Evaluation of Aeromonas Hydrophila Virulence Factors: Role in Natural Epizootics and Their Use as Potential Immunogens.

Larisa Ann Ford

Louisiana State University and Agricultural & Mechanical College

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Evaluation of *Aeromonas hydrophila* virulence factors: Role in natural epizootics and their use as potential immunogens

Ford, Larisa Ann, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1990
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EVALUATION OF AEROMONAS HYDROPHILA VIRULENCE FACTORS: ROLE IN NATURAL EPIZOOTICS AND THEIR USE AS POTENTIAL IMMUNOGENS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements of the degree of Doctor of Philosophy in The Interdepartmental Program in Veterinary Medical Sciences

by

Larisa Ann Ford
B.S., Texas A&M University at Galveston, 1982
M.S. Texas A&M University, 1985
May, 1990
DEDICATION

Due to the imperfections of human nature, persons who have the ability to love unconditionally throughout a lifetime are rare - if at all such a person can exist in our society. Society has created the idea and individuals are capable of striving towards the ideal, but individuals may express unconditional love only in moments or perhaps phases of one's life. This dissertation is dedicated to the memory of Mary Anna Fickes Brown, during her life she gave to me many moments of unconditional love from which I draw strength. Even in her death she gave to me a powerful gift of love—the gift to stand alone with confidence of my own self worth. Though I remember the pain and anguish I experienced when she died, I am now able to thank her for her gift. I have the courage to continue to feel, to think, and to express my ideas to my world.
Numerous persons were invaluable to the completion of this project. First, I should express my appreciation to Dr. R.L. Thune for his guidance and support throughout this study. Also, I should thank Dr. Thune for the tickets to the basketball games and for allowing me to continue my graduate studies even though I often proved to be the superior sport fisherperson. Grateful appreciation is extended to Dr. W.E. Kelso, Dr. T.G. Snider (GIG 'EM), Dr. R.L. Siebeling, Dr. K.L. Schnorr and Dr. W.J. Todd for their helpful suggestions in the organization of this study and the writing of this dissertation. I would also like to thank Drs. Newton, Issel and Stewart for the use of equipment, blatant displays of affection, and an education in the wonders of parasitology, respectively.

Maureen Johnson was more than patient while I struggled to orient myself and to complete laboratory tasks without mishap. She also served as a confidante, fellow doormat and mother hen all for which I am grateful. I thank Larry Hanson (and his wife Lora), John Hawke (and his family), Katherine Byrne' (and her houseful of kittens), Debbie Lea (and her family) and Bernhard Kaltenboeck (including Milla and the kids) for their unyielding support and their friend-no, familyship. I am also most grateful to Gioia Capelli and Cliff Monahan for their contributions in improving my emotional, mental and nutritional status when I had sunk to the Hadal Zone of life.

I am indebted to Drs. Pete Bullock and Rocco Cipriano and the entire staff of the National Fish Health Research Laboratory for gainful employment and their patience with the new kid in Bacteriology.

Finally, I must thank my big brother, John R. Ford, Jr., for allowing me the chance to shame him ONCE AGAIN!!! Yes, Little Sister has received her terminal degree prior to her older brother and even has a real job. Life is good when you can beat your older brother at anything--sibling rivalry is alive and well!
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ABSTRACT

Aeromonas hydrophila and A. sobria have been identified as major causes of mortalities among commercially raised channel catfish and are the cause of septicemic disease in a wide variety of other animals. At present, antibiotic therapy is the only prescribed treatment for Motile Aeromonad Septicemia (MAS) in channel catfish. Antibiotic treatment, however, is expensive and often not effective due to the development of resistance in the bacterial strains. Development of immunoprophylactic methods for prevention of MAS would be of value to the commercial catfish industry. Several studies have demonstrated that whole cell vaccine preparations do not confer protection to serologically heterologous strains of motile aeromonads. A common antigen needs to be identified among virulent strains of motile aeromonads in order for an efficacious vaccine to be developed.

The objective of this study was to determine the role of motile aeromonad virulence factors in natural epizootics of MAS and to determine if particular virulence factors could be used as immunogens. Bacterial isolates collected from channel catfish during MAS epizootics were screened for production of hemolysins, proteases and surface layer (S-layer) protein. Hemolysin and protease activity were variable and limited among the isolates, but 93% of these isolates produce S-layer protein. Furthermore, serum antibodies to the S-layer protein from fish immunized with S-layer preparations were detected by western blot analysis. Channel catfish immunized with crude S-layer preparations were
protected against subsequent challenge with both the homologous and a heterologous bacterial strain. The S-layer protein may be the common antigen necessary for vaccine development.
INTRODUCTION

Motile aeromonad septicemia (MAS) is a disease of aquatic animals caused by members of the motile aeromonad complex, *Aeromonas hydrophila* and *A. sobria*. These species of *Aeromonas* are facultative pathogens that can be routinely isolated from water and dead, moribund and asymptomatic fish.

Since 1982, *A. hydrophila* and *A. sobria* have accounted for 21% of the total bacterial-related fish disease cases reported by the Fish Disease Committee of the Southern Division of the American Fisheries Society. Outbreaks of MAS are often associated with stressors such as overcrowding of fish, reduced oxygen levels in pond water, poor nutritional status of the fish, elevated water temperatures in the pond and excessive or improper handling of the fish (MacMillian, 1985). Commercially raised channel catfish, *Ictalurus punctatus*, are most commonly affected by MAS epizootics. Other vertebrates (including humans) and invertebrates have been reported to have motile aeromonad infections (Lewis and Bendor, 1960; Rock and Nelson, 1965; Shotts et al., 1972; McCoy and Seidler, 1973; Amborski et al., 1974; Glorioso, 1974; Ramsey et al., 1978).

Clinical signs of MAS in fishes include lethargy and cessation to feed. Often the fish will have frayed fins and open ulcers that expose muscle tissue. Internal organs are often discolored, hemorrhagic or show signs of necrosis (Plumb, 1979).
At present, therapy with antibiotic treated feed is the only prescribed treatment protocol for fish with MAS. This alternative, however, is expensive and often ineffective due to lack of feeding activity of infected fish and/or development of antibiotic-resistant strains of bacteria. The problem of antibiotic resistance is compounded by the limited number of antibiotics that have been approved for use in food fish, and leaves the fish culturist no legal alternative treatment when epizootics involving bacterial strains resistant to approved antibiotics develop.

An alternative method in order to prevent rather than treat the disease, would be to develop a MAS vaccine for fish. Vaccines prepared from somatic antigens are available for prevention of *Yersinia ruckeri*, *Vibrio anguillarum* and *Aeromonas salmonicida*, pathogens of coldwater fish (Paterson et al., 1974; Sawyer and Strout, 1977; Austin and Rogers, 1981; Ellis, 1988). Unfortunately, somatic antigens of the motile aeromonads are more complex than those of coldwater pathogens and confer protection only to channel catfish challenged with the homologous strain (Takahashi and Kusuda, 1977; Fliermans and Hazen, 1980; Thune, 1980).

In order for a vaccine to be commercially applicable, the vaccine should induce protection to fish against all virulent strains of motile aeromonads. Differential virulence among strains of motile aeromonads is well documented and variations in the quality and quantity of extracellular toxins such as proteases and hemolysins have been associated with the degree of virulence of a particular strain (Allan and Stevenson, 1981; Thune et al., 1982a and b). Recently, a S-layer
protein has also been reported as a potential virulence factor of motile aeromonads (Dooley and Trust, 1988). These virulence factors may serve as the common antigen(s) necessary for development of an efficacious vaccine. Studies investigating the nature of these virulence factors have used a limited number of strains, and often conclusions are based on information from strains that have been maintained in the laboratory for extended periods of time. Little information exists concerning the role of these virulence factors in natural epizootics and their ability to induce a specific antibody response and protection against MAS in fish.
Bacteria belonging to the genus *Aeromonas* are among the most common bacteria isolated from freshwater habitats (Allen et al., 1983a and b; Allen-Austin et al., 1984). Several species of *Aeromonas* have been recognized as pathogens of aquatic animals. Due to the heterogenous nature of the biochemical, genetic and serological characteristics of the *Aeromonas* genus, taxonomic position of this genus has been the subject of scientific debate and confusion.

The genus *Aeromonas* belongs to the family Vibrionaceae. Members of this genus are Gram negative rods (1.0-3.5 um in length) that occur singly, in pairs or in chains. Most members of this genus are motile by a single polar flagellum. One species, *A. salmonicida*, is non-motile. Aeromonads are facultative anaerobes that are able to ferment glucose with or without production of gas. Aeromonads are oxidase positive and are resistant to the vibriostatic agent 2,4-diamino-6,7-diisopropylpterdine (0/129) (Eddy 1960; Eddy, 1962; Eddy and Carpenter, 1964; Schubert, 1974).

Currently, *Berger's Manual of Systematic Bacteriology* (Krieg and Holt, 1984) separates the genus *Aeromonas* into two distinct groups. The first group includes *A. salmonicida*, a nonmotile psychrophilic aeromonad that is a well known obligate pathogen of salmonids. The second group
includes the motile aeromonads *A. hydrophila*, *A. sobria* and *A. caviae* (Table 1). In earlier literature, members of the motile aeromonads were described under various names including *Aeromonas liquefaciens*, *A. punctata*, *Bacillus punctatus*, *B. ranicida*, *Bacterium punctatum*, *Proteus hydrophilus*, *Pseudomonas punctata*, *Aerobacter* sp. and *Achromobacter* sp. (Bullock, 1964; Bullock and Snieszko, 1971; Snieszko and Axelrod, 1971; Cipriano et al., 1984).

Kluyver and van Niel (1936) as cited by Schubert (1974) first placed isolates of *Bacillus*, *Pseudomonas*, *Proteus* and *Aerobacter* that had been associated with hemorrhagic disease in fish into one new genus, *Aeromonas*. This genus included bacteria that were Gram negative, motile rods with a single flagellum and were able to ferment glucose with or without production of gas. The genus was subdivided by Snieszko (1957) to include 3 species *A. hydrophila*, *A. punctata* and *A. liquefaciens*. In Snieszko's classification *A. liquefaciens* was considered to be the only species pathogenic to fish. Schubert (1967a, 1976b) and later Popoff and Veron (1976) invalidated the division of the genus into the 3 species proposed by Snieszko (1957). Today, *A. hydrophila*, *A. sobria* and *A. caviae* are considered to comprise the motile aeromonad complex (Lallier et al., 1981; Popoff and Veron, 1981; Waltman et al., 1982).
TABLE 1: Biochemical characteristics used to differentiate species of aeromonads (after Popoff, 1984)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>A. hydrophila</th>
<th>A. sobria</th>
<th>A. caviae</th>
<th>A. salmonicida</th>
</tr>
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<tbody>
<tr>
<td>Brown water soluble pigment</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Growth in nutrient broth, 37°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Esculin hydrolysis</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose utilization</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gas from glucose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>H₂S from cysteine</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</table>
DISTRIBUTION OF MOTILE AEROMONADS

The motile aeromonads are distributed worldwide in ubiquitous habitats of surface waters and may be present in high numbers with density dependent upon water temperature and degree of eutrophication (Hazen et al., 1978a). High numbers of Aeromonas hydrophila and A. sobria have been reported in activated sludge samples, sewage effluents, rivers and domestic tap water (Fliermans et al., 1977; Neilson, 1978; Aruajo, et al., 1989). Hazen et al. (1978a) surveyed 147 natural aquatic habitats throughout the United States and Puerto Rico and noted higher densities of motile aeromonads in slightly saline habitats but were not able to isolate motile aeromonads from extremely saline waters. Although aeromonads comprise part of the normal intestinal flora of fish and amphibians (Trust et al., 1974; Bullock and Stuckey, 1975; MacMillan, 1985), motile aeromonads have been associated with hemorrhagic diseases of aquatic animals and probably have been a common disease syndrome of aquatic species since the Middle Ages (Otte, 1963). The first case of hemorrhagic septicemia in fish was reported by Schaperclaus (1930). He reported Pseudomonas punctata (which is now considered to be one of the motile aeromonads) as the cause of disease in carp. Aeromonas hydrophila and A. sobria have now been reported to cause hemorrhagic septicemia in frogs ("red leg" disease), reptiles and a variety of fish including golden shiners (Notemigonus crysoleucas), gizzard shad (Abramis brama), goldfish, striped bass, channel catfish and salmonids
Motile aeromonads, including \textit{A. caviae}, have been associated with wound and burn infections of humans and recently have been reported as the cause of pneumonia, sepsis, diarrhea and ocular infections especially in immunocompromised individuals or individuals exposed to water containing high numbers of the bacteria (von Gravenitz and Mensch, 1968; Bullock and Snieszko, 1971; Davis et al., 1978; Smith, 1980; Rienes and Cook, 1981; MacMillian, 1985; Janda and Duffey, 1988).

\textbf{MOTILE AEROMONAD SEPTICEMIA OF CHANNEL CATFISH}

\textit{Aeromonas hydrophila} and \textit{A. sobria} are causative agents of Motile Aeromonad Septicemia (MAS), a hemorrhagic disease of fish. MAS epizootics are a significant disease problem in the commercial catfish industry. Channel catfish infected with motile aeromonads display a wide range of pathological conditions and the severity of the disease is dependent on environmental and physiological parameters such as stocking density of the affected fish, quality of the pond water, overwintering conditions, presence of other pathogens, degree of injury due to handling, genetic resistance of the host fish and virulence of the particular bacterial strain (Cipriano et al., 1984; MacMillian, 1985).

Portals of entry utilized by motile aeromonads in order to invade host fish are not well understood though the bacteria are suspected to enter fish via open wounds, the gills and stomach epithelium (Ventura and Grizzle, 1987). The disease may manifest itself as an acute or chronic infection in fish. In the acute form of the disease,
### TABLE 2: Species of invertebrates and vertebrates in which motile aeromonads have been reported (adapted from Newman, 1982).

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<td>Shrimp (<em>Machobrachium ohioni</em>)</td>
<td>De Figueiredo and Plumb, 1971</td>
</tr>
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<td>Snails (<em>Achatina futlica.</em>)</td>
<td>Mead, 1969</td>
</tr>
<tr>
<td>Earthworm (<em>Lumbricus terristris</em>)</td>
<td>Marks and Cooper, 1977</td>
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<td>Striped mullet (<em>Mugil cephalus</em>)</td>
<td>Soliman et al., 1989; Callinan and Keep, 1989</td>
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<tr>
<td>Golden shiners (<em>Notemigonus crysoleucas</em>)</td>
<td>Lewis and Bender, 1960; Meyer, 1964; Johnson, 1974</td>
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<td>Channel catfish (<em>Ictalurus punctatus</em>)</td>
<td>Rock and Nelson, 1965; Plumb, 1975; Plumb et al., 1976; MacMillian, 1985</td>
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<td>Gizzard shad (<em>Dorsoma cepedianum</em>)</td>
<td>Rock and Nelson, 1965; Toranzo et al., 1989</td>
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<td>Threadfin shad (<em>Dorosoma petenense</em>)</td>
<td>Haley et al., 1967</td>
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<td>American shad (<em>Alosa sapidissima</em>)</td>
<td>Haley et al., 1967</td>
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<td>Bream (<em>Abramis brama</em>)</td>
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<td>Eel (<em>Anguilla anguilla</em>)</td>
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<td>Ayu (<em>Plecoglossus altivelis</em>)</td>
<td>Vezina and Desrochers, 1971</td>
</tr>
<tr>
<td>Perch (<em>Perca florescens</em>)</td>
<td>Shotts et al., 1972; Esch and Hazen, 1981</td>
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<tr>
<td>Bluegills (<em>Lepomis macrochirus</em>)</td>
<td>Esch and Hazen, 1981; Hazen et al., 1978b; Shotts et al., 1972</td>
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<td>Bass (<em>Morone spp</em>)</td>
<td>Collins, 1970; Wood, 1968; Arkwright, 1912; Marsh, 1902</td>
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<td>Trout (<em>Onchorynchus mykiss</em>)</td>
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<td>Salmon (miscellaneous species)</td>
<td>Larsen and Jensen, 1977</td>
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<td>Cod (<em>Gadus morhua</em>)</td>
<td>Kou, 1972</td>
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<td>Goldfish (<em>Carassius auratus</em>)</td>
<td>Balarin, 1979</td>
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<td>Tilapia (<em>Tilapia aurea</em>)</td>
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<td>Rigney et al., 1978; Grahman,</td>
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<td>Rigney et al., 1978; Marcus, 1971;</td>
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<td>Alligators (Alligator</td>
<td>Shotts et al., 1972; Gordon</td>
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<td>mississippiensis)</td>
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<td>Snakes (several species)</td>
<td>Esterabadi et al., 1973</td>
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<td>Cattle</td>
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exophthalmia and distention of the abdomen may be apparent, although septicemia in the acute form is often fatal with no apparent gross clinical signs. Internally, the liver and kidneys are swollen, friable and hemorrhagic. The chronic form of MAS is characterized by formation of deep dermal ulcers associated with hemorrhage and inflammation. Dermis, epidermis and musculature in the area of the ulcer are often necrotic. Petechial hemorrhages may also be apparent throughout the musculature and peritoneum. Histologically, motile aeromonads cause congestion and necrosis of hepatocytes and hemopoietic tissue. Bach et al. (1978) experimentally infected channel catfish with *A. hydrophila* and demonstrated pathology that included bacterial colonization and increased macrophage infiltration in the reticular sheaths of the splenic ellipsoids. Physiological changes of channel catfish infected with motile aeromonads have not been extensively studied or documented. Anemia may or may not be noted in channel catfish infected with motile aeromonads, and largemouth bass have decreased red blood cell and white blood cell counts with an increase in cortisol levels during MAS epizootics (Esch and Hazen, 1980). Similar to other fish infected with gram negative bacteria, carp infected with *Aeromonas* exhibit a decrease in blood glucose, albumin, globulin, total protein and cholesterol. Uric acid and bilirubin values are usually elevated in fish with gram negative infections (Amlacher, 1961; Gaines, 1972; Huizinga et al., 1979; MacMillian, 1985).
Motile aeromonads are isolated from approximately 25% of the commercially raised channel catfish dying from bacterial infections. Most mortality occurs in April, August and September when the water temperatures are high and the dissolved oxygen concentrations of the pond water are low (Meyer, 1970; MacMillian, 1985). The mortality rate of channel catfish with aeromonad infections vary from a few sporadic deaths to 100% depending on virulence of the bacterial strain, physiological condition of the fish, and environmental conditions in the pond. The economic value of fish that succumb to MAS is a significant cost for the industry and warrants the development of practical, efficacious prevention and treatment protocols.

**TREATMENT OF MOTILE AEROMONAD SEPTICEMIA**

The only method of treatment for channel catfish diagnosed as having MAS is to offer antibiotic-supplemented feed to the fish. Two antibiotics are currently available in commercially prepared feeds and both are USDA/FDA approved for use in food fish. Terramycin® (oxytetracycline) is available in a sinking commercial diet containing 84 to 117 g of active antibiotic/100 lbs of feed. Romet® is a potentiated sulfonamide that is incorporated into a floating pellet. The disadvantages of feeding Terramycin® are twofold: the fish can not be observed feeding, and the bacterial strain causing the epizootic has often developed antibiotic resistance to Terramycin® (Hedges et al., 1985). In fact, even in natural environments such as the Chesapeake Bay,
Terramycin\textsuperscript{R} resistant motile aeromonads have been isolated (Aoki and Egusa, 1971; McNichol et al., 1980; Rahim et al., 1984). Feed containing Romet\textsuperscript{R} is advantageous since the antibiotic can withstand the processing necessary for incorporation of the drug into floating pellets, thus infected fish can be observed eating medicated feed. Unfortunately, Romet\textsuperscript{R} is approved for use against enteric septicemia of catfish (ESC), a disease caused by \textit{Edwardsiella ictaluri}, and can not legally be used to treat MAS unless ESC is also diagnosed (MacMillan, 1985). In order to be efficacious both antibiotic feeds must be used while the fish are still feeding. One of the first clinical signs of bacterial infection that a commercial farmer notices is that fish stop feeding, so antibiotic feed is not useful in these situations. Medicated feeds are also expensive and the cost of feed may out weigh loss of marketable fish if the mortality rate is low. The limited number of antibiotics available to the commercial farmer and inherent problems of using medicated feeds effectively encourage fish health professionals to advise culturists to employ methods to prevent rather than to treat MAS.

**VACCINATION AGAINST MOTILE AEROMONAD SEPTICEMIA**

Stress often contributes to the onset of MAS in commercial catfish ponds. Therefore, maintenance of optimal water quality, stocking density, feeding rates, and minimization of handling or transport of fish are emphasized in fish health management plans for commercial
catfish farms. Because commercial catfish operations are extensive, stress is an inherent problem for even the best managed facilities. In order to prevent outbreaks of MAS, vaccination of catfish could prove to be efficacious because of the limited success of antibiotic therapy.

Currently, there are no commercially available vaccines for channel catfish. Several studies have focused on development of a vaccine/bacterin for MAS but have reported only limited success. To date, two types of vaccines have been investigated as to their potential efficacy in conferring protection to fish against natural MAS epizootics. Monovalent vaccines were among the first approaches to be tested. Khalifa and Post (1963) and Post (1966) demonstrated protection of fish exposed to a monovalent vaccine if fish were subsequently challenged with a homologous strain. Mortality of Japanese eels (Anguilla japonica) was reduced below control levels after they were vaccinated for MAS with a monovalent vaccine and subsequently challenged with the homologous strain (Song, et al., 1976). Protection was also conferred in salmonids vaccinated with a monovalent preparation by the intraperitoneal (IP) route or by hyperosmotic infiltration (HI) (Udey and Fryer, 1978; Acuigrup, 1980). Monovalent vaccines confer protection to challenge with the vaccine strain, but commercial catfish ponds may be populated by several potentially pathogenic strains are likely to be present. Monovalent vaccines would have limited commercial application, but might be used if a single enzootic strain was used to produced a monovalent vaccine for fish at that facility alone. A second approach that has been investigated employs several strains of motile aeromonads and is referred to as a polyvalent vaccine. Thune and Plumb (1982 and
1984) immersion-vaccinated channel catfish with a polyvalent, sonicated preparation. Fish challenged with the homologous strains were protected, but fish challenged with heterologous strains were not. Schaperclaus (1970) stated that polyvalent vaccines were more effective in conferring protection than a monovalent vaccine, but to be commercially applicable, polyvalent vaccines would have to contain all isolates that a fish would likely encounter during the entire production cycle. This approach is both impractical and quite impossible (Plumb, 1984).

Other considerations or complications for marketing a commercially available MAS vaccine for fish include the method of antigen preparation and the route of administration utilized. Song et al. (1976) noted that attenuated A. hydrophila cells induced a higher antibody response in Japanese eels than either heat-killed cells, formalized cells or soluble extracts from the bacteria. Thune and Plumb (1982) compared heat inactivated whole cells, sonicated and freeze-thawed cell preparations for differences in antibody response of channel catfish. The best antibody responses occurred in fish that had received sonicated A. hydrophila cells with Freund's complete adjuvant (FCA). Isbell and Pauley (1983) injected chloroform killed A. hydrophila alone and with FCA intramuscularly (IM) into brown bullheads (Ictalurus nebulosus) and found that the antibody response resembled the pattern in channel catfish. Bullheads that received the antigen plus FCA responded with the highest titers and their response peaked approximately 5-6 weeks post vaccination.

Route of administration for MAS vaccines has been investigated extensively. In Thune and Plumb, (1982), each vaccine preparation was
administered IM, HI, or in an aerosol spray. The method that best invoked an antibody response in channel catfish was the injected vaccine, regardless of type of antigen preparation. Schachte (1978) used a polyvalent vaccine containing heat inactivated *A. hydrophila* and *Flexibacter columnaris* cells to test the differences between oral, injected and immersion routes of vaccine administration and antibody titers induced in channel catfish. Highest antibody titers against *A. hydrophila* were recorded in fish vaccinated by injection. A high degree of variation in titer was recorded in all groups so there was no statically significant differences in antibody titers of fish vaccinated by different routes of administration. Pyle and Dawe (1985) reported similar results for channel catfish vaccinated with formalin killed *Brucella* cells by IM injection, oral drench or topical application. Antibody titers for fish vaccinated by the three routes all peaked in the third week post vaccination, and the greatest antibody responses were reported in the fish that had been injected IM. Injection methods of administration of vaccines is regarded as the most effective for inducing immune responses in fish and adjuvants can easily be used in conjunction with the antigen of interest to increase the immune response. Injection methods have several disadvantages; young fish are not easily injected, injecting mass numbers of larger fish is laborious, and fish become stressed due to handling. The injection method of vaccination could be applied effectively on a commercial scale if injected vaccine was used, for instance, in broodstock. Oral vaccinations could easily be commercially applied to large number of fish of various sizes without the intensive labor required for injection
methods. Disadvantages of using oral vaccines are that high concentrations of antigen are required, the antigen must retain its immunogenicity after being subjected to the conditions of processing feed stuffs, the dosage of antigen an individual fish receives can not be determined, and microencapsulation of the antigen might be necessary to protect the antigen from proteolytic enzymes and acidic condition in the fish's gastrointestinal tract. Immersion methods of vaccination (HI, direct, or spray) have obvious advantages for large-scale applications involving various sizes of fish. Hyperosmotic immersion methods are reported to induce high levels of protection but fish also experience extreme osmotic shock. Again, high levels of antigen are required for immersion vaccination methods because only 0.01% to 0.2% of the initial bath vaccination antigen concentration is taken up by the fish (Tatner and Horne, 1983; Lamer, et al., 1985a and b).

Because whole cell vaccine preparations do not protect fish against challenge with heterologous strains of motile aeromonads, investigators have begun to assess the value of bacterial components that are considered to be potential virulence factors as vaccine preparations. Components that are generally considered virulence factors for motile aeromonads are discussed in more detail in the following section.

**VIRULENCE FACTORS OF MOTILE AEROMONADS**

As previously stated, motile aeromonads can be routinely isolated from a variety of sources and can cause a wide range of clinical...
symptoms in the infected host. Several investigators have been interested in determining if a difference in virulence among strains could be correlated to the source of origin. De Figueirido and Plumb (1977) reported that motile aeromonads isolated from pond water were significantly less virulent than isolates from diseased fish. In another study, A. hydrophila isolates from diseased fish were more virulent for rainbow trout than A. sobria strains isolated from healthy fish (Lallier, 1980). Mittel et al. (1980) examined 25 strains of motile aeromonads and classified them according to virulence based on LD$_{50}$ determinations. Isolates with a LD$_{50} < 10^{5.5}$ in channel catfish fingerlings were classified as virulent. Clearly, motile aeromonads have differential virulence and the source of the isolate is correlated with pathogenicity of the isolate. Mechanisms and factors involved in determining virulence of motile aeromonads are not clearly understood, but several virulence factors have been proposed for the motile aeromonad complex. Extracellular products (hemolysins, proteases, enterotoxins and siderophores) as well as surface components of the bacteria (endotoxin, pili and the surface layer protein) are factors that have been proposed to have a role in pathogenesis of motile aeromonad infections and have been chosen for further discussion below.

HEMOLYSINS

Since hemorrhage is a characteristic clinical sign of animals infected with motile aeromonads, hemolysin may be an important factor in pathogenicity of these bacteria. Two hemolysins (a and B) have been reported for motile aeromonads (Wretlind et al., 1971; Thune et al.,
1982a; Asao et al., 1986; Chakraborty et al., 1987). The α-hemolysin has a molecular weight (MW) of 65 kd and isoelectric points of 5.2 and 4.8 (Kanai and Takagi, 1986). Heating α-hemolysin to 56°C for 10 min will inactivate enzyme activity. Yield of α-hemolysin activity for the bacteria is greater at an incubation temperature of 22°C than 30°C, but yield is reduced at 37°C incubation temperature. Production of α-hemolysin is stimulated by addition of zinc ions (18 ug/ml) in the growth medium, but production is suppressed by iron (0.5 ug/ml) (Ljungh and Wadstrom, 1986). Crude preparations of α-hemolysin are stable in a wide range of pH (3.5 - 9.5) at room temperature (Ljungh et al., 1981). The α-hemolysin is cytotoxic to HeLa cells and the cytotoxic action is restricted to the target cell membrane. This action, however, of the enzyme is reversible (Ljungh and Wadstrom, 1986).

The B-hemolysin is also known as "aerolysin" (Bernheimer and Avigad, 1974; Buckley et al., 1981). A fibrinolysin (Caelitz, 1966), and a non-enterotoxigenic leucocidin (Scholz et al., 1974; Donta and Haddow, 1978) have been reported but are both now considered to be properties of the B-hemolysin. This hemolysin has a molecular weight (MW) of 50-51 kd and has isoelectric points reported at 4.2, 5.4 and 5.5. The B-hemolysin is heat labile and activity of the enzyme is completely abolished after heating at 50°C for 1 hr. The B-hemolysin does not appear in culture media until the logarithmic growth phase of the bacterium occurs. Activity of this hemolysin is not diminished by pronase or trypsin and the hemolysin is irreversibly cytotoxic. The hemolysins (α and B) are neutralized by antibodies produced to the B hemolysin. Neutralization of both hemolysins by an antibody raised against the B hemolysin
indicates that sample preparations are cross contaminated or that there may be an antigenic relationship between the two proteins (Ljungh and Wadstrom, 1986).

Although Rigney et al. (1978) found that injection of frogs with crude hemolysin preparations from motile aeromonads did not induce clinical signs of "red-leg" disease, an injection of crude hemolysin and endotoxin did induce clinical signs of the disease in frogs. Extracellular products from motile aeromonads containing both hemolysin and proteases injected into trout also cause pathology (Allan and Stevenson, 1980). Thune et al. (1982a) reported that exotoxin preparations from motile aeromonads appeared to have two lethal factors, proteases and hemolysins. Subsequently, the hemolysin fraction was determined to be lethal to channel catfish fingerlings at a level of 0.6 mg of purified protein per gram of fish (Thune et al., 1986). The hemolysin is also lethal to mice, rats and rabbits (Ljungh et al., 1981). These studies indicate that hemolysin, particularly the B-hemolysin, may have a role in determining virulence of a particular motile aeromonad strain.

PROTEASES

Motile aeromonads vary in both quantity and quality of the extracellular enzymes that they produce. Variation in proteolytic activity of motile aeromonads has been studied by several investigators. Protease is produced by the majority of motile aeromonads. Janda (1985) and Shotts et al. (1985) reported that 99% of the strains they tested were able to digest gelatin and >55% of the
strains were elastase positive. Proteolytic digestion of albumin, fibrogen and casein were also reported as well as the presence of trypsin-like activity in a majority of the strains investigated. Chabot (1987) noted that these investigators did not distinguish between proteases produced extracellularly and those associated with the bacterial cell surface nor proteases released upon cell death. Extracellular proteases produced by motile aeromonads have been reported by several investigators (Wilkes et al., 1969; Dahle, 1971a and b; Denis and Veillet-Procet, 1980; Allan and Stevenson, 1981; Thune et al., 1982b; Kanai and Wakabayashi, 1984; Ljungh, 1984; Pansare et al., 1985; Chabot, 1987). The quality and quantity of extracellular proteases produced by a particular strain are dependent on the composition of the growth medium and incubation conditions (O’Reilly and Day, 1982). Riddle et al. (1981) described a defined medium (MMGGH) for use in detection of proteases from motile aeromonads. Thompson (1979) reported that varying the amount of iron in a defined medium would maximize growth of motile aeromonads but decrease protease production. Similar effects were noted when the degree of aeration was increased. Optimum growth temperature for two proteases, a heat stable and a heat labile molecule, has been reported to be 30°C. The molecular weight of the heat-stable protease is 22.1 kd and is reported to be both dermonecrotic and lethal to channel catfish (18.0 ug/ g of fish). The heat labile protease has a MW of 43.6 kd and is also dermonecrotic and lethal for channel catfish (LD₅₀ 3.0 ug/g of fish) (Dahle, 1971a and b; Ljungh et al, 1981; Thune et al., 1982b). Amborski et al. (1984), Nieto and Ellis (1986) and Chabot (1987) determined the heat labile protease (19.5 kd)
to be a serine protease (i.e. serine residues are characteristic of the active site of this protease); the heat stable protease included two metalloproteases, one (MW=34-35 kd) that had optimum activity at pH 8.0 and the other (MW=56 kd) being only moderately heat stable and having optimum activity at a wide range of pH (7-11).

Extracellular proteases from A. hydrophila and A. sobria have been shown to be lethal to channel catfish (Thune et al., 1982b), Atlantic salmon (Shieh, 1988) and other salmonids (Shieh and MacLean, 1975; Stevenson and Allan, 1981). Leung and Stevenson (1988) produced an A. hydrophila mutant by transposon mutagenesis. The parent strain was virulent in rainbow trout and produced a single metalloprotease. The mutant was nonvirulent and produced protease at a much reduced level compared to the parent strain. The mutant strain was also determined to be more susceptible to bactericidal activity of normal trout serum and grew poorly in heat inactivated trout serum. The authors concluded that the protease may provide a role in virulence and pathogenicity of the strain by aiding the bacterial cell in overcoming initial defenses of the host and by providing nutrients for bacterial proliferation. Chabot (1987) found no correlation between virulence of motile aeromonads in channel catfish and production of any of the three major motile aeromonad proteases.

ENTEROTOXIN

Enteropathogenicity of Aeromonas hydrophila was first determined in studies in which whole bacterial cells were injected into ligated rabbit intestinal loops. Enteropathogenicity was determined by evidence
of mucosal injury of the intestinal wall and fluid accumulation (Sanyal et al., 1975). Watson et al. (1985) described an enterotoxigenic strain isolated from human fecal samples and concluded that dysenteric symptoms observed in some human clinical cases may be due to aeromonad infections. The enterotoxin has been subsequently described as heat labile when heated at 56°C for 10 min, but the toxic nature is restored upon passage through rabbit intestinal loops. This toxin has a MW of 15-20 kd and isoelectric points of 4 and 6 (Ljungh, 1984), and is stable in pH ranging from pH 4.5 - 10. Optimal yield is produced by bacteria in late logarithmic growth phase incubated at 37°C. The enterotoxin has been described as having both cytotoxic (Cumberbatch et al., 1979; Chakraborty et al., 1984; Shamado et al., 1984) and cytotonic (Rose et al., 1989) characteristics. Another enterotoxin which is heat stable has been reported (Boulanger et al., 1977) but no further studies have described this toxin.

SIDEROPHORES

In the serum and secretions of vertebrates, iron is bound to glycoproteins, transferrin and lactoferrin. These proteins have such a high affinity for iron that serum and secretions are considered an iron limited environment for invading microorganisms. Bacteria must have a means to survive in such an iron depleted environment because iron is an essential nutrient that serves a role in electron transport and as a cofactor of metabolic enzymes. A common strategy employed by bacteria to overcome an iron limited environment is production of siderophores. Siderophores are extracellular, low molecular weight, high affinity iron
chelators that solubilize iron from organic compounds and minerals in iron-limited growth conditions. Some siderophores may also remove iron directly from transferrin and lactoferrin. The iron-siderophore complex is recognized by specific membrane receptors that mediate the transport of iron into the bacterial cell cytosol (Andrus et al., 1983; Crosa, 1989; Griffiths et al., 1988).

A well known marine fish pathogen, *Vibrio anguillarum*, utilizes a plasmid-mediated iron uptake system that involves a water soluble siderophore (anguibactin). Studies have indicated that the presence of anguibactin in *V. anguillarum* isolates is strongly correlated with virulence (Crosa, 1989). Recently, siderophores have been reported in *A. hydrophila* isolates. Barghouthi et al. (1989) reported that out of 25 *A. hydrophila* isolates studied, 70% produced the siderophore, amonabactin. Several of the isolates were reported to also produce enterobactin, a common siderophore of *E. coli*. The authors suggested that production of amonabactin by a particular *A. hydrophila* isolate may indicate the degree of virulence of the isolate. The authors did point out, however, that more studies need to be done in order to verify this hypothesis.

**ENDOTOXIN**

Endotoxin, lipopolysaccharide (LPS) and O-somatic antigen are often used as synonyms. LPS of Gram negative bacteria share a common structure, lipid A, which is covalently bound to the core oligosaccharides and O-specific side chains. The O-chains are composed of polymers of oligosaccharide molecules in repeating units. The core
oligosaccharide is group specific and is a common antigen shared by a single genus of bacteria. The core portion contains 2-keto-3-deoxy-D-manno-octanate (KDO), a unique sugar as well as, hexoses, heptose and phosphoethalamine. The LPS is a high molecular weight compound \((0.2 \times 10^6 - 1.0 \times 10^6 \text{ d})\) that is heat stable at 100°C (Rogers, 1983).

From the bacterial standpoint, LPS probably functions to maintain structural integrity of the cell as well as play a role in permeability of the outer layer of the cell.

The role of LPS in Gram negative infections is not well understood, although endotoxins exhibit highly diversified patterns of pathophysiological effects which are well documented. In mammals, for example, fever, hypotension and changes in leucocyte counts are induced by endotoxic action of LPS (Arbuthnott, 1981; McCartney and Wardlaw, 1985). The portion of the LPS that is responsible for toxicity is the lipid A portion. Again, the mechanism of LPS (lipid A) toxicity is not well understood but is thought to involve one of two means of action. First, the toxin molecule may bind to a membrane receptor of a sensitive host cell thereby triggering a series of cytoplasmic events that lead to impaired metabolism of the host cell. The second mechanism involves interaction of the toxin with lipids and phospholipids of the host cell membrane, changing the permeability in the host cell membrane and causing cell damage (Arbuthnott, 1978; Rigney et al., 1978; Luderitz et al., 1986).

Antigenic heterogeneity of the motile aeromonads is well documented (Liu, 1961; LeBlanc et al., 1981; Dooley et al., 1985; Howard and Buckely, 1985). As early as 1938, Snieszko reported little cross
agglutination among *A. liquefaciens* (now considered to belong to the motile aeromonad complex) strains from diseased carp (Newman, 1982). Miles and Miles (1951) reported that 12 strains had some degree of heterogeneity in their cross agglutination patterns. Ewing et al. (1961) demonstrated twelve O-antigen groups 71 *A. hydrophila* isolates, and 13 strains examined by Takahashi et al. (1977) were found to be heterogeneous. A definitive study by Fliermans and Hazen (1977) demonstrated by immunofluorescence that only 27.5% of 255 isolates reacted with at least one of three antisera prepared from 3 aeromonad strains.

The role of endotoxin in virulence of motile aeromonads is unclear. Investigators have reported that endotoxin must be present in an injection with an exotoxin such as hemolysin in order to induce lesions or death in a susceptible animal (Rigney et al., 1978; Thune et al., 1982a). Endotoxin may have a synergistic role with other factors in virulence of motile aeromonads, but evidence indicates that endotoxin does not have a primary role in pathogenesis of motile aeromonad infections in channel catfish. The great diversity of O-antigens as demonstrated by cross agglutination and fluorescent antibody techniques described above, however does explain the limited success and usefulness of whole cell vaccine preparations in conferring protection to fish.

**FIMBRIAE AND PILI**

Fimbriae or pili are fine hair-like structures (ranging in diameter from 3-14 nm) that radiate out from the surface of bacterial cells. The term "sex pili" refers to thicker, less numerous structures which
radiate from the surface of bacterial cells but are involved in transfer of DNA between bacterial cells (Rogers, 1983).

Pili resemble flagella in terms of structure. Pili are composed of sub-units of ~ 16 kd protein and are produced by several groups of gram negative bacteria including Enterobacteriaceae, Pseudomonadaceae, Proteus spp., and Vibrio spp. (Rogers, 1983). Pili have been correlated with virulence of some groups of bacteria. Lallier and Daigneault (1984) reported that pathogenic strains of A. hydrophila were piliated and that non-virulent strains were not piliated. They also noted that piliated strains represented two antigenic groups and suggested that a particular antigenic group of pili might be virulent for a specific group of fish. Dubourguier et al. (1978) reported E. coli isolates pathogenic for calves belonged to a pili group that was antigenically different from the group of isolates that were pathogenic for both humans and pigs. Recently, Sato et al. (1989) characterized pili from 10 strains of A. hydrophila and found them to be composed of a 17 kd MW protein. They noted serological cross-reactivity among the Aeromonas strains, but these strains were not cross reactive with E. coli pili. They also noted that presence of pili did not correlate with degree of hydrophobicity of the strain or the strain's ability to hemagglutinate.

**S-LAYER**

Houwink (1953) first reported "periodic macromolecular monolayers" as a component of the bacterial cell envelope. Since that time, Houwink's monolayers have been referred to as crystalline surface layers or simply, S-layers. The presence of S-layers as a component of the
bacterial cell membrane is not as rare as once suggested. In fact, S-layers have been demonstrated as part of the bacterial cell structure for members of every taxonomic group of walled bacteria including Gram negative bacteria, Gram positive bacteria, Archaeabacteria, Cyanobacteria and Chlamydia (Smit, 1987; Sleytr and Messner, 1983; Sleytr and Messner, 1988). Several fish bacterial pathogens are known to possess S-layers. Properties of the S-layer of A. salmonicida are well documented, and S-layers have been reported recently in motile aeromonads as well as Vibrio salmonicida (Dooley and Trust, 1986 and 1988; Hjelmeland et al., 1988; Dooley et al., 1989).

S-layers are composed of repeating units of protein or glycoprotein which are aligned in hexagonal or tetragonal arrays. These protein arrays compose the outer most layer of the bacterial cell structure. Often in Gram negative bacteria, carbohydrate chains of the LPS molecule will extend through the S-layer and come in contact with the outer environment. For example, A. salmonicida has been reported to have carbohydrate chains exposed to the outer surface of the bacteria as demonstrated by LPS-specific monoclonal antibody labelling (Chart et al., 1984).

Functions of S-layers are not well understood although several roles have been suggested. First, S-layers may function as a physical barrier against infective agents or other foreign material. The S-layer may also serve as a barrier to protect the cell from bacteriocidal components of the infected host's serum or secretions. Munn et al. (1982) demonstrated that S-layer positive strains of A. salmonicida were more resistant to complement activity than the S-layer negative strains.
S-layer positive strains have been shown to be more resistant to proteolytic enzymes as well (Trust et al., 1982). Maintenance of cell shape and structure has been suggested as a possible role of S-layers. This function, however, is considered to be of little significance to Gram positive and gram negative bacteria because isogenic strains that are S-layer negative show no difference in morphology compared to the S-layer positive strains (Sleytr and Messner, 1988).

S-layers may play a role in cell adhesion and attachment to phagocytes (Atkinson and Trust, 1980; Duguid and Old, 1980; Evenberg and Lutenberg, 1982; Evenberg et al., 1982). For example, S-layers alone or in conjunction with LPS molecules and/or pili are thought to induce autoaggregation of bacterial cells (Dooley et al., 1986) indicating a decrease in surface hydrophilicity and enhanced association with phagocytes (Jiwa, 1983). This theory is supported by evidence indicating S-layer positive *A. salmonicida* strains have an enhanced ability to associate with trout macrophages (Trust et al., 1980). Channel catfish neutrophils have also been reported to phagocytize a S-layer positive strain of *A. hydrophila* more readily than S-layer negative strains (Finco-Kent, 1986).

The S-layer of motile aeromonads is composed of ~52 kd MW protein which is arranged tetragonally. Similar to other S-layer positive bacteria, motile aeromonads that possess an S-layer tend to autoaggregate in static culture and resist bacteriocidal activity of normal fish serum (Dooley et al., 1986). The role of the S-layer protein in determining virulence of *A. salmonicida* strains is well documented and the S-layer must be present in a particular strain for the strain
to be pathogenic to fish (Kay et al., 1984). The role of the S-layer in virulence of motile aeromonads is not as well understood, although researchers have reported that more virulent motile aeromonads are those that are S-layer positive and have been isolated from diseased fish. S-layer negative motile aeromonads and strains isolated from water samples are not as virulent as the S-layer positive strains (Dooley et al., 1986; Thune et al., unpublished data).

Because more specific/defined antigens are being used as vaccines, alternative methods for determining the degree of protection in fish after vaccination need to be further evaluated. Classically, the most common methods of determining induction of fish immune responses after vaccination involve testing sera for agglutinating antibodies or determining the level of protection by comparing mortality rates in vaccinated versus control fish groups. Determining mortality rates between vaccinated and control groups of fish does give a good indication of effectiveness of the vaccine but gives no information concerning the mechanism involved in conferring protection (i.e. humoral/cellular immune responses versus non-specific immune reactions). Determination of agglutinating antibody titer in sera of vaccinated fish is easily performed but is not without problems that can influence interpretation of vaccine effectiveness. First, whole cell preparations of the vaccine strain are usually used as the antigen in determining agglutination titer, and many bacterial cells are known to autoaggregate (especially the aeromonads that have crystalline protein arrays on their surface). Second, the agglutinating titers among fish from a single test group are usually so wide in range that no statically valid conclusions
can be obtained. Finally, several studies have demonstrated that agglutination titers do not correlate to protection of vaccinated fish. For example, Cipriano and Heartwell (1986) reported that brown trout, *Salmo trutta*, were highly susceptible to *A. salmonicida* infections but produced similar agglutinating antibody titers as rainbow trout that were resistant to *A. salmonicida* infections. Clearly, the basic immunology of channel catfish must be considered if these alternative methods are to be evaluated and successfully implemented.

**IMMUNOLOGY OF CHANNEL CATFISH**

Contrary to Metchinkoff's belief (quoted by Finn, 1970) that fish were not compatible with scientific endeavors because they were extremely difficult to maintain in a living state, fish have become important models for immunologists who study the evolutionary aspects of immune reactions. Fish also serve as models for physiological studies of vertebrates and are essential to research in the natural fisheries and aquaculture fields. Most importantly, the study of fish immunology has made production of commercial fish vaccines available to the aquaculture industry, contributed to determination of fish pathogens and has increased knowledge of basic fish health and biology that can be translated into improved management techniques for commercial fish production ventures. Although the subject of intense study in recent years, information available concerning fish immune systems is limited
and poorly understood compared to the wealth of material available concerning the immune systems of higher vertebrates. Generally, variations in immune systems of fish seem to be correlated with the phylogeny of fishes. Fish that are considered to be most primitive on the evolutionary scale have simpler immune systems whereas more advanced teleosts have complex immune systems (Walczak, 1985). The following text will describe what is currently known about the immune system of fish in general and channel catfish in particular.

**FIRST LINE OF DEFENSE**

Foreign material/microorganisms must pass through physical and chemical barriers that compose a fishes' first line of defense against invasion. The first barrier that must be effectively evaded in most instances is the epithelial layers that compose the outer surfaces of the skin, gills and intestinal tract. The epithelia layer itself has a role in osmoregulation and wound healing (Anderson, 1974; Lamers, 1985) and contains goblet cells that secrete mucus. Mucus, it has been suggested, prevents colonization by microorganisms on the epithelial surface of fish, and studies have indicated that fish increase production of mucus after they are subjected to stressful conditions (Anderson, 1974; Pickering and Macey, 1977; Pickering and Richards, 1980). Fish mucus has also been reported to contain lysozyme which is known to have bacteriolytic, anti-viral and anti-fungal properties (Fletcher and Grant, 1968; Ourth, 1980; Lamers, 1985). Mucus has also been reported to have bacteriocidal activity as well as contain C-reactive protein, immunoglobulin (Ig) and complement (Bradshaw et al.,

SECOND LINE OF DEFENSE

Mechanisms in the first line of defense are non-specific reactions that are continually present and provide no memory to the host immune system. Processes that compose the second line of defense, however, are only induced upon invasion. These mechanisms may function due to low levels of specificity to a particular antigen and may provide immunological memory in some instances (Lamers, 1985). Interferon has been confirmed to exist and play an anti-viral role in teleost immunity. C-reactive protein is common in fish serum and mucus and levels of this protein increase after the fish is exposed to bacterial endotoxins. C-reactive protein is believed to have a role in agglutination of pathogenic organisms rendering them unable to establish an infection. The C-reactive protein also activates complement and thereby enhancing phagocytosis (Ellis, 1978). Another mechanism considered to be a second line of defense is the process of natural cytotoxicity, which has been demonstrated in some species of fish. This mechanism is mediated by nonspecific cytotoxic cells (NCC) that are thought to be similar to natural killer cell of mammalian systems (Lamers, 1985). Finally, inflammation and phagocytosis are other mechanisms involving macrophages and granulocyte and similar pathways as those for mammalian systems (Ellis, 1978; Lamers, 1985).

THIRD LINE OF DEFENSE
Mechanisms that comprise the third line of defense are specific immune reactions that invoke memory and primarily carried out by the lymphoid cells of the fishes' immune system. Ellis (1978) reviewed the morphology and function of leucocytes from a variety of fish, and noted the variation between samples was so great that only a few generalizations could be proposed for function of leukocytes in the immune response of fish. Fortunately, T- and B-cell functions of channel catfish have been extensively studied. Clem et al. (1984) and Lobb and Clem (1982) described B-cell like lymphocytes as those cells that expressed a complete immunoglobulin molecule on their surface (sIg+). T-cell like lymphocytes were therefore the cells that carried incomplete immunoglobulin structures on their cell surface (sIg-). This group also reported that after sIg+ cells were depleted from a lymphocyte population by "panning", the response to LPS and Con A were decreased compared to the non-depleted population. In vitro responses to T-independent antigens required presence of only the sIg+ cells, whereas responses to T-dependent antigens required the presence of sIg+ and sIg- cells. The authors concluded that the sIg+ cells represented both morphologically and functionally the B cells described for mammalian species, and the sIg- cells were similar to the mammalian T cells.

Catfish peripheral blood lymphocytes are known to respond to human Interluekin (IL)-1 in vitro and IL-1 has been suggested to play a role in regulating fish immune reactions by directly activating lymphocytes or indirectly by activating pathways that lead to the production of prostaglandins and IL-2, much like the functions of IL-1 in mammalian
systems (Sigel et al., 1986). In fact, Ellasaessar and Clem (1989) and Miller et al. (1989) reported that channel catfish monocytes stimulated with LPS produced products which had IL-1 like activity.

Another cell type which has been studied extensively, especially in relation to parasitic infections of fish, is the melano-macrophage (Ellis, 1978; Lamers, 1985). These cells have been reported in most teleost fish and are found solitary or in clusters throughout the tissues (i.e. they are not strictly located within lymphoid organs). Melano-macrophages appear in hemopoietic tissues of young fish when they first feed; numbers of centers increase with age and often increase during periods of starvation. Melano-macrophages contain melanin, lipofuscin and hemosiderin. Melanin is a result of melanosome ingestion and may serve a role in protecting cells against free-radicals produced by phagocytes during extracellular killing. Lipofuscin is the product of reoxidation of unsaturated lipid and hemoglobin breakdown results in deposition of hemosiderin within the melano-macrophage. Roles in the immune reaction by melano-macrophages have been suggested to include bacterial killing, deposition and scavenging for iron (Ellis, 1978; Lamers, 1985).

Perhaps the most documented affect of the third line of defense in teleosts is that of antibody production. In teleosts only a heavy chain isotype, which is analogous to the mammalian v chain, exists. Teleost Ig is present in a tetrameric form (~700,000 MW) in most bony fishes and in some (including channel catfish), a J-chain has also been described. Common carp, however, do not have a J-chain as part of their Ig structure (Acton et al., 1971; Heartwell, 1975; Ambrosius et al.,
1982; Kobayashi et al., 1982; Lamers, 1985; Lobb, 1986; Van Ginkel and Clem, 1989). Some fish have monomeric or dimeric form of Ig which are often found in skin mucus, intestinal mucus and bile (Lobb and Clem, 1981). Subclasses of the Ig have been described for channel catfish. Lobb and Clem (1981, 1983) and Ghaffari and Lobb (1989) reported that the high molecular weight form (tetrameric) of Ig in the serum of channel catfish exhibited structural variation that was due to variation in the presence of disulfide bridges (covalently bonded) of the tetramers. Catfish Ig dissociates into eight discrete subpopulations when subjected to sodium-dodecyl-sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) analysis, instead of migrating as a homogenous protein at the 700,000 MW area. Subpopulations of ~750, 660, 570, 480, 390, 290, 190 and 90 kd are resolved. Each subpopulation therefore varies in MW by ~90 KD. Presence of these bands at 90 kd intervals suggests that each subpopulation contains a different number of H- and L-chain halfmers (a halfmer is defined as a single combination of an H-chain and L-chain, i.e. one half of a monomer of the Ig molecule) (Lobb, 1986). The catfish gene that codes for the V_H region of Ig has been reported to have structural and regulatory features not unlike mammalian V_H genes. The catfish V_H gene, however, represents a unique family of genes, and does not belong to any known mammalian V_H gene families (Lobb et al., 1989; Wilson, et al., 1989). Lobb et al. (1984) also reported the heterogenous nature of L-chains in channel catfish Igs. L-chains were found to have MW of 22, 24 or 26 kd, although the 22 and 24 kd molecules were antigenically similar to each other, they were each distinct from the 26 kd molecule. After peptide
analysis of these L-chain types it was determined that differences in the L-chains were due to isotypic variation and may be analogous to the kappa and lambda chains of mammalian Igs (Lobb, 1986). Immunization of fish with dinitrophenol (DNF) elicited a preferential response of Igs that were of the 22 and 24 kd L-chain types although no change in relative amounts of H-chain subpopulations were noted (Lobb, 1985).

Kinetics of the humoral response in fish are similar to that of mammalian systems but often have a longer latent period and lower secondary responses than those commonly found in mammals (Heartwell, 1975; Lamers and De Haas, 1983; Lamers et al., 1985a and b).

**IMMUNOMODULATION**

As well documented for mammalian species, the immune reaction of fish can be influenced by a variety of factors including dose, nature of the antigen and route of administration of the antigen (Rijkers, 1982), although some researchers have suggested that various doses of antigen and routes of administration have little effect on the secondary (memory induced) response of channel catfish (Schachte and Mora, 1973; Newmann and Tripp, 1986). Temperature is perhaps the most well documented factor that has demonstrable influences on the immune system of fish (Bisset, 1946; Avtalion, 1969; Avtalion et al., 1973; Collin et al., 1976; Dorson, 1981; Rijkers, 1982; Bly et al., 1986). Clem et al. (1984) reported that channel catfish in vitro antibody response to T-dependent antigens were "good" at 32°C but inhibited by 22°C temperatures. These researchers also noted that the response was
affected by the water temperature in which the channel catfish were held prior to removal of peripheral blood lymphocytes for in vitro studies. Plumb et al. (1986) reported that channel catfish responded with higher antibody titers and increased levels of protection when held at 25°C for 30 days after immunization against Edwardsiella ictaluri and then held for 30 days at 12°C for 30 days prior to challenge, compared to fish held at 25°C for only 5 days after immunization and then moved to 12°C 50 days prior to challenge, or when compared to fish at 25°C or 12°C for the duration of the experiment. Bly et al. (1988) and Bly et al. (1989a) noted that low temperature had an immunosuppressive effect on channel catfish and suppression involved primary T-cell activity rather than memory T-cells of B-cell function. Other factors that influence successful mounting of an immune response in fish include the presence of potential "suppressing factors" such as heavy metals or toxins in the water (Anderson et al., 1984; Hanson and Grizzle, 1985; Walczak, 1985), crowded conditions or handling of the fish (Snieszko, 1974; Ellis, 1981, Bly et al., 1989b), social interactions (Peters, et al., 1988), basic circadian and seasonal rhythms, experimental photoperiod (Zeeman, 1986), and nutritional status of the fish (Li and Lovell, 1985; Navarre and Halver, 1989). Other internal factors that effect the immune response of fish include formation of immune complexes, helper activity, suppressor activity, antibody feed-back inhibition and stage of ontogeny (Lamers, 1985).

The exact age of immunocompetence in channel catfish is not known. Most MAS epizootics occur in the spring and early summer when young fish are abundant in production ponds, so it would be advantageous for a
commercial vaccine to be available in a form that could be applied in mass quantities to small fish. Thune (1980) used a dip method to vaccinate channel catfish sac fry and swim-up fry. The non-vaccinated controls experienced a 45% mortality rate when challenged with the homologous strain. Vaccinated swim-up fry and sac fry had lower mortality rates, 13% and 7.1% respectively. Antibody titers were not obtained in this study but reduced mortality rates in the vaccinated fry indicates that channel catfish become immunocompetent early in their life history.

Few studies have been reported concerning alternative methods of determining immune reactions of fish to bacterial pathogens besides agglutination methods previously mentioned. In one study, mucus precipitin activity determined by single radial diffusion correlated well with the degree of resistance to A. salmonicida infections for several salmonids (Cipriano and Heartwell, 1986). The authors suggested that the mucus precipitin activity could be due to a variety of factors including natural hemagglutins, lysozyme or proteolytic activities or complement activity. In other studies, the defense mechanism against crude LPS in dip immunized carp was found to involve thymus derived sensitized lymphocytes and macrophages (Baba, et al., 1988a and b). Further investigations concerning alternative methods of evaluating vaccine efficacy are needed, especially in light of the fact that commercial vaccines will probably be composed of specific antigens not readily detected by agglutination procedures.
LITERATURE CITED


Cipriano, R.C and C.M. Heartwell. 1986. Susceptibility of salmonids to


Gaines, J.L. 1972. Pathology of experimental infection of Aeromonas hydrophila (Chester) Stanier (Bacteria: Pseudomonadales), in the channel catfish, Ictalurus punctatus (Rafinesque). PhD Dissertation, Auburn University, Auburn, AL.


Johnson, S.K. 1974. Bacterial infection in golden shiner of Texas retailing establishments. Fish Disease Diagnostic Lab #F3, Texas Agricultural Extension Service, College Station, Texas, 77843.

hydrophila produced in vivo. Fish Pathol. 21(4): 245-250.


Snieszko, S.F. 1974. The effects of environmental stress on outbreaks


Thune, R.L. and J.A. Plumb. 1984. Evaluation of hyperosmotic infiltration for the administration of antigen to channel catfish

Aeromonas hydrophila beta hemolysin: purification and 
examination of its role in virulence in 0-group channel catfish, 

Characterization of plasmids in bacterial fish pathogens. Inf. 

aerobic bacteria in the gastrointestinal microflora of the 
grass carp, golfish and rainbow trout. J. Fish Res. Bd. Can. 36: 
1174-1179.

between Hemophilus piscium and Aeromonas salmonicida revealed 
by Aeromonas hydrophila bacteriophage. FEMS Micro. Letters 9: 
199-201.

1982. Properties of a protein, a virulence factor on the surface 
of Aeromonas salmonicida. Dev. Comp. Immunol. Suppl. 2 pp. 175-
180.

Udey, L.R. and J.L. Fryer. 1978. Immunization of fish with bacterins 


of aerobic gram negative bacteria in the cloaca of Rana pipiens. 

Ventura, M.T. and J.M. Grizzle. 1987. Evaluation of portals of entry of 
Aeromonas hydrophila in channel catfish. Aquaculture 65: 205-
214.

chez la perch, Perca flores cens. Can. J. Microbiol. 17: 1101-
1103.

and cytolytic protein in Aeromonas hydrophila from human 
114.

Wakabayashi, H. and S. Egusa. 1979. What is the best organ for isolation 
of eel pathogens? Fish Pathol. 13: 201-203.


Aeromonas hydrophila Virulence Factors: A Field Study

ABSTRACT

In this study, bacterial isolates collected from channel catfish during MAS epizootics were screened for production of hemolysins, proteases and the presence of the S-layer protein. S-layer protein was produced by 93% of the isolates from MAS epizootics. Hemolysin and protease production were more variable and limited among the same isolates. Only 20% of the Aeromonas isolates from epizootics which involved other pathogens as the primary etiology produced the S-layer protein.

INTRODUCTION

The motile aeromonads particularly, Aeromonas hydrophila and A. sobria, are opportunistic pathogens of a wide variety of animals, including commercially-raised channel catfish. Although the pathogenicity of motile aeromonads is not well understood, several extracellular products (ECP) and components of the bacterial cell surface have been proposed as potential virulence factors. Ljungh et al. (1981) and Thune et al. (1986) implicated an extracellular B-hemolysin in the pathogenicity and virulence of motile aeromonads. B-hemolysin
produced by A. hydrophila is lethal to channel catfish at 0.6 mg of purified protein/g of fish (Thune et al. 1986). At least five proteases have been demonstrated in the ECP of motile aeromonads (Thune et al., 1982b; Chabot, 1987), and two of these proteases have been reported to be lethal to channel catfish. The LD$_{50}$ of one protease (heat-labile) is 18.0 ug protein/fish, and the LD$_{50}$ of the heat-stable protease is 3.0 ug protein/fish. (Thune et al., 1982b). Although production of any particular protease(s) by motile aeromonads did not correlate to virulence in channel catfish (Chabot 1987), clinical signs of MAS can be induced in channel catfish injected with sublethal doses of ECP containing hemolysin, proteases or both (Thune et al., 1982b; Thune et al., 1986). Proteases, however, have been implicated in the virulence of some motile aeromonad strains isolated from rainbow trout (Leung and Stevenson, 1988a).

Production of S-layer protein arrays by motile aeromonads has recently been documented (Dooley and Trust, 1988; Thune, unpublished data). S-layers play a role in virulence of other bacterial species, including Aeromonas salmonicida, the causative agent of furunculosis in salmonids (Trust et al., 1982). Although the S-layer protein is not lethal, it may play a role in enhancement of cell adhesive properties or may function as a barrier to the infected host's immune system allowing the bacterial cell to proliferate. In laboratory-LD$_{50}$ experiments, presence of the S-layer has been correlated with virulence of motile aeromonads in channel catfish (Thune, unpublished data).

Many of the strains included in previous studies have been isolated from a wide variety of sources and have often been maintained on
artificial media for extended periods. Artificial media can select for a different bacterial phenotype that has an altered ECP production pattern. Also, production of the S-layer protein can be lost following passage on artificial media, as documented for *Aeromonas salmonicida* (Trust et al., 1982; Cipriano et al., 1984; Dooley and Trust, 1988). Information concerning the production of these virulence factors from natural epizootics is lacking. Therefore, the objective of this study was to evaluate production of B-hemolysin, protease and the S-layer protein by motile aeromonads immediately after primary isolation from catfish during MAS epizootics in commercial ponds.

**MATERIALS AND METHODS**

**Collection of bacterial isolates**

Bacterial isolates were obtained from commercially raised channel catfish collected from ponds experiencing significant epizootics and submitted for diagnosis to the Aquatic Animal Diagnostic Laboratory, School of Veterinary Medicine at Louisiana State University and to the Mississippi Cooperative Research Laboratories in Stoneville and Belzona, MS. Fish were necropsied by routine procedures, including bacteriological and parasitological examination and viral isolation when appropriate. Bacteria isolated from internal organs were presumptively identified as *Aeromonas* sp., restreaked for purity on blood agar plates, transferred to BHI slants and suspended in 0.85% NaCl + 20% glycerol for subsequent storage at -70°C. Care was taken to ensure that isolates were not transferred on artificial media more than two times prior to storage. A corresponding history was obtained for each case from which
Aeromonas sp. was isolated.

Hemolysin Assay

Each isolate was grown in 50 ml of a defined medium containing L-glutamic acid and L-histidine (MMGH) described by Riddle et al. (1981) at 30°C for 48-50 hrs in a shaking water bath. Bacterial cells were pelleted at 3000 rpm for 15 min, the supernatant was collected, and pelleted cells were discarded. The supernatant (containing ECP) was diluted into triplicate tubes containing tris-buffered saline (0.02 M tris + 0.15 M NaCl) to make 5, 10, 20, 40 and 100-fold dilutions. Bovine red blood cells (BRBC) that had been washed 3 times in Alsevers solution and standardized so that 0.5 ml of blood cells diluted with 5.0 ml of distilled H₂O gave a reading of 0.8 at A₅₄₀. A 0.5 ml aliquot of the BRBC suspension was added to the diluted ECP and mixed. After incubation at room temperature for 1 hr, tubes were refrigerated overnight, vortexed and centrifuged. Supernatant from each tube was removed and absorbance at A₅₄₀ was determined. Hemolytic activity was calculated with the following formula:

\[
\text{Activity (HU)} = \text{Dilution} \frac{A_{540} - A_{540\text{ blank}}}{0.4}
\]

Protease Assay

ECP from each isolate was collected as described above except that a medium containing arginine (MMGA) instead of the glutamic acid and histidine was used as the growth medium (Riddle et al., 1981). Protease activity was determined by the method of Kreger and Griffin (1974) with
azocasein as the substrate. Briefly, ECP were diluted in triplicate test tubes with 0.1 M tris, pH 8.0 to make 5, 10, 20, 40 and 100-fold final dilutions. To each tube, 0.5 ml of azocasein (5 mg/ml) was added. Contents of each tube were mixed and incubated for 20 min at room temperature. After incubation, 3.5 ml of 5% trichloroacetic acid (TCA) was added to each tube to stop the reaction. Tubes were refrigerated for 10 min and centrifuged to remove the precipitate. One ml of supernatant was added to 1 ml of 0.5 N NaOH and absorbance determined at A_{440}. Proteolytic activity was calculated with the following formula:

Proteolytic Unit (PU) = \frac{\text{Dilution } A_{440} - A_{440 \text{ blank}}}{1.0}

**Detection of the S-layer Protein**

Each isolate was grown in 50 ml BHI broth at 30°C for 18 hours. Bacterial cells were washed twice with distilled water and resuspended in 10 ml of 0.2 M glycine-HCl buffer (pH 2.2). Cells were mixed by inversion for 15 min at room temperature and then centrifuged for 15 min at 11,000 g. Supernatant was collected and pH adjusted to 4.5. Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the pH-adjusted samples. Production of the S-layer protein for each isolate was confirmed by the presence of a 50,000 MW protein band after the gel was stained with Comassie Brilliant Blue (FIGURE 1). In addition, environmental isolates (ENV) were collected using a selective medium for *Aeromonas* (Shotts and Rimler, 1973), from pond water at the commercial catfish farms and screened for the production of the S-layer protein.
FIGURE 1: A single protein band (the S-layer protein) in acid-extracted preparations demonstrated by SDS-PAGE from motile aeromonad strains (S87-164 and S87-166). Note that isolate B87-885 does not contain S-layer protein. Lane 4 contains the molecular weight standards.
RESULTS

Strains of motile aeromonads isolated in this study can be divided into two major groups, those that produce the S-layer protein and those that do not. Seventeen of the 30 isolates (57%) produced the S-layer protein (TABLE 1). Thirteen of the 30 isolates were determined to be S-layer negative (TABLE 2). In many instances other pathogens or disease syndromes were reported along with the isolation of a motile aeromonad (TABLE 1 and 2). In the majority (22/30) of the diagnostic cases included in this study, external protozoans were found on gills of the channel catfish. Because the intensity of the infestation is difficult to determine and low intensity infections are commonly reported in pond-raised channel catfish, the significance of external protozoan infestation was not considered in this study.

Hemolysin activity from strains isolated from MAS epizootics ranged from non-detectable levels to 10.79 HU with a mean of 7.48 HU. From the WK, CCV and FLEX groups which the motile aeromonad isolation was considered a secondary pathogen, mean HU values were 0.59, 2.83 and 6.18, respectively. Hemolysin activity in these groups was not detected above 9.34 HU. Hemolysin activity by isolates in the WK and CCV groups were significantly lower (p < 0.05) than the hemolysin activity of the MAS isolates as determined by analysis of variance (ANOVA) (TABLE 3).

The protease activity from the MAS group ranged from non-detectable levels to 2.5 PU with a mean of 0.86 PU. Protease activity from the WK, FLEX and CCV isolates had means of 0.54, 0.91 and 1.04, respectively, and ranged from non-detectable levels to 1.53 PU. Statistical differences were not detected between any of the etiological groups.
TABLE 1: Hemolysin and protease production of *Aeromonas* S-Layer positive isolates from commercially raised channel catfish and other concurrently diagnosed pathogens or disease conditions.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>HEMOLYSIN (HU)</th>
<th>PROTEASE (PU)</th>
<th>INTERNAL</th>
<th>EXTERNAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>S86-1129</td>
<td>9.02</td>
<td>0.15</td>
<td>NONE</td>
<td>FLEX</td>
</tr>
<tr>
<td>S86-1326</td>
<td>10.79</td>
<td>0.35</td>
<td>NONE</td>
<td>FLEX</td>
</tr>
<tr>
<td>S87-69</td>
<td>NA</td>
<td>NA</td>
<td>NONE</td>
<td>WK/FLEX</td>
</tr>
<tr>
<td>S87-72</td>
<td>ND</td>
<td>0.59</td>
<td>NONE</td>
<td>WK/FLEX</td>
</tr>
<tr>
<td>S87-74</td>
<td>ND</td>
<td>ND</td>
<td>NONE</td>
<td>NONE</td>
</tr>
<tr>
<td>S87-153</td>
<td>10.16</td>
<td>0.90</td>
<td>NONE</td>
<td>FLEX</td>
</tr>
<tr>
<td>S87-157</td>
<td>7.41</td>
<td>0.29</td>
<td>NONE</td>
<td>FLEX</td>
</tr>
<tr>
<td>S87-164</td>
<td>ND</td>
<td>1.54</td>
<td>NONE</td>
<td>FLEX</td>
</tr>
<tr>
<td>S87-166</td>
<td>8.55</td>
<td>2.50</td>
<td>NONE</td>
<td>FLEX</td>
</tr>
<tr>
<td>S87-316</td>
<td>10.45</td>
<td>0.75</td>
<td>NONE</td>
<td>NONE</td>
</tr>
<tr>
<td>S87-532</td>
<td>5.50</td>
<td>ND</td>
<td>NONE</td>
<td>FLEX</td>
</tr>
<tr>
<td>S87-693</td>
<td>ND</td>
<td>0.35</td>
<td>NONE</td>
<td>FLEX</td>
</tr>
<tr>
<td>S87-749</td>
<td>3.45</td>
<td>0.78</td>
<td>NONE</td>
<td>NONE</td>
</tr>
<tr>
<td>S87-757</td>
<td>5.25</td>
<td>0.40</td>
<td>NONE</td>
<td>WK/FLEX</td>
</tr>
<tr>
<td>LA87-1</td>
<td>4.87</td>
<td>0.57</td>
<td>NONE</td>
<td>NONE</td>
</tr>
<tr>
<td>LA87-278</td>
<td>4.55</td>
<td>1.435</td>
<td><em>E. tarda</em></td>
<td>NONE</td>
</tr>
<tr>
<td>LA87-282</td>
<td>ND</td>
<td>0.71</td>
<td>NONE</td>
<td>NONE</td>
</tr>
</tbody>
</table>

HU=hemolytic units, PU=proteolytic units, NA=not available, ND=not detectable
FLEX-*Flexibacter columnaris*, WK=winter kill, *E. tarda=* *Edwardsiella tarda*
TABLE 2: Hemolysin and protease production of *Aeromonas* S-Layer negative isolates from commercially raised channel catfish and other concurrently diagnosed pathogens or disease conditions.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>HEMOLYSIN (HU)</th>
<th>PROTEASE (PU)</th>
<th>INTERNAL</th>
<th>EXTERNAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>S86-1342</td>
<td>0.22</td>
<td>0.41</td>
<td>NONE</td>
<td>WK</td>
</tr>
<tr>
<td>S87-64</td>
<td>ND</td>
<td>0.57</td>
<td>NONE</td>
<td>WK</td>
</tr>
<tr>
<td>S87-68</td>
<td>0.95</td>
<td>0.57</td>
<td>NONE</td>
<td>WK</td>
</tr>
<tr>
<td>S87-477</td>
<td>8.25</td>
<td>1.14</td>
<td>FLEX</td>
<td>FLEX</td>
</tr>
<tr>
<td>S87-480</td>
<td>1.00</td>
<td>1.06</td>
<td>NONE</td>
<td>FLEX</td>
</tr>
<tr>
<td>S87-481</td>
<td>0.95</td>
<td>0.38</td>
<td>ESC</td>
<td>FLEX</td>
</tr>
<tr>
<td>B87-821</td>
<td>ND</td>
<td>1.53</td>
<td>CCV*</td>
<td>NONE</td>
</tr>
<tr>
<td>B87-822</td>
<td>9.07</td>
<td>0.70</td>
<td>CCV*</td>
<td>NONE</td>
</tr>
<tr>
<td>B87-842</td>
<td>0.93</td>
<td>0.90</td>
<td>CCV</td>
<td>NONE</td>
</tr>
<tr>
<td>B87-880</td>
<td>0.65</td>
<td>ND</td>
<td>CCV*</td>
<td>NONE</td>
</tr>
<tr>
<td>B87-881</td>
<td>0.65</td>
<td>1.03</td>
<td>CCV*</td>
<td>NONE</td>
</tr>
<tr>
<td>B87-884</td>
<td>9.34</td>
<td>0.58</td>
<td>FLEX</td>
<td>FLEX</td>
</tr>
<tr>
<td>B87-885</td>
<td>0.94</td>
<td>1.00</td>
<td>FLEX</td>
<td>FLEX</td>
</tr>
</tbody>
</table>

HU=hemolytic units, PU=proteolytic units, ND=not detectable
FLEX=*Flexibacter columnaris*, WK=winter kill, ESC=*Edwardsiella ictaluri*, CCV=Channel Catfish Virus, *=diagnosed by clinical signs only.
### TABLE 3: The means and standard deviations for hemolysin and protease production for each of the primary etiology groups.

<table>
<thead>
<tr>
<th>Primary etiology</th>
<th>HU</th>
<th>PU</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLEX</td>
<td>6.18 ± 4.57</td>
<td>0.91 ± .29</td>
</tr>
<tr>
<td>CCV</td>
<td>2.82 ± 3.82*</td>
<td>1.04 ± .56</td>
</tr>
<tr>
<td>ESC</td>
<td>0.95</td>
<td>0.38</td>
</tr>
<tr>
<td>WK</td>
<td>0.59 ± 2.25*</td>
<td>0.54 ± .09</td>
</tr>
<tr>
<td>MAS</td>
<td>7.48 ± 4.17</td>
<td>0.86 ± .67</td>
</tr>
</tbody>
</table>

FLEX = *Flexibacter columnaris*, CCV = *Channel catfish virus*, ESC = *Edwardsiella ictaluri*, WK = *winter kill*, MAS = *Motile aeromonad septicemia*

* = significantly different from MAS (p < 0.05)
The S-layer protein was detected in 3 of 6 cases (50%) of isolates in the WK group and in 14 of the 15 cases (93%) in which MAS was the primary etiology. The MAS groups had significantly greater proportion of S-layer producing isolates than the FLEX and CCV groups (p < 0.001), as well as the WK group (p < 0.05). In only three cases in which MAS was not the primary etiology, isolates were found to produce the S-layer protein (S87-69, S87-72 and S87-757). Each of these three isolates were from cases in which WK was the primary etiology (TABLE 4).

DISCUSSION

Presence of the S-layer protein in 93% of the cases in which MAS was determined to be the primary etiology is strong evidence for the role of the S-layer in pathogenicity of motile aeromonads. Only, one isolate from a MAS epizootic did not produce the S-layer protein (S87-480) (TABLE 2). Dooley and Trust (1988) and Thune (unpublished data) have shown a correlation between virulence and presence of the S-layer protein in motile aeromonads isolated from a variety of sources. Data from the current study further substantiates the role of the S-layer in pathogenicity of isolates collected from natural MAS epizootics in channel catfish.

Pathogens associated with motile aeromonad isolates in this study can be grouped into those found internally and those found externally. Columnaris disease, caused by *Flexibacter columnaris*, is another disease of channel catfish that is associated with stress. *F. columnaris* is often present in external lesions and on gills of fish that have been...
TABLE 4. Percentage of the isolates from diagnostic cases that did produce the S-layer protein according to the primary etiology group listed.

<table>
<thead>
<tr>
<th>Primary etiology</th>
<th>TOTAL # ISOLATES</th>
<th>S-layer + ISOLATES</th>
<th>% S-LAYER +</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENV</td>
<td>3</td>
<td>0</td>
<td>0*</td>
</tr>
<tr>
<td>ESC</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCV</td>
<td>5</td>
<td>0</td>
<td>0*</td>
</tr>
<tr>
<td>WK</td>
<td>6</td>
<td>3</td>
<td>50**</td>
</tr>
<tr>
<td>MAS AND FLEX</td>
<td>20</td>
<td>17</td>
<td>85</td>
</tr>
<tr>
<td>internal FLEX</td>
<td>3</td>
<td>0</td>
<td>0*</td>
</tr>
<tr>
<td>external FLEX</td>
<td>12</td>
<td>11</td>
<td>92</td>
</tr>
<tr>
<td>MAS/external FLEX</td>
<td>17</td>
<td>16</td>
<td>93</td>
</tr>
<tr>
<td>MAS only</td>
<td>5</td>
<td>5</td>
<td>100</td>
</tr>
</tbody>
</table>

ENV= environmental isolate, FLEX=Flexibacter columnaris, CCV=Channel catfish virus, ESC=Edwardsiella ictaluri, WK=winter kill, MAS=Motile aeromonad septicemia

* = significantly different from MAS (p < .001)
** = significantly different from MAS (p < .05)
stressed. The organism has also been isolated from internal organs of fish and has been designated a primary etiology in these cases (MacMillian, 1985). Although external columnaris can easily diagnosed by microscopic examination, the intensity of the infection is sometimes difficult to assess. Thus, the distinction of its role as a primary or secondary invader is difficult to make. Data from this study supported this assumption because in 11/12 of the isolates in which external FLEX and MAS was diagnosed, the aeromonad produced the S-layer protein. Internal isolations of *F. columnaris* (FLEX), the diagnosis of channel catfish virus (CCV) or Enteric Septicemia of Catfish (ESC) were considered primary etiologies and the isolation of motile aeromonads in these groups was considered secondary. In the FLEX, CCV and ESC groups, none of the aeromonad isolates produced the S-layer protein (TABLE 4).

At present, the condition known as winter kill is poorly understood. The condition is usually diagnosed in the late fall or early spring and is often characterized by loss of mucus of the fish surfaces and external mycosis. A variety of external protozoans, bacterial septicemia due to *Aeromonas* sp., and *Flexibacter columnaris* infections have been reported, but these findings have been inconsistent (MacMillian, 1985). For these reasons, the WK group was also designated as a primary etiology. The production of S-layer protein by 50% (3/6) of the motile aeromonads in WK cases in this study is of interest and may be a result of the motile aeromonads ability to tolerate and cause disease in a wider range of environmental conditions than other bacterial pathogens of channel catfish. *Aeromonas* may simply take
advantage of the poor condition of the fish because the aeromonads tolerate lower water temperatures than other catfish pathogens.

The presence of the S-layer in more virulent isolates has two potential applications that warrant further research. First, the development of rapid diagnostic tests that utilize the S-layer as the target antigen could aid in distinguishing S-layer positive aeromonads of potential primary etiology from S-layer negative aeromonads that are secondary or incidental isolations. A quick determination of S-layer production could aid the diagnostician in choosing the best treatment protocol for a given situation. Often aeromonads are isolated concurrently with other pathogens (as demonstrated in the data included in TABLES 1 and 2) and the most effective and economical treatment regime may be difficult to determine. For example, when a S-layer positive strain is isolated the use of antibiotic feed may be indicated more strongly than when an S-layer negative strain is isolated.

A second potential application of the S-layer is in vaccine development. Antibiotic therapy is expensive and only two antibiotics are available to the commercial catfish farmer. Since the aeromonads are often resistant to the antibiotics that are available, the development of vaccines for the prevention of MAS is indicated. Unfortunately, whole-cell, lipopolysaccharide (LPS) based vaccine preparations do not protect fish against serologically heterologous strains. Due to the extremely heterologous nature of the motile aeromonads, researchers have tried to define an antigen or combination of antigens that would protect fish against heterologous challenge (Post, 1966; Song et al., 1976; Thune and Plumb, 1982). The S-layer protein may provide the common
antigen necessary for an efficacious vaccine that would protect fish against all virulent strains of motile aeromonads.

In this study, the production of protease and hemolysin was varied and limited among the isolates, indicating that these virulence factors might not be good candidates for vaccine development. Several studies, however, have shown that these extracellular products have a role in virulence (Thune et al., 1986; Leung and Stevenson, 1988 a and b). Data from the present study did show that 20% of the isolates from MAS cases had hemolysin production > 10 HU where as isolates from the other primary groups had lower hemolysin production (TABLE 5). Thune et al. (1986) reported HU values of greater than 50 in 3 of 6 isolates studied and also reported maximum hemolytic activity for one strain to be in excess of 1800 HU. Therefore, all isolates from the present study had much lower hemolysin production than what has been reported as maximum levels for other strains. Thune et al. (1986) also reported that 4 of 6 isolates studied had proteolytic activity greater than 1.40 PU with the highest being 2.49. Again, the isolates in the present study have more limited proteolytic activity as compared to strains described by Thune et al. (1986), even though 3 of the 30 isolates did have PU values of >1.40. Although not clearly demonstrated in the present study, the potential synergistic role in virulence between hemolysin production, protease production and S-layer proteins should not be overlooked.
TABLE 5. Summary data comparing virulence factors produced by motile aeromonads from primary and secondary infections in channel catfish (numbers represent percent of total isolates from that particular group)

<table>
<thead>
<tr>
<th></th>
<th>Hemolytic Units (HU)</th>
<th>Proteolytic Units (PU)</th>
<th>S-Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt; 10</td>
<td>&gt; 1</td>
<td>+</td>
</tr>
<tr>
<td>Primary Infections</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(total cases=15)</td>
<td>20</td>
<td>26.6</td>
<td>93</td>
</tr>
<tr>
<td>Secondary Infections</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(total cases=15)</td>
<td>0</td>
<td>26.6</td>
<td>20</td>
</tr>
</tbody>
</table>
LITERATURE CITED


**Aeromonas hydrophila**: effect on age-0 channel catfish. Trans. Fish. Soc. Ill: 404-408.


Motile Aeromonad Virulence Factors: Their Use as Potential Protective Immunogens

ABSTRACT

Recent studies have focused on the potential role of extracellular enzymes and S-layer protein as a virulence factor for motile aeromonad species, and the presence of an S-layer is prevalent among clinical isolates from Motile Aeromonad Septicemia (MAS) epizootics. Channel catfish were immunized with a variety of crude extracellular products and S-layer preparations with and without adjuvant. Although no evidence of protection for inactive hemolysin and protease was found, data indicated that catfish immunized with a crude acid-extracted preparation of the S-layer plus Freund's incomplete adjuvant (FICA) were protected against subsequent challenge with the homologous strain of Aeromonas.

INTRODUCTION

Vaccination of catfish could prove to be efficacious for prevention of Motile Aeromonad Septicemia (MAS) epizootics in view of the limited success of antibiotic therapy. Previous studies have demonstrated that monovalent, whole-cell, lipopolysaccharide (LPS)-based vaccines are protective for fish after subsequent challenge with the homologous strain, but not heterologous challenge strains (Post, 1966; Song et al., 1976; Acuigrup, 1980). Because LPS-based vaccine preparations do not protect fish against heterologous challenge, investigators have
considered using specific bacterial products other than LPS as antigens in vaccines. Hemolysins and proteases are produced by motile aeromonads and both are lethal to channel catfish (Thune et al., 1982a and b; Thune et al. 1986; Amborski et al., 1984; Nieto and Ellis, 1986). In fact, both enzymes induce clinical signs of MAS in channel catfish injected with sublethal doses (Thune et al., 1982a and b; Thune et al., 1986). In laboratory challenge studies, an S-layer protein has been correlated with virulence in motile aeromonads (Dooley and Trust, 1988; Thune, unpublished data) and is also prevalent in clinical isolates from MAS epizootics (Ford and Thune, 1989). For these reasons, hemolysin, protease, and the S-layer protein were chosen for testing as protective immunogens in channel catfish.

MATERIALS AND METHODS

Antigen preparation for cage study

*Aeromonas hydrophila* strain 21, a hemolysin-producing but protease-deficient strain, was grown in a defined medium containing L-glutamic acid and L-histidine (MMGH) (Riddle et al., 1982a) for 50 hr at 30°C in a 2 L fermentor. Strain 22, a protease-producing, hemolysin-deficient strain 22, was grown in a modified medium that contained arginine (MMGA) instead of glutamic acid and histidine (Riddle et al., 1981). The supernatant containing the extracellular products (ECP), was collected after centrifugation for 15 min, and the pelleted cells were discarded. The ECP assayed for hemolytic activity and proteolytic activity as previously described (page 62), and subsequently inactivated by the addition of 0.5% formalin. S-layer protein was obtained by growing
strain S87-166 (166) in 50 ml aliquots of BHI broth at 30°C for 18 hours. Bacterial cells were washed twice with distilled water and resuspended in 10 ml of 0.2 M glycine-HCL buffer (pH 2.2). Cells were mixed by inversion for 15 min at room temperature and then centrifuged for 15 min at 11,000g. Supernatant was collected and pH adjusted to 4.5. Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the pH-adjusted samples. Production of the S-layer protein for each sample was confirmed by the presence of a 50,000 MW protein band after the gel was stained with Commassie Brilliant Blue. The Bradford method (Bradford, 1976) was used to determine total protein concentration of the acid-extracted sample prior to being aliquoted for fish immunizations.

Immediately prior to injection of these antigens into channel catfish, one aliquots of each antigen was alum precipitated (Garvey et al., 1977) and another was mixed 1:1 with FICA. The third aliquot of each antigen did not contain any adjuvant.

**Immunization of catfish maintained in cages**

For each treatment, 240 fingerling channel catfish were injected intraperitoneally (IP) and divided into 3 groups of 80 fish. Each group of 80 fish were placed into separate, randomly-selected cages (TABLE 1). After injection, fish were tattooed in order to keep a record of the injection treatment that each fish received. During week 1, all protease preparations were injected IP, and fish receiving hemolysin and S-layer preparations were injected during weeks 2 and 3, respectively. Three
TABLE 1: Experimental design for channel catfish immunized with various bacterial preparations.

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>TREATMENT</th>
<th># OF FISH INJECTED</th>
<th>CAGE #'s</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROTEASE</td>
<td>CRUDE ECP</td>
<td>240</td>
<td>5, 9, 17</td>
</tr>
<tr>
<td></td>
<td>CRUDE ECP + FICA</td>
<td>240</td>
<td>2, 3, 16</td>
</tr>
<tr>
<td></td>
<td>CRUDE ECP + ALUM</td>
<td>240</td>
<td>1, 12, 18</td>
</tr>
<tr>
<td>HEMOLYSIN</td>
<td>CRUDE ECP</td>
<td>240</td>
<td>7, 8, 9</td>
</tr>
<tr>
<td></td>
<td>CRUDE ECP + FICA</td>
<td>240</td>
<td>3, 4, 11</td>
</tr>
<tr>
<td></td>
<td>CRUDE ECP + ALUM</td>
<td>240</td>
<td>1, 10, 15</td>
</tr>
<tr>
<td>S-LAYER</td>
<td>CRUDE S-LAYER</td>
<td>240</td>
<td>5, 7, 14</td>
</tr>
<tr>
<td></td>
<td>CRUDE S-LAYER + FICA</td>
<td>240</td>
<td>2, 6, 11</td>
</tr>
<tr>
<td></td>
<td>CRUDE S-LAYER + ALUM</td>
<td>240</td>
<td>12, 13, 15</td>
</tr>
<tr>
<td>CONTROLS</td>
<td>FPBS</td>
<td>240</td>
<td>8, 14, 17</td>
</tr>
<tr>
<td></td>
<td>FICA</td>
<td>240</td>
<td>4, 6, 16</td>
</tr>
<tr>
<td></td>
<td>ALUM</td>
<td>240</td>
<td>10, 13, 18</td>
</tr>
</tbody>
</table>

CRUDE ECP = crude extracellular products preparations
CRUDE S-LAYER = crude acid extracted S-layer preparations
FICA = Freund's Incomplete Adjuvant
ALUM = alum precipitation
FPBS = fish phosphate buffered saline
weeks after the initial injection (weeks 4, 5 and 6), fish were removed from their respective cages and given an identical booster injection.

**Challenge of immunized fish**

Approximately 3 weeks post-booster (weeks 7, 8 and 9 respectively), fish from the appropriate treatments were brought into the laboratory and 10 fish transferred to each of 50 flow-thru tanks. Fish were challenged by intramuscular (IM) injection with 0.1 ml of a standardized dilution of the homologous strain for each treatment group. Mortalities were recorded until no fish were found dead or moribund for 3 consecutive days. Analysis of variance (ANOVA) was performed on the arcsine transformed mortality data to compare percent mortality between treatments.

**RESULTS**

Data from the cage study were inconclusive for trials with protease preparations and hemolysin preparations. In preparation of immunogen for the protease groups, the initial batch of ECP used in the first immunization was found to have no protease activity due to use of incomplete MMGA medium. Therefore, groups immunized with the protease treatments received only one injection of protease (the booster injection). Protection was not significant for protease injected compared to over control groups (TABLE 2). Protection conferred to hemolysin-injected groups could not be determined because the challenge organism was avirulent (TABLE 3). Most of the mortality in the hemolysin-immunized group was recorded in treatments that had been immunized with crude ECP + ALUM or ALUM alone. Mortality, however, was
## TABLE 2: Total percent mortality recorded for fish immunized with various protease preparations and challenged with strain 22.

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>CHALLENGE DILUTION</th>
<th>4.24 X</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^7</td>
<td>10^6</td>
</tr>
<tr>
<td>FPBS</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>ALUM</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>FICA</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>CRUDE ECP</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>CRUDE ECP + ALUM</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>CRUDE ECP + FICA</td>
<td>-</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>90</td>
</tr>
</tbody>
</table>

CRUDE ECP=crude extracellular products preparations  
FICA=Freund's Incomplete Adjuvant  
ALUM=alum precipitation  
FPBS=fish phosphate buffered saline
TABLE 3: Total percent mortality recorded for fish immunized with various hemolysin preparations and challenged with strain 21.

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>CHALLENGE DILUTION</th>
<th>10^7</th>
<th>10^6</th>
<th>10^5</th>
<th>10^4</th>
<th>SALINE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.50 X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPBS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ALUM</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>80</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>FICA</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>CRUDE ECP</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CRUDE ECP + ALUM</td>
<td>20</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td>80</td>
<td>20</td>
<td>30</td>
<td></td>
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<tr>
<td></td>
<td>30</td>
<td>30</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>CRUDE ECP + FICA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>10</td>
<td>30</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

CRUDE ECP=crude extracellular products preparations
FICA=Freund’s Incomplete Adjuvant
ALUM=alum precipitation
FPBS=fish phosphate buffered saline
not correlated with dilution of the challenge organism. In fact, fish receiving only a saline-challenge injection had 70% mortality, indicating that alum may have reduced resistance of fish to stress of the challenge procedure. Fish immunized with crude-acid extracted S-layer preparation had lower mortality (60-80%) than the control groups (p < 0.10) at the $10^5$ challenge dilution. At this same challenge dilution, fish immunized with CRUDE S-LAYER + FICA also had lower mortality (40-60%) than the control groups (p < 0.01) (TABLE 4).

DISCUSSION

Although results from this study indicate that proteases and hemolysin do not confer protection to channel catfish, the data in both cases is incomplete. First, as already stated, the group immunized with protease preparations received only one injection containing detectable levels of proteases. If two injections had been successfully given to these fish, significant levels of protection may have been apparent between treatment and control groups. Although fish immunized with hemolysin preparations did receive two full doses of antigen, the challenge strain was not virulent enough to cause mortality consistently greater than 30%, even in the control groups. If another strain that produces primarily hemolysin in its ECP is characterized and determined to be virulent ($<10^5 \text{ LD}_{50}$), a similar experiment could be performed and the protection conferred to channel catfish from hemolysin preparations more reliably tested. When injected IP, the S-layer
TABLE 4: Total percent mortality recorded for fish immunized with various S-layer preparations and challenged with strain 166.

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>CHALLENGE DILUTION 3.26 X</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^6</td>
</tr>
<tr>
<td>FPBS</td>
<td>100</td>
</tr>
<tr>
<td>ALUM</td>
<td>100</td>
</tr>
<tr>
<td>FICA</td>
<td>100</td>
</tr>
<tr>
<td>CRUDE S-LAYER</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>CRUDE S-LAYER</td>
<td>90</td>
</tr>
<tr>
<td>+ALUM</td>
<td>100</td>
</tr>
<tr>
<td>CRUDE S-LAYER</td>
<td>50</td>
</tr>
<tr>
<td>+FICA</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

CRUDE S-LAYER=crude acid extracted S-layer preparations
FICA=Freund's Incomplete Adjuvant
ALUM=alum precipitation
FPBS=fish phosphate buffered saline

* At this challenge dilution, the CRUDE S-LAYER +FICA had significantly lower mortality than the control groups (FPBS, ALUM, and FICA) at p < 0.01.
confers protection to channel catfish after subsequent challenge with the homologous organism (p < 0.01). Because the LPS of gram negative bacteria is closely associated with the S-layer protein and LPS-based vaccines confer protection to fish challenged with homologous strains, these results are not surprising. The S-layer of motile aeromonads has been correlated with virulent strains and is prevalent in strains isolated during MAS epizootics (Ford and Thune, 1989). Therefore, the S-layer may be a common antigen among virulent strains of motile aeromonads and may serve as the common antigen necessary for vaccine development. The ability of S-layer immunizations to confer protection to channel catfish after subsequent challenge with serologically heterologous, virulent motile aeromonads needs to be investigated.

LITERATURE CITED


Infect. Immun. 9: 828-834.


Role of the S-layer Protein in Humoral Response and Protection of Channel Catfish to Motile Aeromonads

ABSTRACT

The S-layer protein of motile aeromonads appears to play a role in virulence of bacteria during epizootics of Motile Aeromonad Septicemia (MAS) and can confer protection to channel catfish after challenge with the homologous strain of Aeromonas. To evaluate the S-layer protein as an immunogen, channel catfish were immunized with formalized whole cells of an S-layer producing strain, a crude acid-extracted S-layer preparation, an electroeluted S-layer preparation. Agglutinating antibody responses were greatest in fish immunized with the crude, acid-extracted preparations injected plus Freund’s incomplete adjuvant (FICA). Immuno-dot-blot and western blot analysis indicated that channel catfish were able to specifically recognize the S-layer protein. Fish immunized with formalized whole-cells and crude, acid-extracted preparations from strain 166 + FICA were protected (LD₅₀>10⁷) compared to control groups (LD₅₀>10⁶) after challenge with the vaccine strain. Fish immunized with the electroeluted S-layer preparations were not protected. In subsequent immunization trials, immunization of the crude, acid-extracted S-layer + FICA conferred protection to catfish after subsequent challenge with both serologically homologous and heterologous strains.
INTRODUCTION

An S-layer protein has been identified as a potential virulence factor in motile aeromonads isolated from MAS epizootics (Ford and Thune, 1989). The S-layer protein is also present in virulent strains of motile aeromonads as determined by LD_{50} laboratory studies (Dooley and Trust, 1988; Thune, unpublished data). Evidence points to a significant role of the S-layer in pathogenicity of motile aeromonad infections. Although the S-layer appears to be a candidate for vaccine development, simple techniques to evaluate induction of immune responses of fish by such an antigen yield inconclusive results. Agglutination titers of trout sera against whole cells typically target LPS antigens on whole cells (Dooley et al, 1986). Due to the inability of whole-cell, LPS-based vaccines to confer protection to fish challenged with heterologous motile aeromonads (Thune and Plumb, 1982 and 1984), an antigen common to virulent motile aeromonads is needed that will also confer protection against both homologous and heterologous strains. The objective of this study was to develop a method for evaluating the antibody response of channel catfish to specific S-layer immunogens as well as to determine if fish immunized with the S-layer could be protected against both homologous and heterologous challenge.

MATERIALS AND METHODS

Homologous Immunization and Challenge

Channel catfish fingerlings (~10 g) were acclimated to 35 L tanks with a continuous flow of 25° C water. Fifteen fish were placed into
Each of forty tanks. Once the fish were observed to actively feed for a minimum of one week the experiment was initiated. Fish in each of three tanks were immunized with one of eight immunogens prepared from strain S87-166 (166) including formalized whole cell (WC), WC + Freund's Incomplete Adjuvant (FICA), crude acid extracted preparation of the S-layer protein (CRUDE), CRUDE + FICA, electroeluted S-layer protein (ELUTED), ELUTED + FICA, FICA alone, and fish phosphate buffered saline (FPBS). The S-layer protein was obtained by growing strain 166 in 50 ml aliquots of BHI broth at 30°C for 18 hours. The bacterial cells were washed twice with distilled water and extracted in 10 ml of 0.2 M glycine-HCl buffer (pH 2.2). The cells were mixed by inversion for 15 min at room temperature, centrifuged for 15 min at 11,000 g, and the supernatant was collected and adjusted to pH 4.5. Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the pH adjusted samples and production of the S-layer protein for each sample was confirmed by the presence of a 50,000 MW protein band after staining with Commassie Brilliant Blue. Electroelution of the acid extraction preparation was performed using an ElutrapR (Schleicher and Schuell, Inc., Keene, NH) with a 37.6 mM tris + 40 mM glycine buffer. The Bradford (1976) method of protein determination was used to determine total protein concentration of the CRUDE and ELUTED samples prior to use for fish immunizations.

Each fish was injected intraperitoneally (IP) with 0.1 ml of each immunogen and immunogen + adjuvant combination and boosted with the same preparation 21 days later. Prior to boosting, 5 fish from each tank were bled from the caudal artery and blood collected in heparinized
hematocrit capillary tubes. Hematocrits were determined and serum was collected from the centrifuged capillary tubes for determination of agglutinating antibody titers to the homologous strain (166) by microagglutination method in 96-well plates. Ten days post-booster, remaining fish were challenged with a standardized suspension of strain 166. Mortalities were recorded until no fish were found dead or moribund for 3 consecutive days and LD$_{50}$ determinations were calculated using the method of Reed and Muench (1936).

**Heterologous Challenge**

Fifteen channel catfish fingerlings were transferred into each of 54 flow-through tanks and acclimated as previously described. Each fish was injected IP with 0.1 ml of CRUDE + FICA prepared according to the methods described for homologous challenge. After 21 days triplicate tanks were challenged with 4 log dilutions of a standardized culture of 166 or the heterologous strain 50. Mortalities were recorded and LD$_{50}$ determinations were calculated as previously described. Strain 50 was chosen as a heterologous strain because it produced S-layer protein but had a serologically distinct LPS as determined by transblot analyses of whole cell lysates from strains 166 and 50 with anti-strain 166 whole cell antiserum. Briefly, the S-layer protein was detected from both strains but the typical LPS ladder was only present for strain 166 (FIGURE 1). Although a co-migrating, cross-reactive minor protein would not be readily distinguished from the S-layer protein in this western blot, previous studies indicated that rabbit anti-strain 166 antiserum cross reacted with the acid-extracted S-layer protein of strain 166 and
FIGURE 1: Western blot analysis of whole-cell lysates from two motile aeromonads with rabbit anti 166 as the primary antibody.
strain 50 as demonstrated by a single band of identity in Ouchterlony gel diffusion experiments (Thune, unpublished data).

Production of Rabbit Anti-Catfish Whole Serum Antiserum

Channel catfish were seined from a 0.2 acre pond at the LSU Ben Hur Aquaculture Research Facility, anesthetized and bled via the caudal artery. The blood was allowed to clot and the separated serum was aliquoted and stored at -70°C for future use.

Serum was thawed, mixed with an equal volume of FICA (1 ml +1 ml) and injected subcutaneously (SC) into duplicate New Zealand White rabbits at 5 sites along the spinal column. Intravenous (IV) boosts were administered at one week intervals for 3 weeks, after which 20 mls of blood was drawn from each rabbit. Blood was allowed to clot, refrigerated overnight and the separated serum was aliquoted and frozen at -70°C.

Isolation of Channel Catfish Ig

Channel catfish (50 g) were injected IP with bovine serum albumin (BSA) in FICA, boosted after 21 days, and at weekly intervals thereafter for three weeks. Fish were bled via the caudal vein, the blood was allowed to clot and the serum collected. Serum was titered using a standard hemagglutination method (Garvey et al., 1977), aliquoted and stored at -70°C until needed.

Catfish immunoglobulin was purified on a BSA-Sepharose affinity chromatography column by a standard method (Garvey et al., 1977). Briefly, cyanogen bromide activated Sepharose Cl-4B was washed twice with alternating solutions of 0.1 M NaHCO₃ + 0.5 M NaCl and water at 4°C. A BSA solution (3 mg/ml) was added to the beaker containing the
washed Sepharose and allowed to magnetically stirred for two hours at room temperature. BSA molecules now coupled to the Sepharose, was transferred to a 9 ml column attached to a fraction collector, washed with 250 ml of Borate Buffered Saline (BBS), followed by 50 ml each of 0.1 M NaOAc + 0.5 M NaCl, 2 M urea + 0.5 M NaCl and 0.1 M NaHCO₃ + 0.5 M NaCl. The acetic acid -urea- carbonate wash cycle was repeated, and the column was finally washed with 250 ml of BBS. Three 2 ml aliquots of catfish anti-BSA antiserum were loaded onto the Sepharose column, and incubated for one hour at 4° C. The unadsorbed serum was eluted with BBS and bound anti-BSA antibodies were eluted with 3 M NaSCN as described by Lobb and Clem (1983). The eluted fraction was confirmed as the immunoglobulin fraction of catfish serum by immunoelectrophoresis against rabbit anti-catfish whole serum (FIGURE 2).

Production of Rabbit Anti-Catfish Ig Antiserum

One ml containing 240 ug of purified catfish immunoglobulin mixed with 1 ml FICA was used to immunize two New Zealand White rabbits by subcutaneous injection in 5 injection sites along the rabbit’s spinal column. Each rabbit was boosted IV after 3 and 4 weeks with 0.3 ml containing 80 ug of protein. One week later, 10 ml of blood was collected from each rabbit, allowed to clot and refrigerated overnight. The serum was collected and aliquoted for storage at -70°C for subsequent immuno dot-blot and western blot analyses.

Immuno-Dot-Blot

Crude acid extraction of strain 166 S-layer was placed into the
FIGURE 2: Immunoelectrophoresis results showing rabbit anti catfish antiserum reaction with whole channel catfish serum, fraction 2 that contains catfish immunoglobulin, and fraction 1 that contains diluted material that was washed from the column prior to elution with Ig buffer.
wells of a Bio-dot™ apparatus (BioRad, Richmond, CA) in replicate 2-fold dilutions for each of the twelve rows of the apparatus. Protein concentrations ranged from 80 ug - 0.625 ug. Vacuum pressure was applied to the apparatus, allowing any proteins in the preparation to adhere to the nitrocellulose (NC) sandwiched between the upper and lower portions of the apparatus. The NC was removed, cut into twelve strips corresponding to the 12 columns and washed three times with a wash buffer (Tris 10 mM/NaCl 0.9% and 0.05% Tween 20). Catfish sera from fish immunized with the various S-layer immunogen preparations were diluted 1:5 and 2 ml of this serum was incubated with a strip of the NC for 1 hr. Strips were washed with wash buffer 3 times for 10 minutes and incubated with a 1:50 dilution of rabbit anti-catfish antisera for 1 hr. Strips were again washed 3 times with wash buffer, and incubated with a 1:3000 dilution of commercially prepared goat anti-rabbit Ig for 1 hr. After washing in the wash buffer, strips were immersed in color developer (4-chloro-1-napthol in methanol). The color development was stopped by washing with distilled H₂O. Catfish antibodies to the S-layer protein in the catfish serum samples were indicated by the development of a purple dot in the appropriate wells.

Western Blots

Crude acid-extracted S-layer protein preparations were separated by SDS-PAGE and transferred overnight onto nitrocellulose with a tris (25 mM)-glycine (192 mM) transfer buffer. The nitrocellulose was placed into a Miniblotter® (Immunetics®, Cambridge, MA) and individual catfish serum samples from previously immunized fish were placed into individual wells
of the Miniblotter\textsuperscript{R} and incubated for 1 hour. The NC was washed three times with the same wash buffer used in the bio-dot experiments. The NC was removed from the Miniblotter\textsuperscript{R} and the same procedure as described for the immunodot experiments was used to incubate the NC with the secondary and tertiary antibodies.

RESULTS

Channel catfish immunized with various preparations of S-layer protein produced agglutinating antibodies to the formalized whole cells of the homologous strain (166). Titers for both replications of the immunization experiment are tabulated together in TABLE 1. Antibody titers did not exceed a titer of 1:32. Antibody titers of fish immunized with FPBS and FICA alone did not exceed a titer of 1:8, and many of the fish in each immunization group had no detectable agglutination titers. The relatively high titer (1:8) in FICA immunized fish may be due to aggregation of the bacterial cells with another component (i.e. albumin) of catfish sera or may indicate that the fish were previously exposed to the same bacterial serotype. Fish immunized with the crude preparation and the crude + FICA had the highest mean titers of all the immunized groups.

Results from the immuno-dot-blot experiment showed that fish immunized with crude S-layer preparations produced antibodies to the preparation which contain LPS and the S-layer protein, which is the predominant protein that is detected in the S-layer preparation when analyzed by SDS-PAGE and stained with CBB. Catfish immunized with the control preparations (FPBS and FICA) did not show antibody production to the S-layer protein or LPS by immuno-dot-blot analysis. These results further
TABLE 1: Reciprocal agglutination titers from fish immunized with various S-layer preparations.

<table>
<thead>
<tr>
<th>TITER</th>
<th>FPBS</th>
<th>FICA</th>
<th>WC</th>
<th>CRUDE</th>
<th>ELUTED</th>
<th>FICA</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2</td>
<td>17</td>
<td>8</td>
<td>10</td>
<td>11</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>2</td>
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<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>6</td>
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<td>16</td>
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<td>0</td>
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<td>2</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>32</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

\[ \bar{x} \text{ of titers (log)} \]

0.09* 0.43 0.35 0.27 0.79 0.50 0.28 0.33

FPBS=fish phosphate buffered saline
FICA=Freund's Incomplete adjuvant
WC=standardized whole cells of 166
CRUDE=crude acid extracted S-layer preparations
ELUTED=electroeluted S-layer preparation

*significantly lower mean titer than all other groups (p > 0.05)
corroborate the possibility that the FICA fish with 1:8 titers did not produce detectable and specific agglutinating antibodies to the vaccine strain, but rather aggregated due to some other serum component. Pre­bled serum from the rabbit that contributed the secondary antibody (rabbit anti-catfish Ig) in the immuno-dot-blot did not show color development (FIGURE 3). Western blot analysis demonstrated that fish vaccinated with WC + FICA produced antibodies to the S-layer protein as well as higher molecular weight LPS molecules of the crude S-layer preparation (FIGURE 4). Also, note that the fish vaccinated with the crude S-layer preparation and the eluted S-layer preparation produced antibodies to the S-layer protein but also to higher molecular weight molecules (FIGURE 4).

In immunization trials using only S-layer preparations, the lethal dose of 166 cells needed to kill 50% of the population (LD 50) was higher for the CRUDE+FICA groups ( > 5.0 X 10⁶) and the WC+FICA ( > 10⁷) groups than any of other immunized or control groups (p < 0.10) (TABLE 2). In the heterologous challenge trial using fish immunized with CRUDE+FICA, both the homologous and heterologous challenge groups were significantly protected as determined by statistically analysis of the arc-sine transformed data (TABLE 3).

DISCUSSION

Currently, there are no commercially available vaccines for channel catfish. Several studies have focused on development of a
FIGURE 3: Results of immunodot blot utilizing immune catfish sera as the primary antibody and rabbit anti-catfish IgM as the secondary antibody. The results with normal catfish sera are shown on the right. Results with pre-bled rabbit sera are shown in the center lane, and results with immune catfish sera are shown on the left.
FIGURE 4: Results of western blot analysis utilizing catfish immune sera for the primary antibody and rabbit anti-catfish Ig as the secondary antibody. The lanes are labelled corresponding to the type of immune catfish sera used for the particular lane.

- Lane 1: CRUDE + FICA (1-10)
- Lane 10: ELUTED + FICA (21-30)
- Lane 20: WC + FICA (35-39)
- Lane 30: FICA (41-45)
- Lane 40: FICA

FICA = Freund's Incomplete adjuvant
WC = Standardized whole cells of 166
CRUDE = Crude acid extracted S-layer preparations
ELUTED = Electroeluted S-layer preparation
TABLE 2: LD$_{50}$ values for fish immunized with various S-layer positive preparations of S87-156 and challenged with the homologous organism.

<table>
<thead>
<tr>
<th>TREATMENTS</th>
<th>LD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPBS</td>
<td>1.16 X 10$^6$</td>
</tr>
<tr>
<td>FICA</td>
<td>1.24 X 10$^6$</td>
</tr>
<tr>
<td>WHOLE CELLS</td>
<td>3.23 X 10$^6$</td>
</tr>
<tr>
<td>WHOLE CELLS + FICA</td>
<td>1.86 X 10$^7$*</td>
</tr>
<tr>
<td>CRUDE S-LAYER</td>
<td>1.16 X 10$^6$</td>
</tr>
<tr>
<td>CRUDE S-LAYER + FICA</td>
<td>9.50 X 10$^6$*</td>
</tr>
<tr>
<td>ELUTED S-LAYER</td>
<td>1.87 X 10$^6$</td>
</tr>
<tr>
<td>ELUTED S-LAYER + FICA</td>
<td>1.84 X 10$^6$</td>
</tr>
</tbody>
</table>

FPBS=fish phosphate buffered saline  
FICA=Freund's Incomplete adjuvant  
CRUDE S-LAYER=crude acid extracted S-layer preparations  
ELUTED S-LAYER=electroeluted S-layer preparation  

*significantly higher protection (LD$_{50}$) than FICA control group (p < 0.10)
### TABLE 3: Total percent mortality recorded for fish immunized with crude, acid-extracted S-layer preparations and challenged with the vaccine strain 166 or the heterologous strain 50.

<table>
<thead>
<tr>
<th>STRAIN AND CHALLENGE DILUTION</th>
<th>CRUDE + FICA</th>
<th>FICA</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRAIN 166</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.18 X 10⁷</td>
<td>100 80 60</td>
<td>100 100 100</td>
</tr>
<tr>
<td>10⁶</td>
<td>70 50 20</td>
<td>90 90 90</td>
</tr>
<tr>
<td>10⁵*</td>
<td>0 0 0</td>
<td>40 50 30</td>
</tr>
<tr>
<td>10⁴</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>saline</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>STRAIN 50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.86 X 10⁸</td>
<td>100 100 100</td>
<td>100 100 90</td>
</tr>
<tr>
<td>10⁷</td>
<td>100 100 100</td>
<td>100 100 90</td>
</tr>
<tr>
<td>10⁶**</td>
<td>30 20 20</td>
<td>30 80 60</td>
</tr>
<tr>
<td>10⁵</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>saline</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
</tbody>
</table>

CRUDE + FICA = crude, acid-extracted S-layer preparation + Freund’s Incomplete adjuvant, FICA= Freund’s Incomplete Adjuvant

*significantly lower mortality in vaccinated group versus control group (p < 0.05)

**significantly lower mortality in vaccinated group versus control group (p < 0.10)
vaccine/bacterin for MAS but have reported only limited success. To date, two types of vaccines have been investigated as to their potential efficacy in conferring protection to fish against natural MAS epizootics. Monovalent vaccines were among the first approaches to be tested. Khalifa and Post (1963) and Post (1966) demonstrated protection of fish exposed to a monovalent vaccine if fish were subsequently challenged with a homologous strain. Protection was also conferred in salmonids vaccinated with a monovalent preparation by the intraperitoneal (ip) route or by hyperosmotic infiltration (HI) (Udey and Fryer, 1978; Acuigrup, 1980). Monovalent vaccines confer protection to challenge with the vaccine strain, but commercial catfish ponds may be populated by several potentially pathogenic strains of motile aeromonads. Monovalent vaccines, therefore would have limited commercial application. Thune and Plumb (1982 and 1984) immersion-vaccinated channel catfish with a polyvalent, sonicated preparation. Fish challenged with the homologous strains were protected, but fish challenged with heterologous strains were not. In order to be commercially applicable, polyvalent vaccines would have to contain all pathogenic strains. Due to the heterogeneity of the motile aeromonad, this approach is both impractical and quite impossible (Plumb, 1984). Because whole cell vaccine preparations do not protect fish against challenge with heterologous strains of motile aeromonads, investigators have begun to assess the value of bacterial components that are considered to be potential virulence factors as vaccine preparations. The S-layer protein is a promising candidate for vaccine development since it is present in isolates from natural MAS epizootics.
Thune, 1989), present in virulent strains determined by LD_{50} analysis and can be recognized by the catfish humoral immune system.

In each trial crude S-layer preparations administered with FICA conferred protection to channel catfish challenged with either the homologous or heterologous strain making the S-layer protein the first component of motile aeromonads to confer protection to fish challenged with a heterologous strain. The crude, acid-extracted S-layer preparations contained other substances (i.e. LPS) that could have contributed to the protection conferred to immunized channel catfish in this study. In fact, the western blot analysis demonstrates that the fish respond primarily to the S-layer and higher molecular weight LPS. Although the degree of protection may have been influenced by LPS thru non-specific induction of the immune response, this remains the first report of protection conferred to channel catfish by any type of *Aeromonas* vaccine after subsequent challenge with a known heterologous strain. One must note, however that only one heterologous strain was used as a challenge organism. Prior to further vaccine development, immunization trials that include a larger number of heterologous challenge organisms would be advantageous in confirming the ability of the S-layer to confer protection against the wide range of virulent motile aeromonads that are present in the environment.

LITERATURE CITED


SUMMARY

The role of virulence factors from the motile aeromonad complex in the pathogenicity of Motile Aeromonad Septicemia has been the subject of extensive research in recent years. The results obtained from this study indicate that the S-layer protein is a promising candidate for vaccine production.

Further work now needs to be pursued in two main areas. First, the S-layer protein immunization trials need to be tested against a wider range of challenge strains to confirm the ability of the S-layer to confer protection against heterologous motile aeromonads. Second, an immunogen preparation and optimum dosage of the S-layer protein will have to be determined taking into consideration the needs of mass vaccination of aquatic animals. Also, the optimum age of fish to be immunized and route of administration of the immunogen need to be determined to facilitate the production of an efficacious vaccine for commercially raised channel catfish.
Larisa Ann Ford, daughter of John Robert and Rebecca Turner Ford, was born in Charleston, South Carolina on April 22, 1961. In 1979 she graduated as Valedictorian from Flour Bluff High School in Corpus Christi, Texas and entered Tulane University, New Orleans, Louisiana.

In 1980, the author entered Texas A&M University and received her B.S. degree in December, 1982 and her M.S. degree in August, 1985. The author entered the graduate program in the Department of Veterinary Microbiology and Parasitology at Louisiana State University in August, 1985. The author is currently employed by the National Fish Health Research Laboratory, US Fish and Wildlife Service, Leetown, West Virginia.

The author is a member of the American Fisheries Society, International Association of Aquatic Animal Medicine, World Aquaculture Society and the American Society for Microbiology.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate:  Larisa Ann Ford

Major Field:  Veterinary Medical Sciences

Title of Dissertation:  Evaluation of Aeromonas hydrophila Virulence Factors: Role in Natural Epizootics and Their Use as Potential Immunogens.

Approved:

[Signatures]

Major Professor and Chairman
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Kenneth L. Schenck

Date of Examination:

April 19, 1990