes but also the genes for the cognate membrane receptor.

Analysis of the outer membrane proteins produced under mild iron limitation at 2 µM iron (the lowest iron concentration at which the iron uptake deficient mutants or plasmidless strains can grow) shows that the OM2 protein is induced in the insertion mutant 775-6:Tn1 (pJHC91) but is absent from the deletion mutant 775-5:Tn1 (pJHC9-8) (Table 1). The chromosomally mediated OM3 protein is present in both mutants. Although still circumstantial these results strongly support the contention that the plasmid-mediated OM2 protein might be the putative receptor for iron/plasmid-mediated-siderophore complexes.

Figure 5 summarizes our findings. V. anguillarum 775 possesses a plasmid that codes for an iron uptake system which consists of two components. One is a siderophore which can out-compete the vertebrate serum transferrin for bound iron and the other is a specific membrane receptor for the iron-siderophore complex. We believe the receptor is the outer membrane protein OM2. At this time we do not know if the chromosomally mediated OM3 protein plays a role in this process. The derivative carrying the pJHC91 plasmid is thus blocked in step 2 (thus not producing a siderophore) and the derivative containing the pJHC9-8 plasmid is

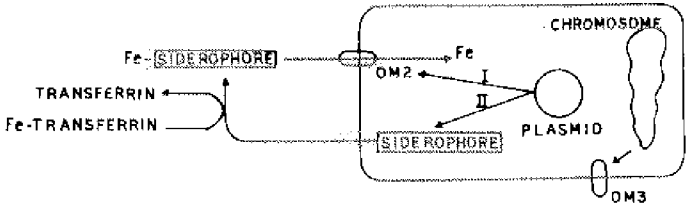

FIGURE 5. Model of the plasmid-mediated iron uptake system in V. anguillarum

blocked in both steps 1 and 2 because it cannot cross-feed or be cross-fed by the parent strain. These findings suggest a problem in both production of a siderophore and presence of a specific active receptor for this mutant.

Our mutant analysis also demonstrates that the iron transport genes are apparently the only virulence determinants carried by the pJM1 plasmid in V. anguillarum.

Of course,the ability of bacteria to cause disease is the result of a constellation of bacterial properties which ultimately lead to their establishment in the vertebrate host. For instance, in the case of V. anguillarum we have recently demonstrated that the ability to withstand the bactericidal activity of normal (non immune) fish serum appears to be another important component of the virulence repertoire of these bacteria (Trust et al, 1981). Other factors may play a role at different stages during the invasion process.

With respect to the location of the virulence genes in V. anguillarum, a plasmid-mediated system of iron-uptake is the mechanism most frequently found in virulent strains of this bacterium isolated in the Pacific Northwest. Conversely serum resistance is a chromosome-mediated trait in all the strains examined so far. Nonetheless, our recent work indicates that in certain cases a chromosome-mediated system can be responsible for iron-uptake in V. anguillarum (Toranzo et al, in press, 1983 and Crosa et al, in press 1983).

Further insight into the molecular aspects of the pJM1 plasmid-mediated iron uptake system has recently been obtained by restriction endonuclease analysis of plasmid DNA obtained from the

iron-uptake deficient transposon-generated mutants. Comparison of restriction endonuclease patterns has allowed us to localize the iron-uptake region within a specific 15 kilobase pairs (Kbp) region of the V. anguillarum virulence plasmid pJM1 DNA (M.A. Walter, S.A.Potter, and J.H. Crosa, manuscript in preparation).

Further dissection and molecular cloning of restriction fragments within this region is currently being carried out in a program to isolate the plasmid genes involved in the iron uptake process and high-virulence phenotype of V. anguillarum 775.

Acknowledgments

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Vibrio ordalii as a Cause of Vibriosis In Salmonid Fish

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Vibriosis is among the most prevalent of marine fish diseases with outbreaks reported in at least 16 countries and in 42 species of teleost fish (Anderson and Conroy, 1970). In the Pacific Northwest, vibriosis was recognized early as a major obstacle to the development of a viable salmonid mariculture industry: a fact that has, in recent years, catalyzed an intensive research effort to better define the nature of the causative agents, gain a better understanding of the pathogenesis of the syndrome, and develop more effective means of preventing and treating the disease.

Prior to this increase in the research effort, most outbreaks of vibriosis had been attributed to a single marine bacterium, Vibrio anguillarum. However, a number of subsequent studies have clearly documented the existence of a marked heterogeneity among the pathogenic vibrios isolated. In addition to a bacterium conforming to the classical description of V. anguillarum (Evelyn, 1971), a culturally, biochemically, and serologically distinct vibrio is routinely isolated in the Pacific Northwest and Japan (Harrell et al., 1976; Ohnishi and Muroga, 1976; Ezura et al., 1980).

A variety of laboratory epithets have evolved to describe this second pathogenic vibrio including Vibrio sp. 1669 (Harrell et al., 1976), Vibrio sp. RT (Ohnishi and Muroga, 1976), V. anguillarum biotype 2 (Schiewe et al., 1977), Beneckea anguillara biotype II (Baumann et al., 1978) later amended to V. anguillarum biotype II (Baumann et al., 1980), and V. anguillarum phenon II (Ezura et al., 1980). Recently, however, Schiewe et al. (1977) and Schiewe and Crosa (1981) have shown the genotypically distinct nature of this vibrio and, moreover, a degree of within-group genetic relatedness characteristic of a separate species. Based on these data the new species Vibrio ordalii has been proposed (Schiewe et al., 1981; effective publication Schiewe et al., 1982).

The purpose of the present communication is to review and summarize the current knowledge of V. ordalii as a cause of vibriosis in salmonid fish. The cultural, biochemical, and serological properties that distinguish V. ordalii and V. anguillarum are

reviewed and results of molecular-genetic characterizations of this important fish pathogen are summarized. Finally, the present, albeit limited, information on the pathogenesis of disease produced by V. ordalii is reviewed.

Cultural and Biochemical Properties

Vibrio ordalii and V. anguillarum are both Gram-negative, asporogenous, straight or slightly curved rods which are motile by a single polar flagellum. Both microorganisms are oxidase-positive, facultatively anaerobic, and sensitive to the vibriostatic compound 2,4-diamino-6,7-diisopropyl pteridine (O/129).

A detailed treatment of the phenotypic characteristics of V. ordalii and V. anguillarum can be found in a number of papers including Evelyn (1971), Ohnishi and Muroga (1976), Baumann et al. (1978), Ezura et al. (1980), and Schiewe et al. (1981). Baumann et al. (1978), using a battery of tests specifically formulated for use with marine eubacteria (Baumann and Baumann, 1981), demonstrated considerable phenotypic dissimilarity between V. ordalii and V. anguillarum. Major differences were shown in nutritional versatility with V. ordalii able to utilize substantially fewer organic compounds as sole or principal sources of carbon and energy. These differences were reflected in the results of a numerical analysis which segregated V. ordalii and V. anguillarum into two clusters linking at 70% similarity (Baumann et al., 1978). Ezura et al. (1980), using more conventional biochemical tests, also compared V. ordalii and V. anguillarum, and the results of their numerical analysis indicated linkage at the 61% similarity level.

Vibrio ordalii and V. anguillarum are readily distinguishable by as many as 17 tests routinely used in a clinical laboratory (Schiewe et al., 1981). Particularly useful in making the distinction are the 12 tests listed in Table 1. Typical results of these tests again attest to the lack of physiological and nutritional versatility by V. ordalii. These differences suggest a more host-dependent mode of existence by V. ordalii; a premise that is supported by the isolation of V. ordalii only from moribund fish. In contrast, V. anguillarum has been isolated from several environmental sources including seawater, sediment, and net-fouling material.

Serological Relationships

A number of investigators have examined both intraspecific and interspecific serological relationships within and between V. ordalii and V. anguillarum. Harrell et al. (1976), using immunodiffusion and antibody absorption techniques, demonstrated antigenic cross-reactivity between V. ordalii and V. anguillarum. Additionally, they showed the presence of antigens unique to each bacterium. Gould (1977), also employing immunodiffusion, demonstrated the antigenically distinct nature of the lipopolysaccharide extracted from V. ordalii and V. anguillarum.

TABLE 1. Tests useful in distinguishing V. ordalii and V. anguillarum, and typical results. Data from Schiewe et al. (1981).

Test	V. ordalii	V. anguillarum
Voges-Proskauer	-	+
Arginine, alkaline reaction	-	+
Citrate, Simmons	-	+
Citrate, Christensen	-	+
Starch hydrolysis	-	+
ONPG	-	+
Lipase	-	+
Growth at 37°C	-	+
Acid production from		
Cellobiose	-	+
Glycerol	-	+
Sorbitol	-	+
Trehalose	-	+

Ezura et al. (1980), in an examination of over 200 strains of bacteria isolated from outbreaks of vibriosis in Japan, found the V. ordalii strains belonged to a single serotype, J-O-1, based on somatic antigens. In contrast, the V. anguillarum strains were divisible into at least three O serotypes; however, 50% of these strains also belonged to the J-O-1 serotype.

In vivo analyses have demonstrated that V. ordalii and V. anguillarum each possess protection-inducing, heat-stable antigens that are unique, and others that are cross-protective (Schiewe and Hodgins, 1977). As shown in Table 2, coho salmon (Oncorhynchus kisutch) immunized with heat-stable components of either bacterium showed a high degree of protection against challenge with the homologous bacterium and a lesser degree of protection against the heterologous organism. In both cases, survival of heterologously challenged fish was higher than the nonimmunized controls.

TABLE 2. Comparison of percent mortality among groups of coho salmon injected with heat-killed preparations of V. ordalii, V. anguillarum, or saline (control) and subsequently challenged with live V. ordalii or V. anguillarum. Data from Schiewe and Hodgins (1977).

	Percent Mortality		
	Immunized with V. ordalii	Immunized with V. anguillarum	Saline-injected control
Challenged with V. ordalii	2	19	57
Challenged with V. anguillarum	29	0	40

The results of these serological studies have been put to considerable practical use. For example, the demonstration of antigenic differences have been exploited in the preparation of diagnostic antisera. In the Pacific Northwest, commercially prepared anti-*V. ordalii* and anti-*V. anguillarum* sera are readily available for use in the diagnosis of vibriosis and for distinguishing between causative agents (Novotny et al., 1975). Moreover, the demonstration of antigenic differences *in vivo* has provided clear evidence of the need to combine antigenic elements from both bacteria in the production of vibrio vaccines for use in those locations where both organisms are endemic.

Molecular-Genetic Characterization

The division of *V. ordalii* and *V. anguillarum* into separate bacterial species was based on genotypic as well as phenotypic differences (Schiewe et al., 1981). Deoxyribonucleic acid (DNA) hybridization analyses (Schiewe et al., 1977; Schiewe and Crosa, 1981) confirmed that *V. ordalii* strains formed a highly related group that showed greater than 83% within-group homology and less than 69% relatedness to *V. anguillarum* (Table 3). In contrast, strains classified as *V. anguillarum* were shown to form a somewhat more heterogeneous group (70% or greater within-group homology) which was, nonetheless, clearly distinct from *V. ordalii* (less than 67% related).

TABLE 3. Summary of polynucleotide sequence relationships among selected marine vibrios. Data from Schiewe et al. (1977) and Schiewe and Crosa (1981)

	Percent Homology	
	V. ordalii	*V. anguillarum*
Species		
V. ordalii	83-100	53- 67
V. anguillarum	58- 69	70-100
V. parahaemolyticus	<4	<4
V. alginolyticus	<4	<5

Additional molecular characterization of *V. ordalii* (Schiewe and Crosa, 1981) has revealed the universal presence of an extrachromosomal element, or plasmid, with a molecular mass of about $20X10^6$ daltons (Figure 1). The uniform molecular nature of these plasmids has been demonstrated by DNA-DNA hybridization and the mole-percent guanine plus cytosine ratio of a representative plasmid, pMJ101 from *V. ordalii* strain DF3k, has been estimated at 44 (Schiewe and Crosa, 1981). Although technical difficulties have prevented the determination of a precise plasmid copy number, the broad and dense nature of the ethidium bromide-stained bands in Figure 1 suggest these plasmids represent a major contribution to the whole-cell DNA pool.

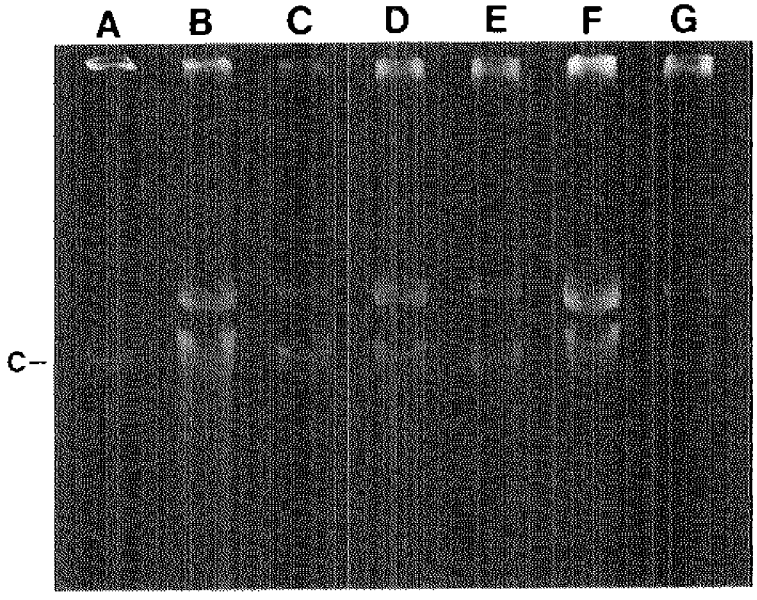

FIGURE 1. Agarose gel electropherogram of crude lysate DNA prepared from representative strains of V. ordalii. A. Molecular mass standards of 60, 38, and 5.5 Mdal; B. strain 241-S; C. strain C74-48; D. strain DF$_1$k; E. strain 45-S; F. strain 95-S; G. YAV5053. C in margin indicated the position of chromosomal DNA. From Schiewe and Crosa (1981).

The carriage of a single homologous plasmid by each of the V. ordalii strains examined is in contrast to the situation observed in V. anguillarum. Crosa et al. (1977) have shown V. anguillarum strains from diverse geographic and host origins to show substantial heterogeneity with respect to plasmid carriage. Several strains were found to be devoid of extrachromosomal DNA while others were shown to harbor one or more plasmids characterized by differing molecular mass. One of the most interesting of the V. anguillarum plasmids is a 47 x 10^6 dalton species, designated pJM1, which has been shown to play a role in virulence (Crosa et al., 1980). Genes residing on pMJ1 encode proteins that are part of a high affinity iron-sequestering system that allows this pathogenic bacterium to compete with the host under iron-limited conditions (Crosa, 1980). As such, the iron-sequestering system is an important determinant of virulence.

The possibility that pMJ101 also encodes proteins involved in the sequestration of iron and thus plays a role in the virulence of V. ordalii has not been thoroughly assessed. However, the apparent

lack of homology between pMJ101 and pJM1 (Schiewe and Crosa, 1981) suggests this is not the case. In any event, the role of pMJ101 in the biology of V. ordalii is an important question yet to be answered. Clearly the isolation of plasmidless isogenic derivatives of V. ordalii would increase the probability of resolving this question.

Pathogenesis of Infection

The pathogenesis of disease produced by V. ordalii in salmonid fish is poorly understood. Only recently have kinetic aspects of disease progression been described. Moreover, of the growing list of bacterial properties known to function as determinants of virulence, few have been studied in V. ordalii.

Ransom (1978), using standard histopathological techniques, followed the progression of naturally-acquired and experimentally-induced vibriosis caused by both V. ordalii and V. anguillarum in Pacific salmon (Oncorhynchus spp.). The results of his studies showed a marked tendency of V. ordalii to form microcolonies in vivo and preferentially colonize skeletal muscle, cardiac muscle, gill tissue, and both the anterior and posterior gastrointestinal (GI) tract. In contrast, V. anguillarum was not observed in colonies but rather was found dispersed in the tissues with the highest concentrations in the blood, loose connective tissue, kidney, spleen, gills, and posterial intestinal tract. The observation of both V. ordalii and V. anguillarum in tissue sections of the posterior GI tract and rectum early in the course of infections led Ransom to conclude that the infection process probably begins with colonization of these sites. In addition, V. ordalii was also observed in tissue sections of skin collected during the initial stages of infection, thus suggesting that this bacterium may also enter the host by direct invasion of the integument.

The fate and organ distribution of V. ordalii and V. anguillarum in juvenile coho salmon has also been followed using bacteriological culture techniques (Schiewe, 1980). As shown in Figure 2A, salmon parenterally exposed to 7.7×10^6 colony forming units (CFU) of V. ordalii rapidly developed a systemic infection with bacteria readily demonstrable in the liver, kidney, spleen and blood. Bacterial concentrations remained elevated in the kidney, spleen and blood through the first 22 hr post challenge while the concentration in the liver declined to less than 10 CFU/g. At 22 hr a gradual increase in the number of bacteria was evident in each tissue examined and by 162 hr all fish were dead. The demonstration of high bacterial concentrations in blood, spleen and kidney of V. ordalii-infected fish is somewhat in conflict with the results of Ransom (1978). However, this disparity probably resulted from the different methods of bacterial challenge used in these studies (subcutaneous injection vs. waterborne or naturally acquired infection).

Parenteral challenge of juvenile coho with approximately 1000-fold fewer viable V. anguillarum (3.2×10^3 CFU) also resulted in a systemic infection. However, bacterial proliferation occurred more

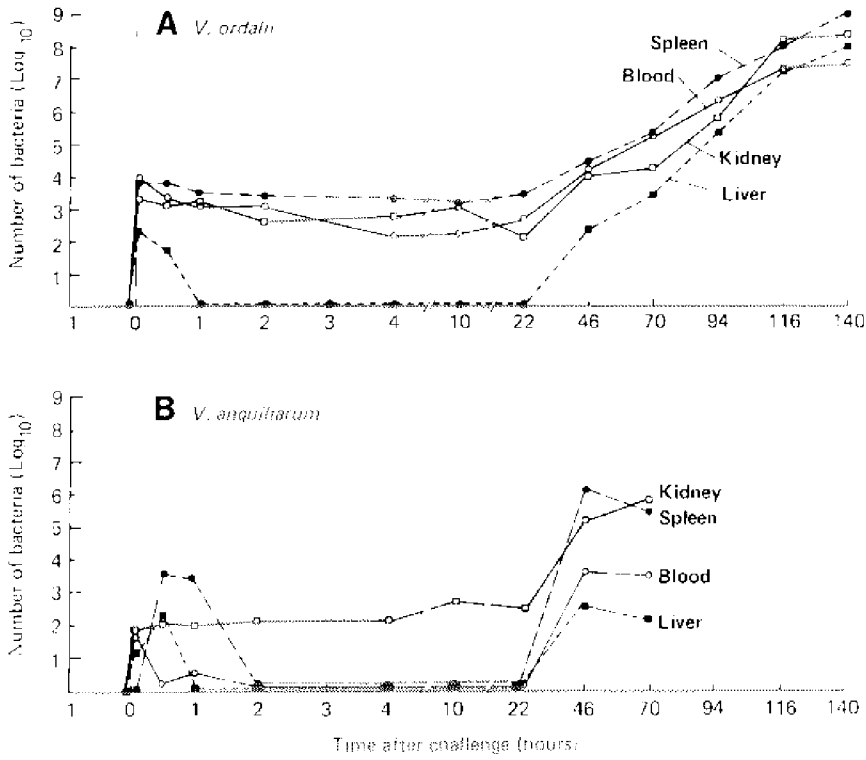

FIGURE 2. Comparison of the fate and organidistribution of V. ordalii (panel 2A) and V. anguillarum (panel 2B) in juvenile coho salmon at 15°C. Infection was initiated at time 0 with the subcutaneous injection of 7.7×10^6 CFU of V. ordalii or 3.2×10^3 CFU of V. anguillarum. One fish was sacrificed 5 min prior to bacterial challenge and one each at 5 min, 30 min, 1 hr, 2 hr, 4 hr, 10 hr, 22 hr, and subsequent 24 hr intervals post challenge. Numbers of viable bacteria per ml of blood and per g of kidney, liver, and spleen were determined by spread-plate techniques.

rapidly than was the case with V. ordalii; all fish were dead within 94 hr (Figure 2B). In addition, an eclipse-like period was observed between 2 and 22 hr post-challenge in which V. anguillarum could not be cultured from liver, spleen or blood.

A particularly noteworthy observation made during the course of several investigations on the progression of vibriosis caused by either V. ordalii or V. anguillarum has been that of the development of a marked leukopenia in moribund fish (Harbell et al., 1979; Ransom, 1979). For example, leukocyte counts as low as 10% of

normal have been recorded in chinook salmon (O. tshawytscha) experimentally infected with V. ordalii (Ransom, 1978). This observation suggests the elaboration of a powerful leukocytolytic factor by these pathogenic vibrios but to date the production of such a factor has not been confirmed. A similar leukopenic response has been documented in fish infected with Aeromonas salmonicida (Klontz et al., 1966) and a leukocytolytic factor has been characterized (Fuller et al., 1977).

A variety of bacterial properties have been shown to contribute to the virulence of some of the more thoroughly characterized microorganisms of medical importance. Researchers have only recently begun to evaluate bacterial fish pathogens for some of these same properties. For example, the ability of V. ordalii to resist the bacteriocidal properties of normal (nonimmune) fish serum has recently been examined by Trust et al. (1981). Representative strains of V. ordalii isolated from moribund fish during several outbreaks of vibriosis were first shown to be serum resistant based on their ability to survive and grow in vitro in normal rainbow trout (Salmo gairdneri) sera. Spontaneously arising serum-sensitive mutants of many of these strains were subsequently isolated and, when tested in vivo for pathogenicity, were found to be of markedly lower virulence. Thus clearly suggesting an important role for serum resistance in the virulence of V. ordalii.

The ability of V. ordalii to agglutinate erythrocytes from a variety of animal species has also been recently examined (Trust et al., 1981). Hemagglutination is generally recognized as an in vitro assay that can be used to assess the ability of bacteria to attach to eucaryotic cells, presumably an important first step in pathogenesis of any infection. The results indicated that a correlation between the virulence of V. ordalii and the ability to agglutinate fish erythrocytes could not be readily made. Although each strain of V. ordalii tested agglutinated at least one of the eucaryotic cells tested, only one out of the 6 virulent strains agglutinated rainbow trout erythrocytes under the in vitro conditions employed.

Conclusion

In summary, V. ordalii is a major cause of vibriosis among cultured salmonids in the Pacific Northwest and Japan. Although many aspects of the biology of V. ordalii remain to be examined, particularly those relating to the pathogenesis of disease, progress is being made. It is anticipated that new knowledge can be readily applied in the development of more effective means of treating and preventing this important marine fish disease.

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Furunculosis: Pathogenicity, Mechanisms of Bacterial Virulence, And the Immunological Response of Fish to *Aeromonas salmonicida*

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Introduction

More than any other fish pathogen, *Aeromonas salmonicida* has become a model for the study of bacterial virulence mechanisms and the immune response in fish. Although this bacterium was originally considered to cause serious mortality in only cultured salmonids, it actually infects and causes diverse pathological conditions in many families of fishes (McCarthy, 1975). *Aeromonas salmonicida* is commonly associated with typical salmonid furunculosis but atypical variants of this bacterium also cause carp erythrodermatitis (Bootsma et al., 1977; Bootsma and Blommaert, 1978), goldfish ulcer disease (Elliot and Shotts, 1980*a*,*b*), ulcer disease of trout (Paterson et al., 1980), and septicemic infections in nonsalmonid (McFadden, 1970) and marine fishes (Evelyn, 1971, Fluchter, 1979). Several reviews have been prepared that comprehensively describe salmonid furunculosis and other associated diseases caused by *A. salmonicida* (Herman, 1968; McCraw, 1953; McCarthy and Roberts, 1980). In this paper, the molecular basis of virulence of *A. salmonicida* and the immunological response of fish to this pathogen will be discussed.

Pathogenicity and Virulence Mechanisms

The elucidation of virulence mechanisms of *A. salmonicida* has evolved from reports on the descriptive pathology associated with furunculosis. Although this disease derives its name from characteristic boil-like lesions or furuncles induced by localized infections of *A. salmonicida* in the dermis of chronically infected fish, these lesions are not always present. The bacterium often produces a generalized systemic infection causing expressed pathology in vascular tissues (Mackie et al., 1930). Because no pathological changes occurred in healthy fish injected with culture and lesion filtrates from *A. salmonicida*, Mackie et al. (1935) attributed the gross pathology and mortality associated with furunculosis to a massive bacteremia that interfered with the blood supply at foci of infections and resulted in extensive tissue necrosis. Field et al. (1944) also observed that injections of virulent bacteria into

experimental fish induced a rapid hypoglycemia. These authors suspected that mortality in fish acutely infected with *A. salmonicida* resulted from bacterial utilization of host blood sugars and resulted in fatal hypoglycemic shock. However, elevated levels of amino acids, nitrogenous compounds, aldolase, and creatine phosphokinase derived from muscle proteins, and an increased level of carbamyl transferase derived from liver cells in the serum of infected fish suggested that the initial problem associated with furunculosis was not hypoglycemia but proteolysis of host tissues by bacterial enzymes (Shieh and MacLean, 1976; Shieh, 1978). Although Shieh and MacLean (1975) extracted and purified an extracellular protease from spent cultures of *A. salmonicida*, experiments were not conducted to determine if the protease induced pathogenic changes in healthy fish. This protease was shown to be a single protein with a molecular weight of 11,000 that was inhibited by phenylmethane sulfonyl-fluoride and, therefore, contained serine at the active site. The protease had a temperature optimum of 60°C and pH optimum of 10.5.

Because *A. salmonicida* is a gram-negative bacterium, the role of bacterial endotoxin in the pathology of furunculosis was examined. Anderson (1972) indicated that quantitatively virulent strains of *A. salmonicida* produced more lipopolysaccharide than avirulent bacteria. However, unlike their mammalian counterparts, fish were later shown to tolerate high concentrations of injected *A. salmonicida* endotoxin and have limited detrimental cardiovascular effects due to the release of circulating histamine (Wedemeyer et al., 1968; Paterson, 1972).

Consistent with descriptions of furunculosis is the absence of a pronounced leukocyte response and subsequent phagocytosis of bacteria. Blake (1935) described the presence of free bacteria and little phagocytosis in the blood of diseased fish and a definite leukocytic infiltration was not present at the foci of infection. To account for the absence of a leukocytic infiltration at the foci of infection, Griffin (1953) postulated that *A. salmonicida* produced a leucocidin. Klontz et al. (1966) indicated that a transient bacteremia and marked inflammatory response occurred within 8 h after virulent bacteria were injected intramuscularly into fish. However, by 72 h after injection the bacteremia persisted and had caused severe leukopenia in the kidneys, spleen, and site of inoculation and the tissues became progressively necrotic. The ability of sonicated bacteria to lyse steelhead trout (*Salmo gairdneri*) leukocytes provided further evidence that *A. salmonicida* produced a leukocytolytic component (McCoy, 1973). Fullet er al. (1977) then extracted a glycoprotein from culture supernatants of *A. salmonicida* that initiated cytolysis of rainbow trout leukocytes, produced pronounced leukopenia in fish and enhanced the LD-50 values of bacterial strains when injected with viable bacteria. Although these workers indicated that more leukocytolytic factor was extracted from cultures of a virulent strain than from an avirulent strain, other researchers have found that the leukocytolytic potential was not related strictly to the virulence of a given strain (Cipriano et al., 1981). In the latter study, the relationship of leukocytolytic properties of *A. salmonicida* to the virulence of different

strains of bacteria was determined by direct incubation of trout leukocytes with bacteria, quantification of the amount of leukocytolytic factor produced by each strain, and by incubation of the leukocytolytic factor derived from each strain with trout leukocytes. No positive correlation between the virulence of *A. salmonicida* strains and leukocytolytic activity was established. However, the characteristic inhibition of a leukocytic response among fish infected with *A. salmonicida* suggests that the leukocytolytic factor is an important bacterial aggressin. McCarthy (1976) also observed that virulent and avirulent strains of *A. salmonicida* had common aggressins and did not differ in their ability to invade host tissues. Therefore, much of the research concerning the elucidation of virulence mechanisms of *A. salmonicida* has again concentrated on the proteolytic nature of this bacterium.

After inducing protease-deficient strains of bacteria that were treated with N-methyl-N'nitro-N-nitrosoguanidine and EDTA, Sakai (1977) observed that the mutant derivatives were not pathogenic in fish. Fish that were injected with culture filtrates of protease-deficient mutants did not develop furuncle-like lesions at the site of injection whereas fish that were similarly injected with culture filtrates of the protease-positive precursors developed lesions (Sakai, 1978). Ellis et al. (1980) further indicated that cell-free culture extracts of virulent *A. salmonicida* contained extracellular products (ECP) that induced most of the pathological conditions observed in diseased fish. Although the ECP was leukocytolytic, hemolytic and proteolytic within in vitro assays, only proteolytic activity was observed in vivo. In these studies, ECP caused severe proteolysis of muscle and collagenous tissues, and the authors further suggested that leukocytolytic activity probably resulted from localized concentrations of ECP on phagocytes.

Cipriano et al. (1981) resolved the ECP of *A. salmonicida* into four constituent fractions by ion exchange chromatography (Fig. 1). The fraction eluted from DEAE Sephadex A-25 columns with 0.3 mol/L NaCl was equivalent to the leukocytolytic factor previously isolated by Fuller et al. (1977). However, this fraction also had proteolytic activity, was toxic to RTG-2 and CHSE monolayers, and induced pathologic changes, lesions and mortality, when injected into rainbow trout (Fig. 2), brook trout (*Salvelinus fontinalis*), and Atlantic salmon (*Salmo salar*). The proteolytic and cytolytic activity of this fraction correlated with the virulence of the isolate from which it was derived. SDS polyacrylamide gel electrophoresis studies indicated that this fraction was not homogenic and consisted of at least six different proteins. No determinations have yet been made to identify the band(s) responsible for proteolysis, leukocytolysis, and cytolysis.

Although protease is prominently involved in the pathology associated with furunculosis, the presence of an A-layer microcapsule or additional cell envelope protein exterior to the outer membrane of bacteria (Fig. 3) has become recognized to be a definite requisite for virulence (Udey, 1977). The A-layer is responsible for the autoagglutination or clumping that is characteristic of virulent cells of *A. salmonicida* and facilitates their attachment to fish

and human tissue culture monolayers (Udey and Fryer, 1978; Udey, 1978; Evenberg, 1982). The A-layer was shown to have a molecular weight range between 49,000 and 54,000, and was composed of repeating tetragonal subunits. (Kay et al., 1981; Evenberg et al., 1983). The amino acid sequence of this protein closely resembled that of the K88 adhesive fimbriae of enteropathogenic *Escherichia coli* (Evenberg and Lugtenberg, 1982).

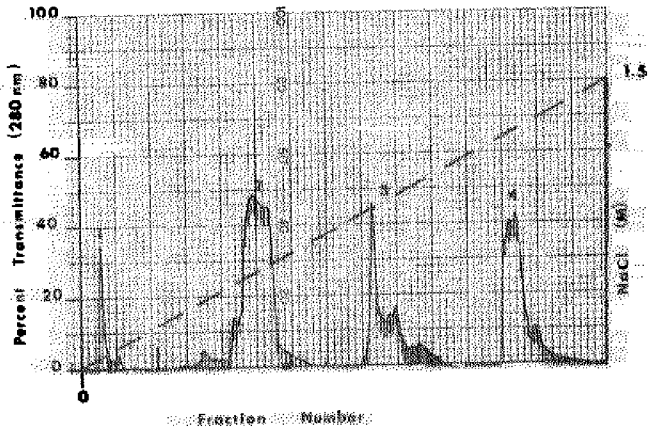

Figure 1. Typical elution profile of the extracellular material precipitated by bringing spent culture media of *Aeromonas salmonicida* to 0.8% saturation with $(NH_4)_2SO_4$ from DEAE Sephadex A-25. After equilibrating the column with three void volumes of 0.01 M K_2HPO_4 (pH 8.0), adherent proteins were eluted with a 0 to 1.5 M NaCl discontinuous concentration gradient. Flow rate of the column was 240 mL/h, fractions were collected in 4.0 mL volumes and recorded with an LKB UA-5 absorbance monitor (chart speed = 1.5 cm/h).

Immunological Response of Fish to *A. salmonicida*

Since the Furunculosis Committee first asked whether fish that survived natural epizootics of furunculosis acquired immunity to subsequent infections (Mackie et al., 1933), many researchers have examined the immune response of fish to *A. salmonicida*. Although the goal of these workers has been to develop an effective vaccine, practical hatchery immunizations of fish against furunculosis remains relatively unsuccessful. Notwithstanding concentrated research efforts, methods used to vaccinate fish against other bacteria (i.e., *Vibrio anguillarum*, *Yersinia ruckeri*) have not protected fish against *A. salmonicida*.

In the initial attempt to vaccinate fish against furunculosis, Duff (1942) orally immunized trout against *A. salmonicida* with a chloroform-inactivated bacterin. However, later attempts to vaccinate fish by similar methods produced equivocal results (Snieszko and

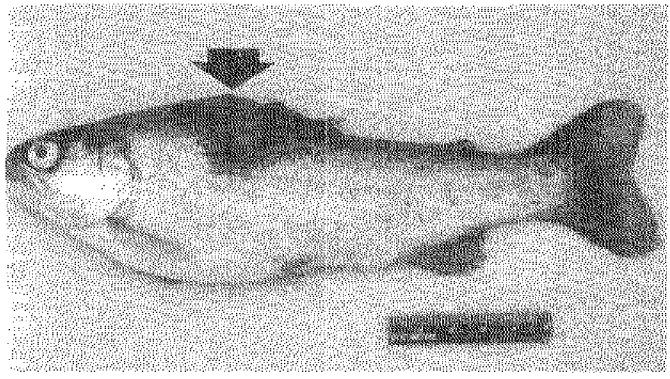

Figure 2. Pathology induced in rainbow trout injected intramuscularly with 80 µg protein of the leukocytolytic and proteolytic fraction 7 that was eluted from DEAE Sephadex A-25 with 0.3 M NaCl. Within 48 h after injection there was marked inflammation at the site of injection. By 92 h post-inoculation there was localized necrosis and hemorrhage of tissues.

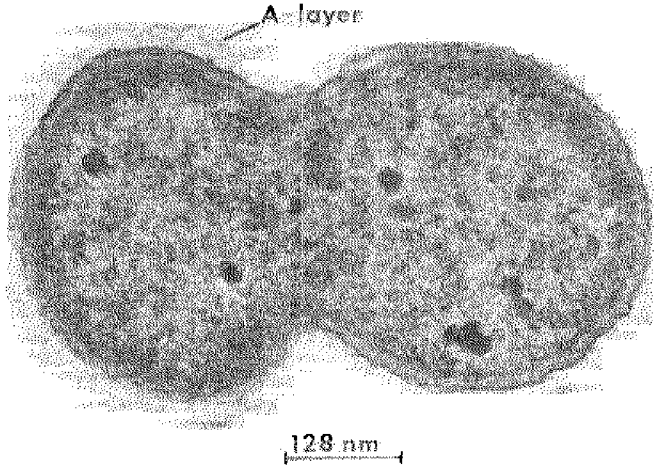

Figure 3. Transmission electron micrograph of virulent cells of *Aeromonas salmonicida* undergoing active division. This particular strain had an LD-50 value of 38 cells in juvenile brook trout, the bacteria autoagglutinated in broth culture and have a characteristic cell envelope A-layer protein. Photo by J. Morrison.

Friedle, 1949). Fish that were immunized by injections of virulent bacteria emulsified in an adjuvant developed antibodies that resulted in protective humoral immunity (Krantz et al., 1964*a*; Paterson and Fryer, 1974*b*). Although injections of endotoxin were highly antigenic and stimulated persistent agglutinin production in juvenile coho salmon (*Oncorhynchus kisutch*), the immunogenic ability of this antigen was not established (Paterson and Fryer, 1974*a*). Because fish can be successfully vaccinated by injections with formolized virulent cells in adjuvant, such a vaccine has become commercially available. However, except for use in brood stocks, injection of a vaccine into large quantities of small fishes has little practical and economical application within routine hatchery immunization programs.

Other attempts to deliver vaccines against furunculosis and produce protective immunity by either oral (Krantz et al., 1964*b*; Klontz and Anderson, 1970; Udey and Fryer, 1978), hyperosmotic immersion (Palmer and Smith, 1980), or spray deliveries (Could, 1977), were either ineffective or produced inconclusive results. The failure of these programs was partly attributed to an inability to induce the disease experimentally and thus to accurately assess efficacy.

Furunculosis is easily induced by injecting susceptible fish with virulent cells of *A. salmonicida*, but there are specific problems with such challenges. Although *A. salmonicida* can invade fish by a percutaneous entry (Miyazaki and Kubota, 1975; Cipriano, 1982*a*), injection of bacteria is not natural and bypasses many defense mechanisms of the host. The standardization of small volume inocula used to inject fish is also complicated because *A. salmonicida* cells autoagglutinate. Finally, the strong pathogenicity of even small numbers of virulent *A. salmonicida*, when injected into fish, may overwhelm the host's immune response. In fact, Michel (1979) vaccinated rainbow trout with formolized bacterins that evoked a strong production of humoral antibodies. However, despite the presence of these antibodies, the fish were not protected against intramuscular injections of virulent bacteria.

At the National Fish Health Research Laboratory (Kearneysville, W. Va.), a contact-exposure challenge, in which OWNI brook trout are maintained in 12.5°C flowing spring water, and then exposed for 60 s to a viable bacterial culture containing 10^8-10^9 virulent cells of *A. salmonicida* per milliliter, is used to induce furunculosis. Although this is not a standardized challenge, we have had consistent results using challenge isolates that have an LD-50 value in the range of 10^1 cells for juvenile OWNI brook trout. Using this particular strain of trout, we have not noted any variations in susceptibility of fish to the challenge, and expect an 80% average mortality within 14 days after exposure to the pathogen (Cipriano, 1982*a*).

Bath challenges in which fish are first tempered to an elevated water temperature ($\geq$ 15°C) and then exposed for 30 to 60 min to reduced concentrations of bacteria (10^5 cells), are also effective (McCarthy et al., in press). The development of such challenges

has facilitated our ability to study the immune response of fish to *A. salmonicida* and its constituent antigens.

Spence et al. (1965) first showed that coho salmon injected with immune serum from rainbow trout were passively protected against experimental infections. Although passive immunization has little practical value as a management tool, such studies have provided important information about the antigenicity of *A. salmonicida*. Previous vaccination attempts indicated that virulent strains of *A. salmonicida* are generally more potent immunogens than their avirulent counterparts (Peterson, 1981). This suggests that virulent bacteria have additional antigenic components that are not found in avirulent cells. As already mentioned, the most significant difference between virulent and avirulent strains of *A. salmonicida* is the presence of an additional cell envelope A-layer protein exterior to the outer membrane of virulent bacteria.

To demonstrate the immunogenic nature of A-layer protein, McCarthy et al. (in press) prepared a rabbit antiserum against a virulent A-layer (+) strain of *A. salmonicida*. The antiserum was then absorbed with avirulent A-layer (-) bacteria until only the antibodies developed against A-layer protein remained. The resulting antiserum protected sockeye salmon (*O. nerka*) from experimental challenges and thereby indicated that the A-layer protein was immunogenic. Most importantly, however, these researchers also showed that treatment of virulent cells with EDTA released the A-layer protein and that EDTA-treated vaccines protected sockeye salmon against experimental and field challenges.

Studies conducted at the National Fish Health Research Laboratory, however, have indicated that avirulent strains of *A. salmonicida* can also be immunogenic and evoke prophylactic immunity in fish (Cipriano, 1982*b*). In these experiments, antisera were produced in adult rainbow trout against sublethal doses of viable virulent or avirulent bacteria. Living cells were injected so that the trout would respond immunologically to both somatic and extracellular bacterial antigens. After two booster injections, given at 28 day intervals, the antisera prepared against either the virulent or avirulent strain had similar humoral antibody titers of 1/512 in pooled serum samples from respective groups of rainbow trout. Juvenile brook trout were injected with these antisera, challenged 72 h post-inoculation, and after 14 days the results indicated that both antisera were equally protective. No statistically different mortality was observed between groups of trout passively immunized with either antiserum. In duplicated experiments, mortality was 17.5% among trout injected with the anti-virulent serum, 20% among trout immunized with the anti-avirulent serum, and 73% among groups of control fish.

Because living cells were used to produce the antisera, studies were then conducted to determine antigens that were immunogenic. In these studies, brook trout were immunized with formalin-killed cells washed free of residual extracellular material, unwashed cells, and the extracellular material produced by these cells. The extracellular material was prepared by precipitating proteins from

spent culture media with $(NH_4)_2SO_4$ (Cipriano, 1982b), and 5 µg protein ECP was injected per fish. In these studies, only those fish immunized with intraperitoneal injections of extracellular antigens resisted experimental challenges. Mortality was 97.5% among brook trout that were injected with either the washed or unwashed cells, 55% among brook trout injected with extracellular antigens, and 100% among control brook trout.

Because an arbitrary dose of ECP (5 µg protein) was used in the previous experiments, further tests were conducted to determine the immunogenicity of ECP injected into fish at different concentrations. ECP was adjusted with phosphate buffer to contain either 140.0, 14.0, or 1.4 µg protein per 0.1 mL inoculum and groups of brook trout were injected intraperitoneally with one of the respective antigen concentrations. Other groups of brook trout were injected similarly with the same concentrations of ECP that had been adsorbed onto DEAE Sephadex to particulate the antigen. Brook trout that were injected with either phosphate buffer or DEAE Sephadex served as controls. After 21 days, all fish were challenged by a 60 s exposure to cultures of *A. salmonicida* containing between 1.6 and 2.3 virulent bacteria per mL. Immunogenicity of the respective ECP treatments was assessed by comparison of the mortality from control and vaccinated groups of fish 14 days after challenge (Table 1). These data indicated that each group of immunized brook trout were protected against experimental challenge (Chi Sq. d.f. =1; P<0.05). Protection was not different among groups of fish injected with the soluble ECP at three different concentrations. On the other hand, immunization of fish with higher concentrations of the particulated ECP enhanced protection. In these studies, the optimum protection was observed among the group of brook trout immunized with 140.0 µg protein of the particulated ECP. Mortality was 8.9% within this group of fish and 91% among controls.

Table 1. Mortality among juvenile brook trout immunized by injection with extracellular material (ECP) from *Aeromonas salmonicida* that was either adsorbed or not adsorbed onto DEAE Sephadex.

ECP Concentration (ug/fish)	Unadsorbed ECP		Adsorbed ECP	
	Mortality	Chi Square[1/]	Mortality	Chi Square
1.4	10/20	15.3	29/45	14.5
14.0	11/20	11.6	19/45	24.2
140.0	12/20	10.0	4/45	67.*
Controls	20/20	----	41/45	----

[1/] Chi Square (d.f.=1); P<0.05

Further experiments were then conducted to compare the antigenicity of ECP in brook trout vaccinated by injection, immersion or oral deliveries. In these tests groups of 100 brook trout were treated as follows:

1. Each fish was injected intraperitoneally with 140 µg protein of the particulated ECP.

2. Fish were given a standard trout ration at 3% body weight daily for 10 days. The diet contained 140 µg protein of the

particulated ECP per gram of food.

3. Fish were immersed for 2 min in 2 L of spring water containing 140 ug protein of the particulated ECP per mL.

After 60 days, each fish was bled, serum was collected and titrated for antibody activity against formalinized cells of *A. salmonicida* by standard microtiter agglutination. Mean serum antibody titers were calculated for each group of brook trout vaccinated by injection, oral, and immersion deliveries of the particulated ECP. A mean titer was also calculated for control brook trout that had been sham-injected with DEAE Sephadex.

These results also indicated that ECP evoked significant levels of antibody production by injection, oral, and immersion deliveries (Fig. 4). The geometric mean serum agglutinin titer was 2458 among brook trout vaccinated by injection with the particulated ECP, 512 among fish immersed in the vaccine, 153.6 among fish orally vaccinated, and 14.4 among control brook trout. Although injection produced the highest levels of agglutinin production, vaccination of fish by injection is neither practical nor economical. Based on these results, therefore, it was concluded to concentrate on an immersion delivery of experimental vaccines.

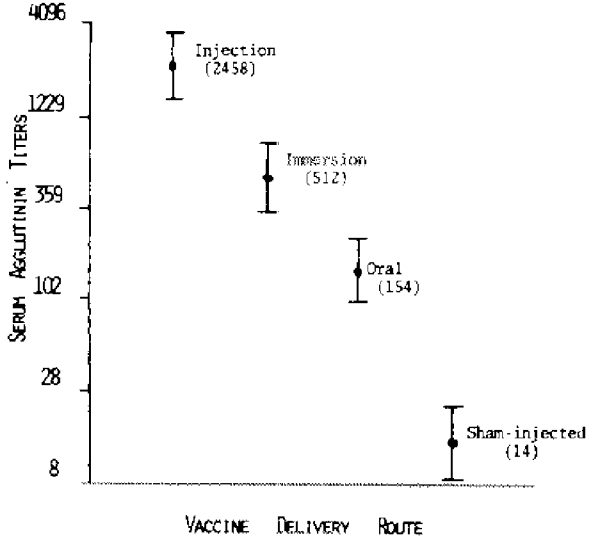

Figure 4. Mean and standard errors of serum antibody titers evoked among groups of 100 brook trout 60 days after vaccination by injection, immersion, or oral delivery of particulated ECP from *Aeromonas salmonicida*. Control brook trout were sham-injected with only the DEAE Sephadex that was used to particulate the ECP antigen.

Unlike the cell-associated immunogen reported by McCarthy et al. (in press), our studies indicated that protective immunity could be induced by an antigen contained in the spent culture media. Although McCarthy et al. (in press) reported that fish vaccinated with spent culture media from *A. salmonicida* did not resist challenge, these workers did not concentrate the extracellular material. The concentration steps involved in my preparation of the extracellular antigen may account for the observed differences on the immunogenicity of the extracellular antigens between the two studies.

Additionally, Austin and Rodgers (1981) indicated that the extracellular antigens concentrated from spent culture media of *A. salmonicida* with polyethylene glycol 6000 was antigenic and successfully immunized brown trout (*Salmo trutta*) against experimental challenges. However, field results with an orally delivered vaccine were negative. All vaccinated trout died within 14 days after the onset of mortality from septicemias induced by *A. hydrophila* and extensive tissue damage of the pancreas, liver, and spleen. Because none of the control fish that were not vaccinated died, the authors speculated that the tissue damage observed histologically in vaccinated fish resulted either directly from a reversion to toxicity of ECP or indirectly from the immunosuppressive effect of ECP on splenic antibody-producing cells that resulted in the rapid onset of septicemias caused by *A. hydrophila*.

Because ECP is hemolytic, leukocytolytic, and proteolytic (Ellis et al., 1980), experiments were conducted to identify the immunogenic components of ECP (Cipriano, in press). The extracellular materials from several strains of *A. salmonicida* were precipitated from spent culture media and this material was resolved by chromatography on DEAE Sephadex A-25 into four constituent fractions. Fraction 1 was eluted from the Sephadex columns with 0.01 mol/L K_2HPO_4 (pH 8.0) and shown to contain endotoxic material sloughed into the culture media by cellular autolysis (Cipriano, in review). Fraction 2, eluted with 0.3 mol/L NaCl from the DEAE, contained both proteolytic and leukocytolytic activity (Cipriano et al., 1981) and induced lesion development and mortality in fish. The third fraction was eluted from the DEAE with 0.6 mol/l NaCl and was hemolytic for sheep erythrocytes (Fig. 5). The brown pigmentation of this fraction also indicated that the fraction contained the melano-pigment often produced by strains of *A. salmonicida*. The fourth fraction was eluted from DEAE columns with 1.2 mol/l NaCl and it was shown to be cytolytic for cultured RTG-2 cell monolayers. However, when this fraction was injected intramuscularly or intraperitoneally into brook trout, rainbow trout, or Atlantic salmon, no gross pathology was observed. Each fraction was then injected intraperitoneally into respective groups of brook trout (5 µg protein per fish), the fish were maintained in 12.5°C spring water for 21 days, challenged, and the efficacy of each vaccination was assessed. Whether these fractions were derived from either a virulent (LD-50 = 3.8×10^1 cells per fish) or an avirulent (LD-0 at 10^7 cells/fish) strain of *A. salmonicida* did not effect the results. Only those groups of brook trout immunized with the cytolytic fraction 4 were consistently and significantly ($P < 0.001$) resistant to experimental challenges (Table 2).

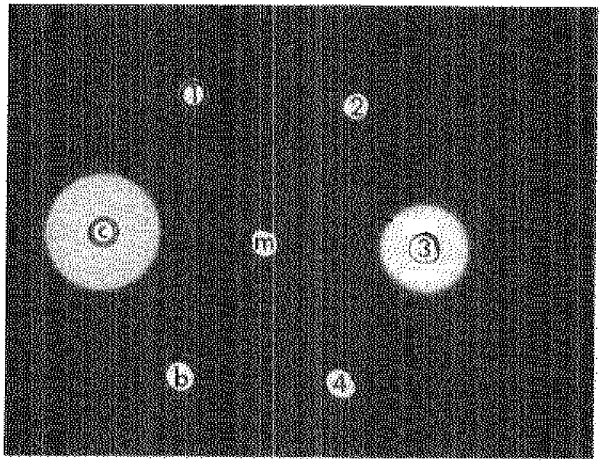

Figure 5. Hemolytic effects of the crude extracellular material (C) from *Aeromonas salmonicida* and its constituent fractions (1, 2, 3, and 4). Control wells contained a 0.8% saturation $(NH_4)_2SO_4$ extract of uninoculated media (M), and the 0.01 M K_2HPO_4 buffer (pH 8.0) against which all test components were dialyzed. Only the crude extract and fraction 3 were hemolytic for a 5% concentration of sheep erythrocytes suspended in a 1% agarose.

Table 2. Percent mortality among juvenile brook trout vaccinated with fractions derived from the extracellular material from *Aeromonas salmonicida* following experimental challenges.

Source	Fraction				Control
	1	2	3	4	
Virulent strain	100	90	97	17	77
Avirulent strain	71	71	50	10	71

When fraction 4 was emulsified in Freund's incomplete adjuvant the protection conferred to brook trout was was highly significant ($P<0.001$) but not different from that observed among fish immunized with fraction 4 alone. This fraction was obtained from four strains of *A. salmonicida*: two virulent, one avirulent, and an atypical strain isolated from diseased goldfish (*Carassius auratus*). The antigen was immunogenic and in each case protected brook trout against experimental challenges. Fraction 4 was shown to consist of a single protein subunit (MW 67,000) and had a 1:1.2 ratio of protein to carbohydrate. Antiserum prepared against fraction 4 extracted from a virulent isolate formed a single band against its homologous antigen on agar gel diffusion plates, but did not react with purified preparations of A-layer protein or endotoxin. These results indicated that virulent, nonautoagglutinating avirulent and atypical strains of *A. salmonicida* share a common extracellular immunogen.

Because avirulent strains of *A. salmonicida* were shown to have a common extracellular immunogen (fraction 4), studies were conducted to determine if fish could be successfully vaccinated by immersion in an attenuated vaccine (Cipriano and Starliper, in press). The strain of *A. salmonicida* chosen for these studies was originally isolated from diseased trout and had an LD-50 value of 1.3 X 10^2 cells in juvenile brook trout. However, following repeated passages and agar storage the isolate lost virulence, did not autoagglutinate in broth, and electron micrographs indicated that no additional cell envelope A-layer protein was present (Fig. 6).

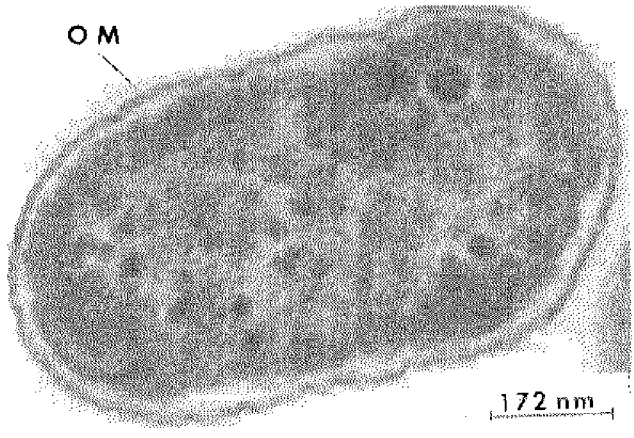

Figure 6. Transmission electron micrograph of an avirulent strain of *Aeromonas salmonicida*. Before repeated subculture and storage on agar, this strain was virulent (LD-50 = 138 cells) and autoagglutinated in broth culture. After subcultures, the strain became attenuated (LD-50 = 10^6 cells), did not autoagglutinate in broth, and no longer produced the cell envelope A-layer protein characteristically present in virulent bacteria exterior to their outer membrane (OM). Photo by J. Morrison.

This strain also had reduced leukocytolytic and proteolytic activity as indicated within in vitro assays, quantification of protein fraction, and visualized by a depletion of fraction 2 eluted from DEAE Sephadex A-25 (Fig. 7). Brook trout were injected intraperitoneally with graded dilutions of these viable bacteria. After 28 days, no mortalities had occurred, the fish were challenged, and the bacteria were shown to have evoked protection. Therefore, subsequent vaccinations were attempted in which brook trout and Atlantic salmon were immersed for 60 s in whole cultures containing about 10^9 attenuated cells/mL. Fish were held for 28 days at 12.5°C, challenged, and the efficacy of vaccination was assessed. As shown in Table 3, immersion of salmonids in the attenuated vaccine conferred protective immunity.

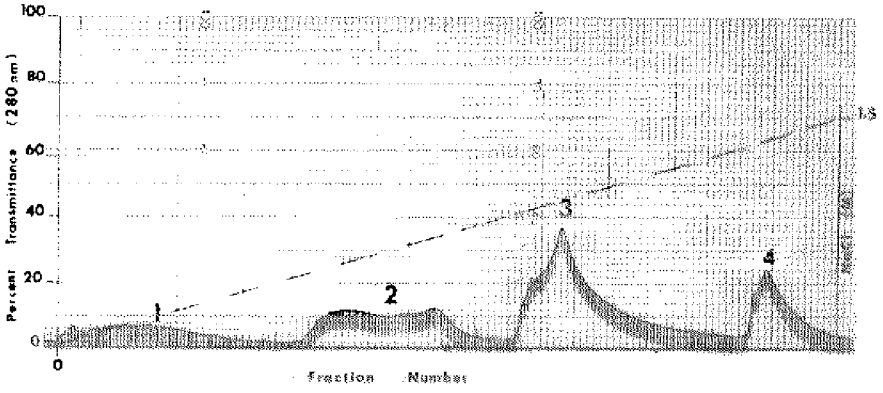

Figure 7. Elution profile of the extracellular material from the avirulent strain of *Aeromonas salmonicida* used to produce the attenuated-furunculosis vaccine. Column procedures were similar to those described in Figure 1 except that the absorbance monitor chart speed was increased to 3.0 cm/h. In its avirulent form, appreciably reduced amounts of the endotoxic-fraction 1 and the proteolytic-fraction 2 were obtained by elution of extracellular material from DEAE Sephadex A-25 (see Figure for comparison). Quantitative protein estimates were also lower than those described previously.

Table 1. Average percent mortality among replicate groups of salmonids immunized by immersion in an attenuated-furunculosis vaccine, following virulent experimental challenges.

Species	Brook trout	Atlantic salmon	
		Trial 1	Trial 2
Vaccinated fish	44	12.5	4
Control fish	94	87.5	92

Concurrent with vaccination trials, studies were conducted to determine the humoral immune response of fish to *A. salmonicida*. Although immunoglobulin-M-like agglutinin antibodies are evoked in trout injected with various antigenic preparations of *A. salmonicida* (Dorson, 1972; Paterson, 1972; Olsen and Fryer, 1974; Ikeda, 1978), the protective nature of such agglutinins has not been established (Michel, 1979; Cipriano, 1982b; McCartny et al., in press). To determine how salmonids responded to antigenic stimulation, we injected rainbow trout with sublethal concentrations of virulent bacteria (Cipriano and Starliper, in review). Serum from fish that had received three booster injections at 28 day intervals responded with the production of agglutinins, precipitins, and serum neutralization activities against certain antigens from *A. salmonicida*. These serum activities were resolved by ion exchange

chromatography on DEAE Sephadex A-25 columns. Only those fractions containing agglutinin and precipitin activity reacted with a rabbit antiserum to trout immunoglobulin. The agglutin fraction showed a beta mobility on immunoelectrophoresis, whereas the precipitin fraction had a beta-to-gamma mobility. Although the fraction of serum containing neutralization activity did not react with the antiserum to trout immunoglobulin, this fraction did react with a rabbit antiserum to normal trout serum and on immunoelectrophoresis the neutralization fraction had an alpha mobility.

To determine which of these activities constituted immunity against furunculosis, respective groups of brook trout were injected with whole immune serum or one of the serum fractions containing agglutinin, precipitin, or neutralization activities. After 72 h each group of fish was challenged and the protection passively conferred to brook trout by the individual inocula was established. Among two replicate studies the mean mortality was 87.5% in trout injected with control serum, 71.8% among those injected with the serum fraction containing precipitin activity, 37.5% among trout injected with whole immune serum, and 25% among trout injected with either of the fractions containing agglutinin and neutralization activities. These results indicated that serum agglutinin and neutralization activities were important elements of the humoral immune response to *A. salmonicida*.

In the same study, we also examined the immune response evoked in serum from convalescent brook trout, rainbow trout, and brown trout against certain antigens of *A. salmonicida*. These results indicated that precipitin titers, as determined by counterimmunoelectrophoresis, were predominantly against fraction 2 that contained the proteolytic and leucocytolytic activities of the extracellular antigen of *A. salmonicida*. It was previously shown that active immunization of fish with fraction 2 did not establish protective immunity (Cipriano, in press). In the current study the serum fraction containing precipitin activity (formed against fraction 2) did not passively protect fish from experimental challenge.

On the other hand, serum neutralization activity was determined to be evoked by the cytolytic glycoprotein (fraction 4). The serum fraction containing neutralization activity passively protected trout against experimental challenges and active immunization of fish with this fraction resulted in prophylactic immunity (Cipriano, in press).

Agglutinin activity in serum of convalescent fishes was significantly lower when washed cells rather than unwashed cells were used as an antigen in standard microtiter agglutination tests. Because the extracellular antigens were probably washed from the cells, each fraction was tanned to sheep erythrocytes and used in passive hemagglutination assays against the sera from convalescent fishes. In these studies, passive hemagglutinin titers were predominantly evoked by fraction 1, which was then shown to be endotoxic materia sloughed into the spent culture media during cellular autolysis. Endotoxin mixed with adjuvant was shown to evoke formation of persistent agglutinins in coho salmon, but the protective ability

of this material was not demonstrated (Paterson and Fryer, 1974a). When we injected fish with fraction I-endotoxin that was emulsified in adjuvant, the preparation was immunogenic and conferred protection against experimental challenges. However, as previously reported (Cipriano, in press), injection of fraction I (endotoxin alone) was not protective. In replicated experiments mean mortality was 94% among control trout injected only with Freund's Incomplete adjuvant, 91% among brook trout injected with fraction I (endotoxin alone), and 31% among fish injected with the endotoxic material emulsified in adjuvant. These studies indicated that endotoxin is also immunogenic in salmonids.

Because we had established that endotoxin and fraction 4 glycoprotein were immunogenic components of *A. salmonicida*, studies were then undertaken to produce an efficacious vaccine that would be safe and practical to use in hatcheries. Endotoxin and fraction 4 were antigenic components of the avirulent strain of *A. salmonicida* used to prepare the attenuated vaccine whereas no A-layer protein was apparent in these bacteria. Furthermore, spent culture media of this strain was shown to be predominently composed of only fractions 3 and 4 (Fig. 7). Our previous work had thus far indicated that the melano-pigment containing fraction 3 was not immunogenic. Therefore, we speculated that a protective response induced by spent culture media from this avirulent strain of *A. salmonicida* would be primarily induced by fraction 4 glycoprotein. Because these nonautoagglutinating avirulent bacteria also lacked a cell envelope A-layer protein, bacteria were used to enrich the spent culture media as a source of endotoxin. Cultures of the attenuated strain of *A. salmonicida* were inactivated with chloroform and three vaccines were prepared: cells alone, spent media alone, and whole cultures (Cipriano et al., in review). No protection was afforded fish immersed in vaccines containing either cells or spent media alone. However, a highly significant level of protection ($P<0.001$) was observed among brook trout and Atlantic salmon immersed in combined vaccines in which the avirulent bacteria were delivered in their culture media. These studies suggest that both immunogens (cellular endotoxin and fraction 4 glycoprotein) were needed to evoke protection among fish immersed in bacterins of this avirulent strain. Limited field trials in which brown trout were vaccinated by a 60 s immersion in the chloroform-inactivated combined whole cell and culture media vaccine were also successful. Among groups of 12,000 brown trout, mortality was 2.1% among vaccinated fish and 28.9% among controls.

These studies and those of McCarthy et al. (in press) are the first reports on the successful immersion vaccination of fish against furunculosis. The apparent differences between these studies suggest that there are several potential immunogens in strains of *A. salmonicida*, and different methods for the preparation of efficacious vaccines. The effectiveness of a specific vaccine is therefore dependent upon the antigenic composition of the immunogen, antigen preparation, and the appropriate delivery of specific protective antigens.

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Synthesis of the Structural Proteins of Infectious Hematopoietic Necrosis Virus

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Introduction

Infectious hematopoietic necrosis (IHN) virus is a member of the Rhabdoviridae family and more closely resembles the rabies virus group than the vesicular stomatitis virus group (McAllister and Wagner, 1975; Lenoir and deKinkelin, 1975; Leong et al., 1981). Five major structural proteins have been identified in purified preparations of IHN virions. These proteins have been designated L for the polymerase, G for the surface glycoprotein, N for the nucleocapsid protein, M_1 and M_2 for the envelope proteins. The glycoprotein nature of the virion protein G has been confirmed by specific labeling of the G protein with ^{3}H-glucosamine and two phosphoproteins have been identified, N and M_1 (McAllister and Wagner, 1975). Estimates for the molecular weights of these proteins have varied between 150,000 to 190,000 for L, 67,000 to 80,000 for G, 38,000 to 40,500 for N, 22,500 for M_1, and 17,000 to 20,000 for M_2. These variations in the reported size of the virion proteins suggested to us that the variations might result from strain differences in the IHN virus used in each study. Thus, the molecular weights of the virion proteins were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for eleven different strains of IHN virus. Striking differences in the migration of the N and G proteins were observed among the virus strains.

In this report, the synthesis of the structural proteins of the Round Butte strain of IHN virus was examined. The time of appearance of each virion protein during the course of infection was determined in Chinook salmon embryo cells. Unlike rabies virus, IHN virus infection results in the inhibition of cellular protein synthesis. Virus protein synthesis was detected in vivo without resorting to hypertonic shock treatment of the infected cells. The time of appearance of each protein in vivo is unique and suggests that each structural protein is synthesized on a monocistronic mRNA species.

Structural Proteins of IHN Virus

The structural polypeptides of the Round Butte strain of IHNV were separated by SDS-PAGE in a mini-slab gel system (Matsudaira and Burgess, 1978) and stained with silver nitrate in a procedure modified from Allen, 1980. The relative mobility of the marker proteins was inversely related to the logarithm of the respective molecular weights in this gel system. Five virion proteins were identified with molecular weights of 150,000 for L (polymerase), 67,000 for G (glycoprotein), 40,500 for N (nucleocapsid protein), 22,500 for M_1 (matrix protein), and 17,000 for M_2 (matrix protein) (Figure 1). In some virus preparations even after several additional centrifugations, there appeared two G proteins at 67,000 and 65,000. In addition, the relative contribution of each protein species to the total protein content of the virus was determined by scanning for optical density (630 nm) absorption peaks in the silver and Coomassie blue stained gels. The data shown in Table 1 is different from that reported by Mcallister and Wagner, 1975 and because of this, both sets of data are included. An approximation of the number of molecules of each protein per virion was made as described by Obijeski et al., 1976. The determination is based upon the assumptions that: (1) the estimates of the molecular weights of the viral proteins are correct; (2) the virions contain only one molecule of viral RNA; and (3) the gram molecular weight of the viral genome is 3.57×10^6 (Kurath and Leong, personal communication).

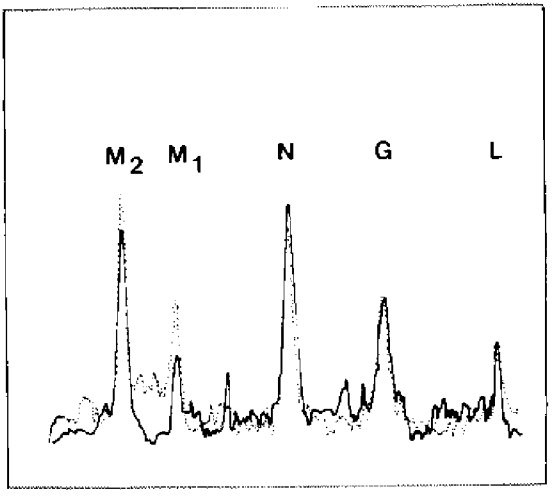

Fig. 1. Densitometer tracings of SDS-polyacrylamide gels of purified IHN virus stained with Coomassie blue, broken line, or silver nitrate, solid line.

In determining the virion ratio of RNA to protein, a suspension of purified virus labeled with (^{3}H) uridine containing 0.48 mg of protein per ml, and a total of 8.36×10^{5} cpm was used. After extraction, the purified RNA had a specific activity of 3.6×10^{4} cpm per µg of RNA. It was calculated from these figures that there was .48 mg of protein to 0.023 mg of viral RNA in the original virus suspension corresponding to a protein-to-RNA weight ratio of 21:1. Similar ratios were obtained in three different experiments.

Structural Proteins of Different Strains of IHNV

When the virion of six other strains were compared in the same manner, major differences in N and G were seen (Figure 2). Striking differences in the apparent molecular weight of the N protein were immediately obvious. The Coleman Hatchery strain from California exhibited a G protein which was considerably larger than the G proteins of the other strains. Since all virus strains were grown in CHSE-21 cells for these studies, the differences in G and N were not due to differences in host cell glycosylation or phosphorylation.

Strain differences in the structural proteins of IHNV were also examined by specifically labelling the viral proteins in vivo with ^{35}S-methionine. Approximately 24 hours after infection at a multiplicity of infection of 5 to 10, the cells were exposed to ^{35}S-methionine for 1 hour. The cells were lysed with SDS and the lysate was applied directly to a polyacrylamide gel. After electrophoresis, the gel was stained and dried before exposure to X-ray film for 24-48 h. A comparison of the silver stained gel and the autoradiogram developed for eleven different strains of IHNV is shown in Figure 3. It is clear that major differences in the apparent molecular weights of the N and G proteins are detectable in the autoradiogram. In fact the same strain differences that appeared in the silver stained gel for purified IHNV also appeared in the autoradiogram of the different infected cell lysates. Thus, direct ^{35}S-methionine labeling and SDS-PAGE of infected cells is a simple method for making comparisons of the IHNV strains.

Intracellular IHN Virion Protein Synthesis

It is clear from Figure 3 that the rate of synthesis of cellular proteins is reduced after IHNV infection. This observation suggested that it should be possible to determine the time of the intracellular appearance for each virion protein during the infectious cycle. At one hour intervals after infection, cultures of CHSE-214 cells were exposed to ^{35}S-methionine for 1 hour and then analyzed by SDS-PAGE. The synthesis of N, M_1 and M_2 is apparently initiated at 6-7 hours after infection (Figure 4A). The G protein appears at approximately 9-10 hours after infection. When earlier samples were taken and an excess of radioactively labeled material was analyzed by SDS-PAGE, N protein

Table 1. Estimated Number and Molecular Weight of IHN Virus Proteins.

Protein Species	Mol. Wt. ($\times 10^3$)[a]	Percentage of Total Virus Proteins		Mol. Wt. of Protein per Virion ($\times 10^{-6}$)[d]		Number of Molecules per Virion[e]	
		Incorporation[b]	Stained[c]	Incorp.	Stained	Incorp.	Stained
L	150.0	4.6	8.1	3.4	6.1	23	40
G	67.0	17.8	26.1	13.3	19.5	198	290
N	40.5	42.0	30.4	31.3	22.7	774	560
M_1	22.5	11.8	15.5	8.8	11.6	391	514
M_2	17.0	23.8	19.9	17.8	14.9	1,044	874

[a]Molecular Weight Estimates were determined as described in the text.

[b]Percentage of each protein determined by using virus labeled with ^{14}C amino acids from McAllister and Wagner, 1975.

[c]Percentage of each protein was calculated four separated determinations of the area under each peak of gels stained with Coomassie blue or silver and scanned at 620 nm. The average percentage of each protein for silver staining and for Coomassie Blue staining was again averaged for the final result.

[d]Total virion protein was derived from a 21:1 ratio of virus protein to RNA and the estimate that the mol. wt. of the IHN viral genome is 3.57×10^6 (Kurath and Leong, unpublished observation).

[e]Number of protein molecules per virion was calculated by dividing the daltons of protein per virion by their molecular wt.

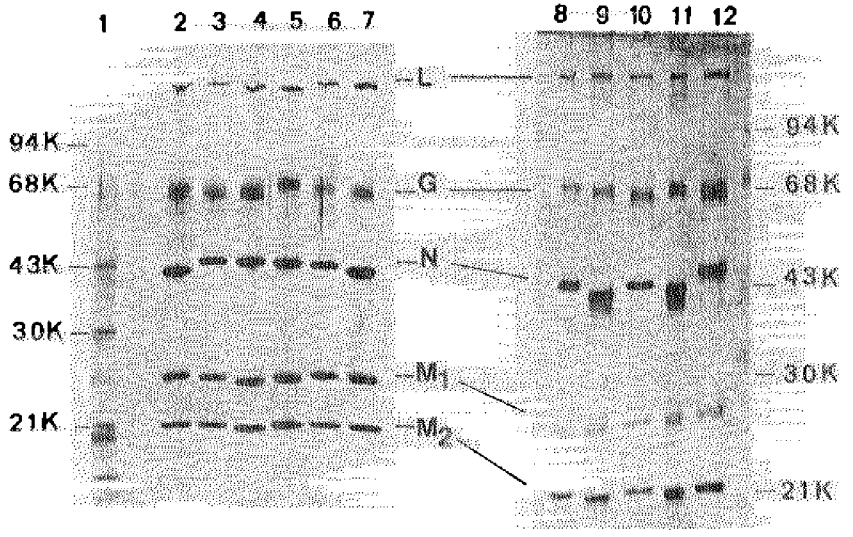

Fig. 2. Electrophoretic profile of different strains of IHN virus. Purified IHN virus was disrupted and electrophoresed in SDS-polyacrylamide gels. Lane 1 contains the molecular weight marker proteins. The other lanes are identified as follows: Lane 2 (Round Butte Hatchery), Lane 3 (Nan Scott Lake), Lane 4 (Elk River), Lane 5 (Coleman Hatchery), Lane 6 (Karluk River), Lane 7 (Suttle Lake), Lane 8 (Coleman Hatchery), Lane 9 (Round Butte Hatchery), Lane 10 (Karluk River), Lane 11 (Round Butte Hatchery), and Lane 12 (Cedar River).

synthesis was observed as early as 2-3 hours in the infection cycle (Figure 4B). Since cellular protein synthesis is not inhibited during early infection, it is difficult to distinguish viral proteins such as M_2 and L from host proteins in these early samples.

Two forms of the glycoprotein G is observed intracellularly (Figure 5A). These two forms, G_2 for the lower band (65,000) and G_1 for the upper band (67,000), appear to have a product-precursor relationship. In an experiment designed to determine the kinetics of intracellular viral protein synthesis and accumulation, cells infected with IHNV (MOI approximately 20) for 24 hours were exposed to ^{35}S-methionine for 1, 5, 10, 30, and 60 min. At the indicated times, the cells were lysed and the lysate analyzed by SDS-PAGE. After one minute, both G_1 and G_2 appeared and after 5 and 10 minutes of label, G_2 was the dominant glycoprotein being synthesized in the cell (Figure 5A). However, at 30 minutes, the amount of radioactivity appeared equally distributed in both bands as labeled G_2 protein accumulated in the cell. By 60 minutes, the G_1 band was the predominantly labeled glycoprotein band.

When infected cells were exposed to ^{35}S-methionine for 5 min

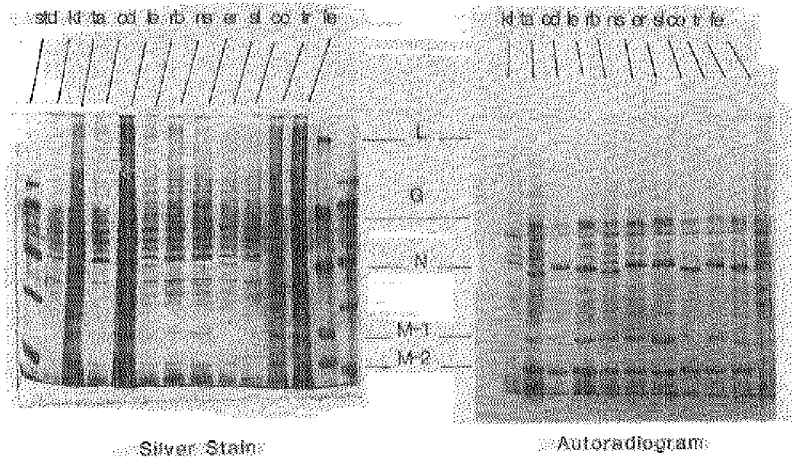

Fig. 3. A comparison of eleven different strains of IHN virus was made by autoradiographic analysis of the intracellular proteins labeled by ^{35}S-methionine. The lanes are marked KL (Karluk River, Alaska), TA (Tamgas Creek, Alaska), CD (Cedar River, Washington), LE (Lewis River, Washington), RB (Round Butte, Oregon), NS (Nan Scott Lake, Oregon), ER (Elk River, Oregon), SL (Suttle Lake, Oregon), CO (Coleman Hatchery, California), TR (Trinity River, California), and FE (Feather River, California).

followed with excess unlabeled methionine for 20, 40, 60, and 120 min, similar results were observed (Figure 5B). A 5 min exposure to the labeled precursor resulted in a predominant G_2 band. After a chase of 20 min with excess unlabeled methionine, the predominantly labeled band was G_1. These results do suggest that G_2 is synthesized immediately and then is further glycosylated to form G_1. Similar results have been reported for vesicular stomatitis virus (Knipe et al., 1977).

In both labeling experiments, comparatively higher rates of synthesis for the N and M_2 proteins were observed. The rates of synthesis for G and M_1 proteins were lower. A labeled band corresponding to the L protein did not appear until 20 min after a 5 min exposure to ^{35}S-methionine (Figure 5B). However, continuous exposure to the labeled amino acid for more than 30 min did not produce a labeled L band on the autoradiogram (Figure 5A). In both experiments, each gel lane received 50,000 cpm of labeled material. This apparent paradox in the results may be explained if L protein is synthesized at a very low rate and constitutes a very small portion of the total virion protein synthesized in the cell. Thus, during continuous exposure to ^{35}S-methionine, L protein synthesis occurs at such a low rate that its presence is undetectable. The appearance of L in the pulse-chase experiment indicates that L protein accumulates at a faster rate than the other virion proteins.

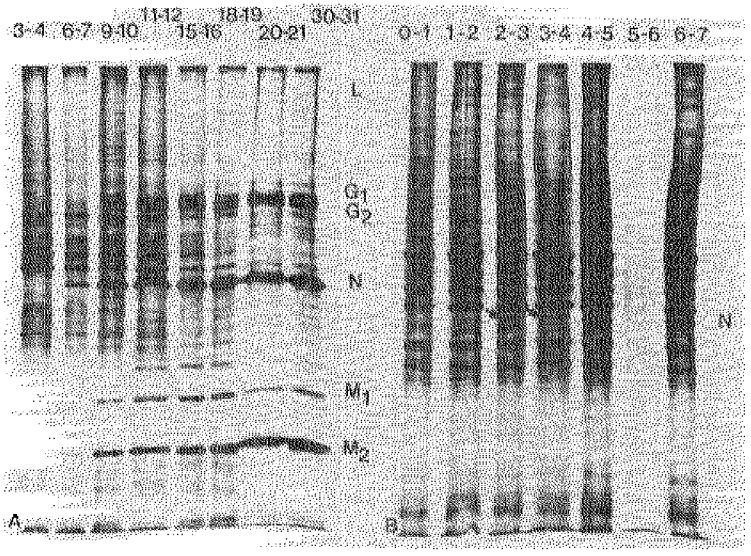

Fig. 4. Gel electropherogram of IHN virus proteins synthesize in CHSE-214 cells at different times after infection. Infected cells were exposed to ^{35}S-methionine for one hour at the times indicated after infection. Each lane received 50,000 cpm. The film in Fig. 4B was exposed for a longer time so that early virus protein synthesis was detectable.

Conclusions

The virion proteins of IHN virus have been identified and their intracellular synthesis has been examined. There are five virion proteins with molecular weights of 150,000 (L); 67,000 and 65,000 (G_1 and G_2); 40,500 (N); 22,500 (M_1); and 17,500 (M_2). The glycoprotein nature of the G proteins was verified by specific labeling with ^{14}C-glucosamine (data not shown) and their location of the surface of the virion was determined by treatment with Triton X-100 (data not shown). The nucleocapsid protein, N, was found to be associated with the viral nucleic acid and specifically labeled with ^{32}P-orthophosphate (data not shown). Thus, the work of McAllister and Wagner, 1975, has been confirmed.

An estimate of the number of molecules per virion for each protein was made for IHNV. The ratio of virus protein to RNA was determined to be 21:1, a very low figure in comparison to VSV and rabies. These viruses have ratios of 92:1 and 72:1 respectively (Bishop and Roy, 1972; Coslett et al., 1980). The low protein to RNA ratio for IHN virus is unusual and may reflect differences in the membrane structure of fish and mammalian cells (Moore et al., 1976). It is similar to that obtained for the bunyavirus, La

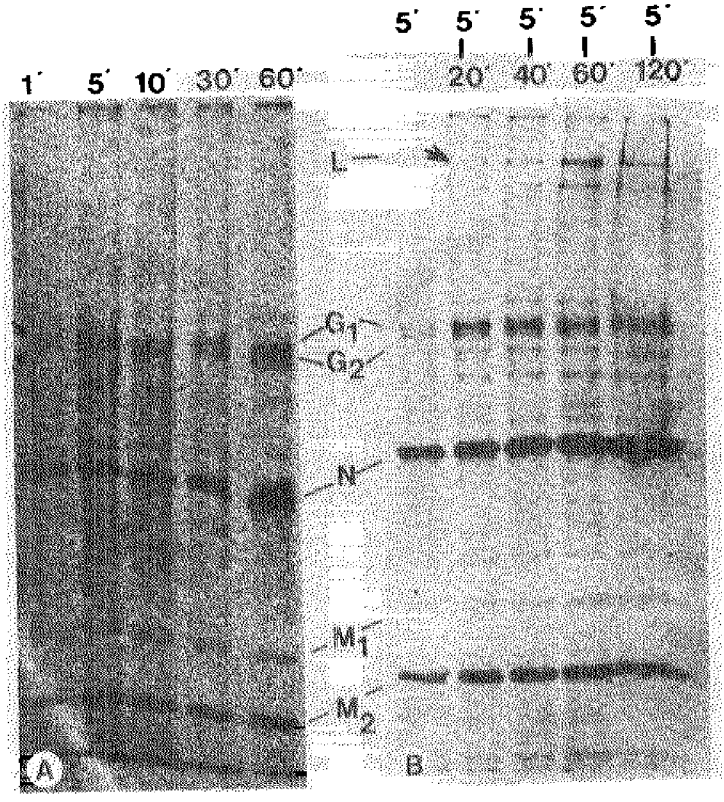

Fig. 5. Gel electropherogram of viral proteins synthesized in CHSE-214 cells after pulse-labeling with ^{35}S-methionine. In 5A, the infected cells were labeled for 1, 5, 10, 30, or 60 minutes before samples were taken for SDS-PAGE. In 5B, the infected cells were labeled for 5 minutes and sampled or the label was removed and fresh media containing excess cold methionine was added for 20, 40, 60, or 120 minutes before samples were taken for SDS-PAGE.

Crosse virus, which has a ratio of 30:1 when grown in BHK/21 cells (Obijeski et al., 1976). This ratio is used for calculating the total molecular weight of the virion protein.

The relative contribution of each protein to the total molecular weight of the virion was estimated from densitometer tracings of SDS-polyacrylamide gels of purified virus. For both silver and Coomassie blue stained gels, the relative proportion of each virion was very different from that reported by McAllister and Wagner, 1975, for ^{14}C-amino acid labeled virus (Table 1). These differences may be a result of differences in the virus strain. More likely it reflects differences in the methods for determining the proportion of each virion protein. For most proteins a nearly

linear relationship exists between the relative density of a band and the micrograms of protein in the band (Switzer et al., 1979). However, variations in staining do exist between different proteins (Oakley et al., 1980). Thus, densitometer tracings of Coomassie blue stained gels were also included in the study. Several investigators have used Coomassie blue stained gels to estimate relative virion protein concentrations (Bishop and Roy, 1972, Coslett et al., 1980, and Obijeski et al., 1976). We found no significant differences in the staining patterns produced by silver or Coomassie blue. Any error in these calculations would be an overestimate of the number of G protein molecules since these stains may bind more extensively to glycoproteins (McKnight, 1977).

The dalton equivalents of protein in IHN virions were calculated by reference to the amount of viral RNA per total viral protein. The ratio obtained (21:1) is equivalent to 4.8% RNA per IHN virion, whereas the VS virion has an estimated 0.7% to 1.09% RNA and rabies is composed of 1.39% RNA. These differences are reflected in the estimated number of virion proteins per IHN virus particle. The numbers of L, G, N, M_1, and M_2 molecules per virion for rabies is reported as 79 (L), 1723 (G_1 and G_2), 1975 (N), 402 (M_1), and 1156 (M_2) (Coslett et al., 1980). In contrast, IHN virions contain approximately a third as many L, a tenth as many G, and a half as many N molecules (Table 1). The remarkable difference is in the number of G molecules per virion. We and many others have found that it is difficult to produce antibody to IHN virus in warm-blooded animals. The poor immunogenicity of G or the poor neutralizing activity of the antisera may be a direct result of the low numbers of G molecules on the surface of the virion.

The nucleocapsid protein, N, is phosphorylated and found in association with the viral RNA and other N proteins inside the virion. In addition, N protein interacts with L and possibly M, proteins in the virion. These interactions should impose strict demands on the N-protein and therefore, this sequence should be conserved among virus strains. That is the case for rabies and VSV. However, this report shows that the apparent size of the N protein varies among the IHNV strains. We are determining whether these variations in electrophoretic mobility result from differences in phosphorylation or size of the polypeptide chain.

Infection of salmon cells with IHN virus results in an inhibition of cellular protein synthesis. In this characteristic, IHNV differs from other members of the rabies virus group of the Rhabdoviridae. Cellular protein synthesis is not inhibited after infection with rabies virus (Coslett et al., 1980) and any study of rabies protein synthesis in the cell requires exposure to hypertonic shock to reduce the background of host protein synthesis. However, it has been possible to examine the synthesis of IHN viral proteins in the cell without resorting to this drastic treatment.

It is clear that there are two species of virion glycoproteins, G_1 and G_2. Differences between these two glycoproteins may reflect incomplete glycosylation or some degradation of the carbohydrate moiety during virus purification. Since SDS-PAGE gels of fresh virus preparations do not exhibit the two glycoproteins, it is more likely that G_2 results from some degradation of G_1 in purified virions. Although peptide mapping data are not available for G_1 and G_2 it is probable that G_1 and G_2 have identical amino acid sequences. The pulse-chase experiment (Figure 6B) suggests that G_2 is synthesized and chased into the G_1 form which migrates slower. The slower form should contain more carbohydrate. Recent work by Dietzchold et al., 1979, has shown that G_1 and G_2 have identical peptide maps but G_2 protein contains less carbohydrate than G_1.

The first protein for IHNV to appear in the course of infection is the N, or nucleocapsid protein, at 2-3 hours after infection. At 6-7 hours after infection, the membrane proteins, M_1 and M_2, can be identified in the autoradiograms. The two forms of the glycoprotein, G_1 and G_2, are found at 9-10 hours after infection. It was not possible to distinguish the virion L protein from other host proteins in the gel until late in infection when cellular host protein synthesis was completely inhibited. The L protein appears to be synthesized at such a low rate that it constitutes a very small portion of the total protein synthesis in the infected cell. However, L protein accumulates faster than the other virion proteins, indicating that L is not turned over as quickly. These experiments show that IHN virus proteins are synthesized independently. Virus production begins at 12-14 hours after infection (Leong et al., 1981).

It appears that IHN virus protein synthesis in salmon cells is similar to that of rabies. Each virion protein seems to be derived from the translation of independently transcribed monocistronic mRNAs. In fact, we find five polyadenylated mRNA species are synthesized in vitro. These mRNA species correspond appropriately in sizes to the RNA species expected for each virion protein.

Acknowledgments

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Oregon Agricultural Experiment Station technical paper no. 6431.

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Molecular Characterization of Infectious Pancreatic Necrosis Virus Isolated from a Marine Fish

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Introduction

Infectious pancreatic necrosis virus (IPNV) was first isolated by Wolf et al. (1960) from diseased brook trout. The virus has since been shown to be worldwide pathogen for young salmonid fish including rainbow trout (Besse and de Kinkelin, 1965; Sano, 1971), Atlantic salmon (Mackelvie and Artsob, 1968), cutthroat trout (Parisot et al., 1963), coho salmon (Wolf and Pettijohn, 1970), and Arctic char (Ljungberg and Jorgenson, 1973). The virus has also been isolated from non-salmonids including bream (Adair and Ferguson, 1981), eels (Sano et al., 1981), pike (Ahne, 1978), white suckers (Sonstegard and McDermott, 1972), and marine molluscs (Hill, 1976). We have previously reported the association of an IPN virus with the annual spring epizootics of the Atlantic menhaden in the Chesapeake Bay (Stephens et al., 1980) and recently McAllister et al. (1982) have reported involvement of an IPN-like virus with Southern flounder mortalities off the North Carolina coast.

The virus is a 60 nm icosahedral particle with a bi-segmented double-stranded RNA genome (Cohen et al., 1973; Dobos, 1976; MacDonald and Yamamoto, 1977). Four structural proteins make up the virion (Dobos et al., 1977), the putative polymerase protein being coded for by one RNA segment and the three smaller proteins being coded by the other RNA segment (MacDonald and Dobos, 1981) whose major reaction product *in vitro* was RNase resistant and co-sedimented with the 14S virus genome (Mertens et al., 1982).

Studies to date concerned with the molecular characterization of IPNV have employed the prototype VR-299 strain isolated by Wolf et al. (1960) or other IPNV isolates made from fish in fresh water. This report compares the molecular characteristics of an IPNV isolate made from a marine fish with those of the VR-299 strain.

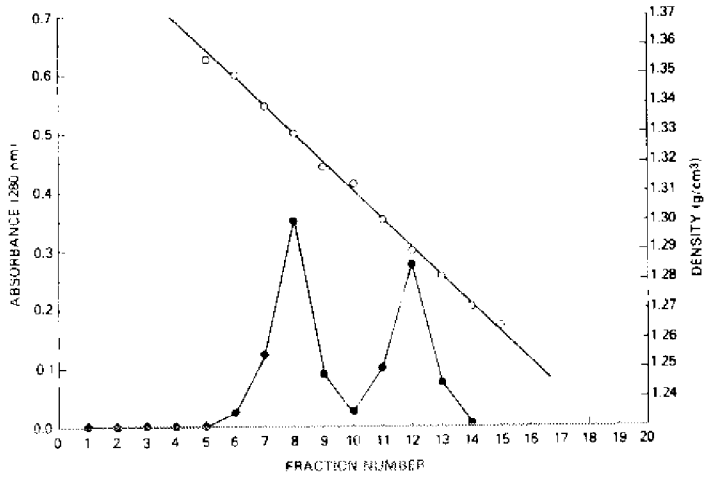

Figure 1. Isopycnic centrifugation in CsCl of harvests from cells infected with a high M.O.I. The virus preparation was centrifuged at 4°C for 20 hours at 35000 rpm using a SW50L rotor. Absorbance was monitored at 280 nm (●—●) and density of the fractions determined in a refractometer (o—o).

Viral Proteins

Harvests of cells infected at a high M.O.I. with the menhaden virus yielded two bands when subjected to equilibrium density gradient centrifugation in CsCl (Fig. 1). The band at a density of 1.33 g/cm^3 contained infectious virus while the band at 1.29 g/cm^3 had little or no infectivity. The two bands were then isolated and purified using the method described by Dobos et al. (1979). When purified virus was subjected to SDS-PAGE analysis (Laemmeli, 1970; Allen, 1980); the infectious virus band revealed 4 polypeptides with molecular weights of 86000, 56000, 30000, and 27000 daltons with the 56000 dalton protein accounting for 65% of the viral protein (Fig. 2). PAGE analysis of the top component (density 1.29 g/cm^3) revealed only the 56000 dalton protein.

Antisera generated in rabbits against both infectious virus and the top component were used in cross-neutralization tests. The results demonstrated that both antisera were protective at a serum dilution of 1-2560 using 1000 $TCID_{50}$ of virus as the test dose.

Since top component contained only the 56,000 dalton protein, this indicates that it is the major immunologic component of the virus. Neutralization testes against infectious hematopoietic necrosis virus showed no protection at a dilution of 1-5, thereby demonstrating specificity of the antiserum.

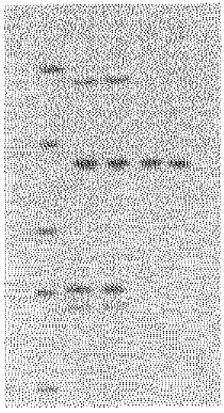

Figure 2. SDS-PAGE analysis of the polypeptides from the menhaden and VR299 strains of IPNV. Lanes: 1, molecular weight standards; 2, Menhaden infectious virus; 3, VR299 infectious virus; 4, Menhaden top component; 5, VR299 top component. Molecular weight markers (Lane 1) were phosphorylase A (90000), bovine serum albumin (65000), ovalbumin (43000), carbonic anhydrase (30000), and trypsin inhibitor (20100).

Virion Polymerase

The results in Fig. 3 show that purified virions possess the ability to incorporate ^{3}H-UTP into an acid precipitable fraction. The incorporation of ^{3}H-UTP began to plateau after 2 to 3 hr incubation.

Replacement of the ribonucleoside triphosphates with deoxyribonucleoside triphosphates (^{3}HdTTP, dATP; dCTP, dGTP) resulted in no significant radioactivity incorporated into the acid precipitable fraction suggesting that the activity was an RNA-dependent-RNA polymerase (Fig. 3).

The polymerase activity associated with the intact particle did not require prior treatment with chymotrypsin for activation as do the reoviruses (Skehel and Joklik, 1969). In fact, treatment with 100 µg of chymotrypsin for 90 min resulted in the loss of the polymerase activity (Fig. 3) as well as a decrease in infectivity from 10^{10} to $<10^{3}$ $TCID_{50}$'s per ml. The enzyme activity was dependent on the presence of Mg^{+2} because its removal resulted in

No incorporation of ^{3}H-UTP in an acid precipitable product (Fig. 4). Replacement of 20 mM Mg^{+2} with 1, 10 or 100 mM $MnCl_2$, $CaCl_2$ or NaCl resulted in no incorporation demonstrating the specficity of this enzyme for Mg^{+2}. The polymerase activity appeared to be active at 20, 25, and 30°C with the only difference being that at 25 and 30°C the curves reached a plateau more rapidly than at 20°C. Actinomycin D and α-amanitin did not inhibit the polymerae activity at the concentrations used (10 µg/ml). Also, the addition of RNase A at a concentration of 10 µg/ml did not degrade the reaction product suggesting that single stranded RNA was not released from the virus.

Viral RNA

The secondary structure of the viral nucleic acid was characterized in several ways. Purified viral nucleic acid

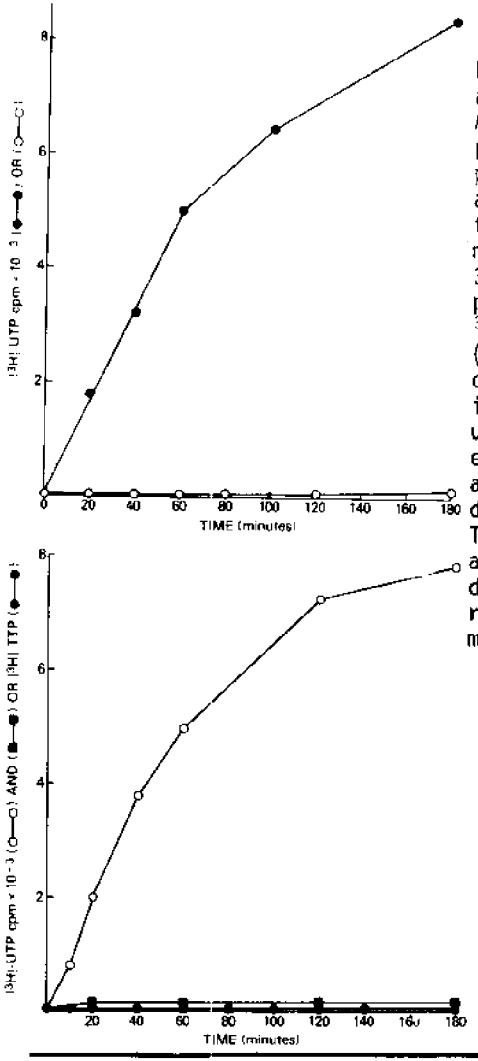

Figure 3. Assay of polymerase activity in purified virus. Assay conditions for the IPNV polymerase were 50 mM Tris-HCl, pH 8.0; MgCl, 20 mM; ATP, GTP, and CTP, 2 mM; ^{3}H-uridine triphosphate, 10 µCi (1 Ci/mmole) and 50 µg of virus at 30° C. Plotted is the TCA precipitable radioactivity of ^{3}H-UTP using untreated virus (o—o) or virus treated with chymotrypsin (■—■) and the incorporation of ^{3}HTTP by untreated virus (●—●). The effect of Mg^{+2} on the polymerase activity of purified virus is depicted on the lower graph. The conditions for polymerization were identical to those described above except that reactions contained either 20 mM (●—●) or 0 mM (o—o) $MgCl_2$.

labeled with ^{3}H-uridine (Dobos et al., 1979) was centrifuged in a self forming $CsSO_4$ gradient for 48 hours. Figure 4 illustrates that the labeled nucleic acid came to equilibrium at a density of 1.60 g/cm^3 whereas single stranded RNA controls, 16S and 23S rRNA from E. coli came to equilibrium at a density of 1.66 g/cm^3. The double stranded RNA from VR299 strain of IPNV labeled with ^{3}H-uridine was also centrifuged in a separate $CsSO_4$ gradient and came to equilibrium at a density of 1.60 g/cm^3.

When RNA samples denatured at 105°C for 5 minutes were centrifuged in $CsSO_4$ gradients, the menhaden virus as well as the VR299 strain of IPNV, demonstrated shifts in their densities from 1.60 to 1.66 g/cm^3. Both 16S and 23S rRNA from E. coli retained a density of 1.66 g/cm^3.

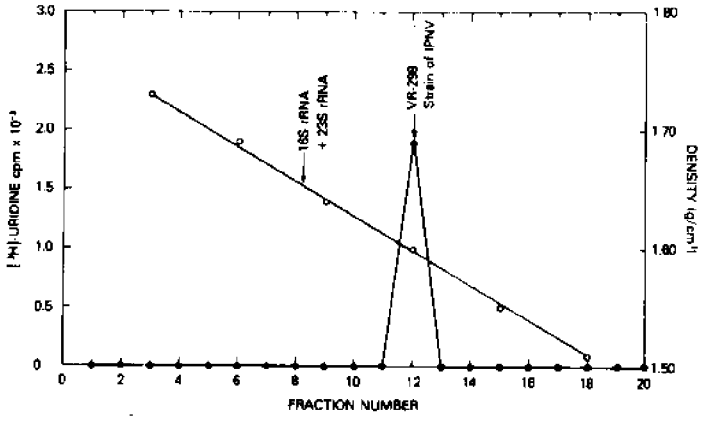

Figure 4. Isopycnic density gradient centrifugation of purified Menhaden virus RNA in a self forming $CsSO_4$ gradient. Gradients were centrifuged for 48 hours at 175,000 x g in a SW50L rotor at 20°C. Markers included were the RNA of the VR299 strain of IPNV and 16S and 23S rRNA of E. coli.

Velocity sedimentation in sucrose gradients

Menhaden virus RNA had a sedimentation value of 14S when centrifuged in a 10-30% sucrose gradient containing 70% formamide. Denaturation of the virus RNA with formamide for 30 minutes at 50°C resulted in a shift from 14S to 24S when centrifuted in sucrose-formamide gradients (Fig. 5). Similar results were obtained with the VR299 strain of IPNV.

Melting point (T_m determination of the viral RNA)

The heat stability of the virus RNA is illustrated in Fig. 6. The virus RNA exhibited a sharp melting profile indicative of a double stranded nucleic acid. The T_m of the viral RNA preparation was 87°C in 0.1X SSC buffer.

Electrophoretic patterns of the viral RNA

To determine if the menhaden virus RNA segments had minor differences in their molecular weights when compared to the VR299 strain, nucleic acids from both strains were subjected to electrophoresis in 7.5% polyacrylamide gels using the buffer system of Loenig (1967). The results in Fig. 7 show two peaks of radioactivity localized at the same position as those for the VR299 strain which have previously been determined to have molecular weights of 2.5 and 2.3 x 10^6 daltons.

Absence of a poly(A) sequence at the 3 terminus

Figure 8 shows that the menhaden viral RNA was not retained by

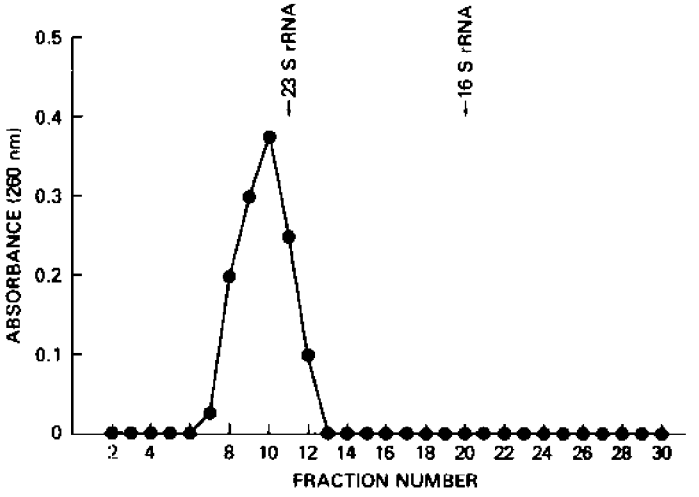

Figure 5. Density gradient centrifugation of denatured viral RNA in a 10-30% continuous sucrose-formamide gradient. Samples were denatured in 98% formamide for 30 minutes at 50°C and centrifuged in gradients for 18 hours at 40000 rpm in a SW41 rotor at 20°C. Markers used were 16S and 23S rRNA from E. coli

oligo (dT)-cellulose columns (Aviv et al., 1972) and was eluted in buffer A as was Reovirus 1 RNA, which is known not to have 3′ poly(A) sequences in its RNA. Poliovirus RNA, which does contain a poly(A) sequence, bound to the oligo (dT)-cellulose column and was eluted with buffer B. Together, these results indicate that the menhaden strain of IPNV does not have a poly(A) sequence at its 3′ terminus.

Cross hybridization studies with isolated RNA segments

The results of electrophoresis of isolated viral RNA in 7.5% acrylamide gels shown in Fig. 9 demonstrate that each ^{3}H-labeled segment was clearly separated from the other. Once it was established that each segment could be separated from each other, viral segments were labeled with ^{125}I using the thallium chloride method and hybridized to excess RNA from the other segment, to both segments, to tRNA from E. coli or itself. The results of the hybridization experiments are shown in Table 1. Segment I RNA hybridized to segment II RNA at a level of only 2.9% whereas it was found to hybridize 80.2% to segment I. The ^{125}I-labeled probe to segment I reannealed at a level of 3.1% and to 16S rRNA at a level of 2.9%. Excess RNA from both segments (I and II) hybridized to the ^{125}I-segment I at a level of 79.8%. Segment Ii showed similar results to segment I. Segment II annealed to excess segment II RNA at a level of 77.9%, to segment I at 4.5%, and to segment I and II at 78.5%. It hybridized to 16S rRNA at only a level of 2.4% and to itself at a level of 4.1%. Taken together, these hybridization studies indicate that nucleotide sequences of segments I and II are not closely related.

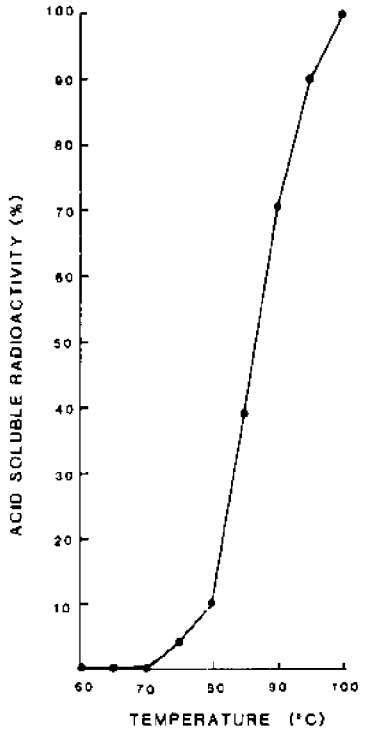

Figure 6. Thermal denaturation of the menhaden viral RNA. H-RNA was resuspended in 0.1X SSC buffer and heated from 60 to 100°C in 5°C increments. At each increment, samples were removed and treated with single strand specific RNase A. Samples were TCA precipitated and the radioactivity determined.

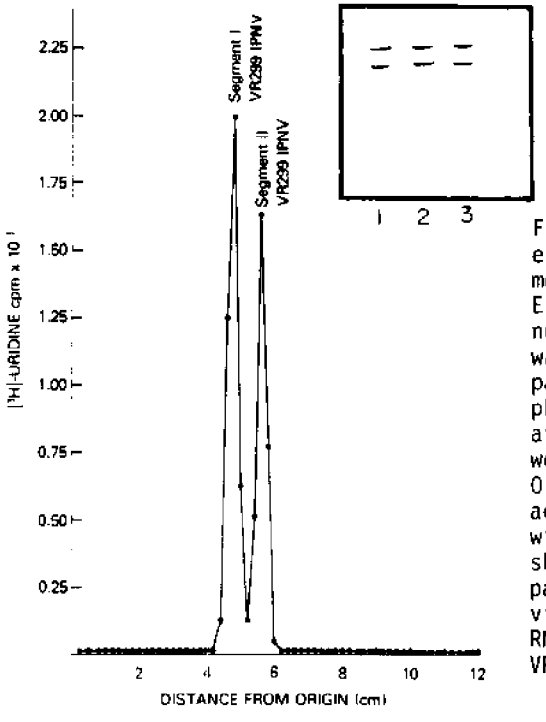

Figure 7. Acrylamide gel electrophoresis of menhaden virus RNA. Electrophoresis of purified nucleic acid from VR299 was performed in a companion gel. Electrophoresis was carried out at room temperature. Gels were either sliced into 0.2 cm pieces and radioactivity counted or stained with silver. The insert shows electrophoretic patterns of: 1-Menhaden virus RNA; 2-VR299 virus RNA; and 3-menhaden and VR299 RNAs.

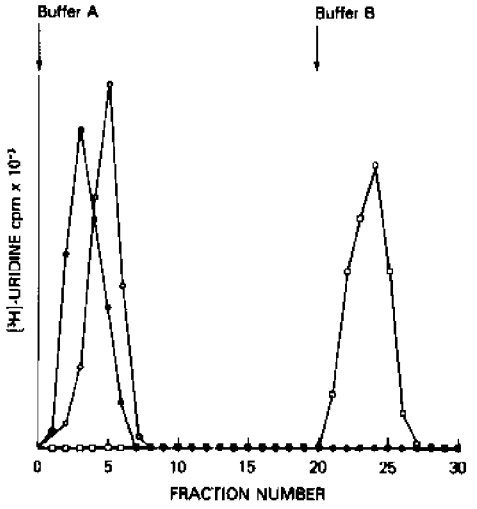

Figure 8. The ability of the menhaden virus RNA (o—o), reovirus RNA (●—●) and poliovirus RNA (□—□) to bind to a 2.0 ml oligo (dT)-cellulose column. Samples were applied to the column in buffer A (0.5 M KCl, 0.01 M Tris-HCl, pH 7.5). Poly(A) material was eluted with buffer B (0.01 M Tris-HCl, pH 7.5).

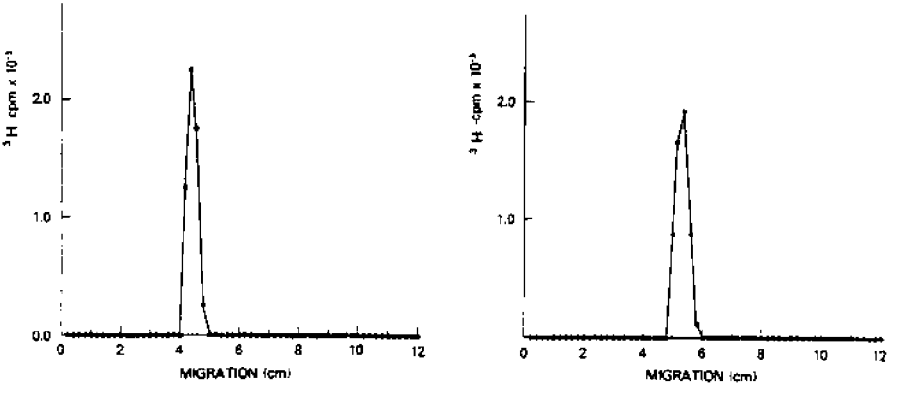

Figure 9. Demonstration of the purity of the viral RNA segments by electrophoresis. Purified ^{3}H-viral RNA was subjected to electrophoresis in 7.5% polyacrylamide gels and stained with ethidium bromide to localize RNA bands. The RNA segments were cut out, eluted from the gel and again subjected to electrophoresis in acrylamide gels. Gels were then cut into 0.2 cm piece and the radioactivity determined.

Table 1. Cross hybridization studies between RNA segments of IPNV[a]

^{125}I viral RNA	Source of Excess of RNA	Percent
Segment I[b]	None	3.1
Segment I	Segment II	2.9
Segment I	Segment I	80.2
Segment I	Segment I and II	79.8
Segment I	t-RNA (E. coli)	2.9
Segment II[c]	None	4.1
Segment II	Segment I	4.5
Segment II	Segment II	77.9
Segment II	Segment I and II	78.5
Segment II	t-RNA (E. coli)	2.4

[a]Hybridization mixtures contained 10000 cpm of ^{125}I-labeled segment I or II, 1.0 absorbance unit of excess RNA, 10 mM Tris-HCl, pH 7.5, 0.4 M NaCl and 0.1% SDS in a 100 µl volume. RNA was denatured at 105°C and allowed to renature at 60°C for 2 hours. Following RNase A digestion of the hybrids TCA precipitable material was counted in a gamma counter.
[b]The molecular weight of segment I = 2.3×10^6
[c]The molecular weight of segment II = 2.5×10^6

Discussion

It is clear from the study that the characteristics of the IPNV isolated from the Atlantic menhaden are quite similar, if not identical, to those reported for IPNV isolates from salmonids. The polypeptide profiles of the menhaden virus and the VR-299 strain of IPNV both show four virion proteins of similar molecular weights, i.e., 86, 56, 30, and 27 x 10^3 for the former and 90, 57, 29, and 27 x 10^3 for the latter (Dobos et al., 1977). Indeed, the data presented are largely confirmatory of previous work done on the molecular biology of IPNV, particularly that published from the laboratory of Peter Dobos at the University of Guelph.

Infection of cells at a high M.O.I. resulted in the increased production of defective virus having a density of 1.29 g/cm^3 in cesium chloride. The SDS-PAGE analysis of this virus band revealed only one polypeptide with a molecular weight of 56,000. Antiserum prepared to this "top component" was found to neutralize viral infectivity to the same extent as antiserum prepared against infectious virus. This indicates that the 56,000 dalton polypeptide is the major antigenic determinant for the virus. Since a similar size polypeptide has been found in other birnaviruses (Dobos et al., 1979), it will be of interest to compare these proteins for antigenic similarities or

differences. Such comparison is currently underway using monoclonal antibodies generated against several IPNV serotypes.

The nucleic acid of the menhaden virus was shown to be double-stranded by centrifugation in $CsSO_4$ gradients, electrophoresis in polyacrylamide gels, sensitivity to RNase and by thermal denaturation profiles. Centrifugation in a self-forming $CsSO_4$ gradient for 48 hr revealed that the menhaden virus RNA had a buoyant density of 1.60 gm/cm^3 as did the RNA from the VR-299 strain of IPNV. This is similar to what has been previously reported for IPNV by MacDonald and Yamamoto (1977) and Cohen et al. (1973), who reported densities of 1.60 and 1.615 g/cm^3, respectively. The single-stranded RNA controls, 16S and 23S RNA from E. coli, came to equilibrium at 1.66 g/cm^3 as did denatured RNA from the menhaden virus. Hence, density gradient centrifugation data indicates that the viral RNA is double-stranded.

Data from digestion with ribonuclease A also provided evidence that the RNA was double-stranded. Both RNA species from the menhaden virus and the VR-299 strains of IPNV were highly resistant to the action of RNase A with 93 and 92% remaining acid precipitable, respectively. This was in contrast to the sigle-stranded RNA from poliovirus which was highly degraded, resulting in only 5% remaining acid precipitable. The final evidence that the viral RNA was double-stranded came from the thermal denaturation profile. Denaturation was assessed by the ability of single-stranded RNA to become sensitive to digestion with RNase A and, therefore, not remain acid precipitable. The virus RNA denatured in a sharp profile characteristic of a double-stranded nucleic acid and had a T_m of 87.0°C in 0.1 x SSC buffer and is similar to the 89°C reported by Cohen et al. (1973) for the VR-299 strain.

It is known that significant variation can occur in the molecular weights of the RNA segments associated with the reoviruses (Ramig et al., 1977) and rotaviruses (Verly and Cohen, 1977; Ridger and Holmes, 1979) containing 10 and 11 segments of double-stranded RNA respectively. To date, electrophoretic analysis of the RNA segments has been applied to only the VR-299 strain of IPNV. Therefore, it was of interest to determine if the molecular weights of the RNA segments of the menhaden strain of IPNV were the same as that of the VR-299 strain. Electrophoresis in 7.5% acrylamide gels demonstrated the appearance of 2 peaks of RNA having molecular weights of 2.5 and 2.3 x 10^6 daltons which agrees with the findings of Cohen et al. (1973) and Dobos et al. (1976) for IPNV.

The viral RNA was then characterized for its ability to bind an oligo(dT)-cellulose column using the procedure outlined by Aviv and Leder (1972). Viruses whose RNA can be utilized as a messenger RNA, the (+) strand viruses such as the picronaviruses, have poly (A) sequences at the 3' terminus while those viruses in which the virion RNA does not serve as a messenger, e.g., reoviruses, lack poly (A) sequences at the 3' end. It was

therefore of interest to compare viral RNA from the menhaden virus to poliovirus and reovirus RNA's in this respect. The results indicated that neither reovirus or menhaden viral RNA would bind to an oligo(dT)-cellulose column under conditions w hich favored binding of poliovirus RNA. Thus, IPNV is similar to the reoviruses with respect to their absence of polyadenylic acid residues at the 3' end.

In order to substantiate the 2-dimensional fingerprint analysis of each RNA segment of IPNV by MacDonald et al. (1977), hybridization reactions involving purified RNA segments were performed. Because the in vivo incorporation of nucleotide precursors into viral RNA was low, RNA segments were denatured and radiolabeled with ^{125}I to a high specific activity. This was performed in order that only a small amount of RNA could be utilized and thereby prevent the reannealing of the RNA probe during the actual hybridization reactions. The results indicated that the two RNA segments were not related at the level of nucleotide sequences. These experiments thus confirm the results of MacDonald et al. (1977) concerning the nucleotide sequence of the 2 segments of RNA by nucleic acid hybridization techniques.

To determine if in vitro transcription was similar to that reported for the reoviruses, studies were performed using the same strategy that was utilized in the study of the RNA transcriptase associated with reoviruses (Skehel and Joklik, 1969; Banerjee and Shatkin, 1970). Several differences were observed between these two types of viruses. The RNA polymerase associated with the intact IPNV particle did not require prior treatment with chymotrypsin for activation as do the reoviruses (Skehel and Joklik, 1969). In fact, chymotrypsin treatment abolished its activity. Secondly, unlike the reovirus enzyme, which can transcribe all ten segments in vitro for 48 hr at 37 C, the polymerase activity associated with IPNV virus was found to plateau at approximately 4 hr.

We have conducted some preliminary experiments into the nature of the polymerase products (data not shown). Purification and subsequent centrifugation of the products from a 1 hr polymerase reaction through sucrose-formamide gradients revealed that the reaction products sedimented to a 14-16S position that subsequently shifted to a 14S position if the reaction was allowed to proceed for 24 hr. There never was a shift to the 24S position as might be expected if mRNA was being synthesized. This suggests that the virus associated enzyme is a RNA polymerase and not a true transcriptase. A similar polymerase activity has been shown for Drosophila X virus by Bernard (1980). When fractions from the 14S peak were pooled and denatured in the presence of 98% formamide at 50°C for 30 min, there was a shift in the sedimentation coefficient from 14S to 24S indicating that the 12S peak was composed of double-stranded RNA. In this regard, the 14-16S peak, as well as the 14S peak, were found to be resistant to the action of RNase A with 93.2 and 94.7% of the incorporated radioactivity remaining acid precipitable. When the 14S product

was denatured at 105°C for 2 min and quickly cooled on ice, the material became sensitive to RNase A with 4.1% remaining acid precipitable.

Pooled fractions from the 14S peak failed to bind to an oligo(dT)-cellulose column. Similarly, denaturation of the 14S peak by heating at 105°C for 2 min and quickly cooling on ice failed to enhance binding to the column. Therefore, it appears that the events in RNA transcription and replication do not resemble those reported in vitro for the Reoviridae.

Recently, Martens et al. (1982) described the in vitro synthesis of RNA by the IPNV polymerase. Approximately 50% of the polymerase product remained associated with the double-stranded template of the virions. The remainder was single-stranded RNA which apparently was broken down to 5-7S fragments by a virus-associated RNase. The use of bentonite as an RNase inhibitor permitted the synthesis of small amounts of single-stranded RNA which when analyzed by sucrose gradient centrifugation was identical to the 24S mRNA produced in IPNV infected cells (Dobos, 1977). The refinement of an in vitro transcription system would have value in the cloning of mRNAs from the IPNV genome which could be used for sequencing studies for the possible production for antigenic material for vaccine purposes.

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