AN ABSTRACT OF THE THESIS OF

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Title: PATHOGENIC, ANTIGENIC, AND IMMUNOGENIC PROPERTIES OF AEROMONAS SALMONICIDA STUDIED IN JUVENILE COHO SALMON (ONCORHYNCHUS KISUTCH).

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An additional layer, exterior to the outer membrane cell-wall layer, was found on Aeromonas salmonicida cells from aggregating strains. Nonaggregating strains were found to lack this layer. The nonaggregating strains were all avirulent (LD_{50} greater than 1×10^8 CFU/fish) while the majority of the aggregating strains were virulent (LD_{50} less than 1×10^4 CFU/fish). Other, yet undetermined virulence factors may also be required for strains to be highly virulent. Cells from an aggregating strain adhered to fish and human tissue culture cell monolayers in significantly larger numbers (30 fold) than cells from nonaggregating strains.

No differences were detected in acetic acid degraded O-antigen polysaccharide or Bovien-type antigens from aggregating and non-aggregating strains of A. salmonicida using double immunodiffusion. Immunodiffusion also failed to detect any antigens in aggregating strains which might be associated with the additional layer. Such an antigen was detected in agglutination assays following agglutinin adsorption procedures using a rabbit anti- A. salmonicida serum. This antigen was not discernable in similar assays with antiserum from juvenile coho salmon.

Thermal stress of the host and dosage of A. salmonicida were important parameters in water-borne challenge experiments. Actively immunized juvenile coho salmon, and those receiving juvenile coho salmon antiserum produced to the challenge strain, were significantly protected from furunculosis when challenged in the laboratory. Under natural challenge conditions (at the Oregon Department of Fish and Wildlife's Siletz River Salmon Hatchery) four A. salmonicida vaccines were evaluated. The parenterally administered vaccine significantly reduced the furunculosis mortality while three different oral vaccines provided no protection.

Pathogenic, Antigenic, and Immunogenic Properties of *Aeromonas salmonicida* Studied in Juvenile Coho Salmon (*Oncorhynchus kisutch*)

by

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Redacted for Privacy

Head of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

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INTRODUCTION

Furunculosis is one of the major infectious diseases of hatchery reared salmonid fish. Immunization of juvenile fish with *Aeromonas salmonicida*, the causative agent of furunculosis, is an attractive means for control of this disease. Prophylactic immunization has been shown to be quite effective in the control of vibriosis in salmonids (Fryer et al. 1972; Fryer et al. 1976). Although similar vaccination trials for the control of furunculosis (Duff 1942; Spence et al. 1965; Overholser 1968; Klontz and Anderson 1970) proved to be encouraging, no suitable immunization procedure has been developed. Many previous investigations were encumbered by a lack of information about the virulence factors of *A. salmonicida* as well as the factors which influence the immune response of fish to this organism.

The purpose of this study was to obtain sufficient information on factors influencing the immunogenicity of *A. salmonicida* so that an effective furunculosis vaccine might be developed. A major goal of this research was to determine the factors associated with the virulence of *A. salmonicida*. Another objective was to identify antigenic and structural differences between virulent and avirulent strains.

Several properties of *A. salmonicida* strains were examined in this study. The cell wall ultrastructure and adhesive properties of

both virulent and avirulent strains were investigated. Differences in the immune response of juvenile coho salmon to antigens of virulent and avirulent strains were examined by passive immunization, cross agglutination, and agglutinin adsorption techniques. Further antigenic studies were made using rabbit serums. The effects of pathogen dose and host stress on the course of furunculosis mortality were also investigated. This study culminated with an evaluation of the efficacy of three different oral vaccines and one administered parenterally. The choice of vaccines and the natural challenge procedure used in their evaluation was based on other results obtained during this study.

LITERATURE REVIEW

Furunculosis of fish, caused by Aeromonas salmonicida, has been the subject of more scientific reports than any other infectious disease of fish. Two excellent reviews (McCraw 1952; Herman 1968) summarize the literature up to 1952 and from 1952 to 1966 respectively.

This dissertation will cover the major published reports (1930 to 1976) on the pathogenicity and virulence of A. salmonicida as well as those on the antigenicity and immunogenicity of the organism.

Pathogenicity and Virulence of Aeromonas salmonicida

Aeromonas salmonicida has been considered of great importance throughout the years because of its ability to severely infect a wide variety of fishes. It has been reported to infect no less than nine species and/or subspecies of Salmonidae. Twelve other species of fish from several families in the class Osteichthyes were also reported susceptible to A. salmonicida (McCraw 1952; Herman 1968). Hall (1963) found that the lamprey (Ichtyomyzon castancus), belonging to the class Agnatha also contracts furunculosis.

The ability ot A. salmonicida to resist and overcome the defense mechanisms of such a wide variety of fish species suggests that the bacterium possesses potent virulence factors.

Furunculosis can be manifested in four clinical forms: (1) acute - sudden mortality with few or no lesions; (2) subacute - increasing mortality with formation of lesions ("furuncles"); (3) chronic - low mortality with intestinal inflammation and some hemorrhage; and (4) latent - no mortality and no symptoms but with A. salmonicida present in vital organs (Herman 1968). The acute and subacute forms are of greatest significance because they are the forms of the disease which can result in massive fish mortalities. Pathological and histopathological changes in the host found in the subacute form are extensive. These changes have been described in detail by Duff (1932), Duff and Stewart (1933), Klontz et al. (1966), Kimura (1970), and Wolke (1975).

Several investigators have studied the virulence of A. salmonicida strains. Duff (1937) reported that goldfish (Carassius auratus) died of furunculosis when injected with approximately 10^8 cells from smooth strains of the bacterium but not when injected with an equal number of rough strain cells. Using juvenile coho salmon, Paterson (1972) showed that a strain (SS-70), which had recently been isolated from a diseased fish, evoked a 50% mortality with less than 100 cells per fish (LD_{50} of 100 cells). This observation was confirmed by McCoy (1973) who also showed that strain

SS-70 became attenuated (LD_{50} of 4.4×10^5 cells per fish) when transferred repeatedly on laboratory media.

Anderson (1972) compared the virulence of "rough" and "smooth" colony variants[1] isolated from cultures stored and transferred every six months over periods of two to eleven years. These variants were distinguished on the basis of "subtle" differences in colony morphology as seen under a dissecting microscope. Each type of variant from five strains was assayed for its virulence in coho salmon by determining the LD_{50} dose (strain 34 had no "smooth" variant and strain 111 had no "rough" variant). Three "smooth" variants had LD_{50} values of 2.0×10^1 to 8.0×10^2 cells per fish (assuming 0.1 ml was injected per fish, although this was not stated). In contrast, "rough" variants had LD_{50} values ranging from 7.0×10^9 to 4.0×10^{11} cells per fish. The "smooth" form of strain 111, however, had an LD_{50} of 4.0×10^{11} cells per fish and was assumed to be a mutant.

Although marked differences in virulence have been noted in strains of A. salmonicida, there has been a paucity of research on the factors associated with these differences. It has been observed in salmonids that even though A. salmonicida is prevalent in the blood during active furunculosis, little or no phagocytosis occurs (Blake, after McCraw 1952; Klontz et al. 1966; McCoy 1973). In addition, McCoy (1973) found that attenuated as well as virulent A. salmonicida strains resisted phagocytosis in coho salmon.

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[1]Quotation marks are placed around the rough and smooth designations because it appears that these distinctions are misleading. Discussions of strains WFDL-S and WFDL-R in this dissertation will clarify this point.

McCoy (1973) and Fuller (1974) independently demonstrated, however, that both virulent and avirulent cells were phagocytized *in vitro* by coho leucocytes.

Saline extractable extracellular material from *A. salmonicida* cells was shown by Klontz et al. (1966) to produce degeneration of hematopoietic tissue in rainbow trout (*Salmo gairdneri*). They suggested that this was due to a leukocidin. A leukocidin was also proposed by Griffin (1954) to explain the lack of leukocytic infiltration during furunculosis infections. Fuller (1974) isolated a glycoprotein leukocidin from autolysates of both virulent and avirulent *A. salmonicida* strains. Evidence was presented which indicated that virulent strains produce quantitatively more leukocidin than avirulent ones. This leukocidin was also active *in vivo*. Injection of the leukocidin into adult rainbow trout resulted in significant reduction of leukocyte counts in comparison with saline injected control fish (Fuller 1974).

Other potential virulence mechanisms have also been investigated. A potent endotoxin for mice was extracted with trichloracetic acid from *A. salmonicida* (Paterson 1972). Quantities as large as 5 mg were injected intraperitoneally (i.p.) into 23 g coho salmon fry, without producing detectable ill effects.

To determine if extracellular aggressins might be correlated with virulence, McCoy (1973) examined both virulent and avirulent strains of *A. salmonicida* for hydrolases and hemolysins. No differences

were detected between strains in assays for hyaluronidase, fibrinolyase, hemolysins (sheep and horse), deoxyribonuclease, ribonuclease, gelatinase, lipase, elastinase, and caseinase.

Although it has never been experimentally demonstrated, Griffin (1954) suggested that metabolic by-products such as agmatine, putrescine, and tryptamine might be produced from arginine, ornithine, and tyrosine, respectively. These compounds are pharmacologically active and may alter the host's metabolism and consequently its susceptibility to A. salmonicida infection.

Antigenicity and Immunogenicity of Aeromonas salmonicida

This section will focus on three aspects of serological and immunological investigations with A. salmonicida. These will include: (1) serology of A. salmonicida antigens; (2) parenteral immunization of fish with A. salmonicida; and (3) enteral, or oral vaccination trials with A. salmonicida bacterins.

Serology

Using the complement fixation technique, Blake and Anderson (1930) conducted the first significant serological study of A. salmonicida. They examined 82 strains (most of which were autoagglutinating) from six countries for their ability to bind complement fixing antibodies from one immune rabbit serum. All strains were

positive but the amount of complement bound varied as much as six minimal hemolytic doses (e.g. 14 vs 20). This variation suggested that not all strains of A. salmonicida were serologically identical.

Duff (1939) investigated the serological relationships of the rough (R) and smooth (S) variants of A. salmonicida. Duff used cross-agglutination and agglutinin-adsorption techniques to determine the characteristics of these strains. He classified the strains (including both the S and the R variants)into two groups. In Group I he placed those strains in which the R variant appeared to possess an antigen or antigens not present in the S variant of the same strain. This group was given the antigenic formula R = S + n. Group II was composed of those strains in which S cells possessed an additional antigen not found on R cells. The antigenic formula suggested for this group was $S_1 = R_1 + n_1$. Duff also found that monospecific "n" antiserums agglutinated either the S or the R cells of every strain but never agglutinated both S and R phases of the same strain.

In these assays, a NaCl concentration of only 0.05% was used to prevent autoagglutination of rough phase cells. Kabat (1961) found this low concentration of salt to be inadequate for the normal agglutination reaction, whereas Duff reported no such problem.

In a study of the extracellular antigens of the genus Aeromonas, Liu (1961) showed that nontoxigenic extracellular antigens were produced by A. salmonicida. Liu also found that of 15 Aeromonas

hydrophilia and *Aeromonas punctata* strains examined, only three produced extracellular antigens which cross reacted with anti -*A. salmonicida* serum.

Karlsson (1964) investigated the serological relatedness of 11 *A. salmonicida* strains from Sweden, Ireland, and Scotland. It was noted that the strains had a strong tendency to autoagglutinate; this could be prevented, however, by subjecting them to 60 watts of ultrasonic energy for two to three minutes. Cross-agglutination assays were made using antiserums prepared to all 11 strains. With any one serum, no more than a four-fold difference in titer was observed between strains. Cross-agglutinin adsorption was performed with three antiserums and their three homologous antigens. Each adsorbing strain removed all the agglutinins from all three serums. Karlsson concluded that the cell wall antigens from these strains were identical. Using gel immunodiffusion, he also distinguished six identical antigens from all 11 strains. These antigens were present in the soluble fraction of ultrasonically disrupted cells.

In a limited study, Paterson (1972) examined the serology of two strains: (1) the autoagglutinating strain, SS-70, which is virulent for coho salmon, and (2) strain SIL-67, which is avirulent and does not autoagglutinate. Paterson was not able to show any serological differences between these two strains using the cross-agglutinin adsorption technique.

McCoy (1973) on the other hand, using gel immunodiffusion, found a band could be detected in SS-70 cell sonicates that was not present in those from SIL-67. McCoy observed only one band with the SIL-67 antigens and only two with SS-70. The results of his study must be somewhat suspect in view of the fact that Karlsson (1964) observed six bands from all strains examined using a similar technique.

Kimura (1970) extensively studied a bacterial disease of the Japanese "Sakuramasa" (Oncorhynchus masou) and of pink salmon (Oncorhynchus gorbuscha) which closely resembled furunculosis. He concluded that the causative agent was A. salmonicida but based on its biochemistry and serology concluded that it should be classified as a new subspecies of A. salmonicida (masoucida).

Parenteral Immunization

Smith (1940) was the first investigator to demonstrate that fish could produce antibodies to A. salmonicida. Both carp (Cyprinus carpio) and rainbow trout were found to produce agglutinins to A. salmonicida in response to parenteral injection of cells, even when held at water temperatures as low as 10°C. Gee and Smith (1941) reported that an antibody response could be induced in nearly all (26 of 27) carp held at 20-23°C.

Krantz et al. (1963, 1964a) demonstrated that both brook trout (Salvelinus fontinalis) and brown trout (Salmo trutta) developed

anti- A. salmonicida agglutinins following a parenteral injection of formalin-killed cells in saline. Agglutinin titers were increased when cells were emulsified in a mineral oil adjuvant before injection. The use of an adjuvant also markedly increased the degree of protection against subsequent parenteral challenge with virulent cells.

Spence et al. (1965) passively immunized juvenile coho salmon with hyperimmune rainbow trout anti -A. salmonicida serum. They found that hyperimmune serum alone could effectively delay the onset and reduce the severity of furunculosis. Normal serum or saline administered similarly had no protective effect. This was the first demonstration that hyperimmune serum components (presumably specific antibody) provided protection in fish.

Overholser (1968) demonstrated that adult chinook salmon produced specific antibodies following injection with formalin-killed cells. Adult fish were inoculated both with and without Complete Freund Adjuvant (CFA). All groups responded with elevated agglutinin titers.

The most significant work to date on the immune response of fish to A. salmonicida has been that of Paterson and Fryer (1974a). They administered whole A. salmonicida cells in CFA to juvenile coho salmon and subsequently investigated the response of the population, duration of the response, immunological competence of juvenile animals, and the protection afforded by vaccination. It was determined

that even juvenile fish as small as 1.2 g each could produce agglutinating antibody titers as high as 1:20,480 and that these could persist for longer than one year. One-hundred percent of the population of both 1.2 g and 20 g fish showed at least some rise in A. salmonicida agglutinin titers at 12° C. Fish vaccinated with killed A. salmonicida (virulent strain SS-70) emulsified in CFA had a significantly lower mortality than those receiving CFA alone when both groups were challenged by injection with SS-70 cells.

Paterson and Fryer (1974b) also determined the effect of water temperature and antigen dose on the immune response of juvenile coho salmon. A trichloroacetic acid (TCA) extract of A. salmonicida cells (endotoxin) was injected either with or without an aluminum hydroxide carrier. Fish which received either preparation at doses from 1 to 5,000 µg produced significant agglutination titers at 12°C. Temperature had a considerable effect on the antibody response to this antigen. The lag time for antibody synthesis was increased with decreasing temperature while both the rate of synthesis and the mean titers decreased with decreasing temperature. The effects were more pronounced in 6.5 g than in 22.6 g fish.

Coho salmon anti- A. salmonicida agglutinins were characterized by Cisar and Fryer (1974). Hyperimmune serum was collected from juvenile coho salmon immunized with A. salmonicida cells emulsified in CFA. Specific antibodies were purified using immunoadsorption onto purified cell walls (from strain SIL-67). The antibody was found to be a 17S macroglobulin composed of four IgM-like subunits

each of approximately 145,000 daltons. It was also restricted in its electrophoretic migration (pH 7.5) to the anodic portion in agar gels. An important finding was that as little as 6 ng antibody N/ml was required to produce agglutination of A. salmonicida cells in the standard tube test. Even with titers as high as 1:20,000 the level of antibody may be as low as 120 ng antibody N/ml of serum.

Oral Vaccination

To date, attempts have been made to orally immunize fish against five bacterial pathogens: A. salmonicida, A. hydrophila, Flexibacter columnaris, Enteric Redmouth (ERM) bacterium, and Vibrio anguillarum. The literature concerned with these attempts has been recently reviewed (Klontz and Anderson 1970; Sneiszko 1970; Rohovec 1975). This review will be limited to those studies dealing with oral vaccination trials with A. salmonicida.

Research on the oral immunization of fish began with the vaccination trials conducted by Duff (1942). Cutthroat trout (Salmo clarki) were fed a diet containing chloroform-killed virulent cells of A. salmonicida. Duff unfortunately did not accurately calculate the amount of vaccine incorporated into the diet. Water temperatures during feeding periods were low (6.7°C and 8.3°C) in the two experiments. Both vaccinated and unvaccinated fish were challenged at 19°C by adding virulent bacteria to the water. Vaccinated fish were not protected after 40 consecutive daily feedings but were

protected after 64 or more. Control groups had a mortality of 75% following a waterborne challenge, while only 25% of the vaccinated fish died. When similar groups were challenged by a parenteral inoculation of A. salmonicida, 90% of the unvaccinated and 63% of the vaccinated fish died. These initial experiments provided convincing evidence that fish could be actively immunized against furunculosis by the oral feeding of killed bacterins.

Only one attempt to orally immunize fish against furunculosis was reported between 1942 and 1964. Snieszko and Friddle (1949) compared the efficacy of feeding a heat-killed A. salmonicida bacterin with the feeding of sulfamerazine in preventing furunculosis in brown trout. The vaccine was incorporated into the diet at a level of 0.5 g cells/kg food. However, the vaccine diet was only fed for eight days before challenge. A parenterally administered challenge dose of 5.0×10^8 cells resulted (not surprisingly) in a 100% mortality in both vaccinated and control groups. Based on this experiment it was concluded that oral vaccination was not effective in preventing furunculosis in fish.

Krantz et al. (1964b) fed chloroform-killed A. salmonicida cells to brown trout on alternate days over a 65-day period. They also fed different age groups of brook and brown trout several doses of viable cells over the same period. None of the groups developed elevated agglutinating antibody titers. Even though

the fish were never challenged, it was concluded that oral vaccination was not an effective means of immunization.

Spence et al. (1965) could not demonstrate protection of coho salmon after oral vaccination with formalin-killed A. salmonicida. Yearling coho were fed vaccine-diet for 98 days at 12°C and challenged in 17°C water using a method similar to Duff's. As in the experiments of Krantz et al., no increase in agglutinin titers were observed in vaccinated fish.

A different type of antigen preparation was developed by Klontz for use as an oral vaccine (Klontz and Anderson 1970). An alum-precipitated antigen prepared from the water-soluble portion of ultrasonically disintegrated cells was lyophilized and called FSA vaccine. In an initial experiment (1965) 100 yearling brook trout were fed FSA at a rate of 60 µg FSA/fish per day for 25 feedings or a total of 1,500 µg per fish. When challenged by means of exposure to a natural epizootic, none of the FSA-fed fish died of furunculosis while 58% of unvaccinated fish died of the disease. Based on this one study, these investigators vaccinated 8,637,000 trout and salmon over the next three years (1966-68). However, none of the field trials could be evaluated because furunculosis mortality levels, even in control groups, were only 2-5%.

The FSA vaccine was further shown to confer protection in a pilot study at the Oregon Department of Fish and Wildlife's (ODFW) Siletz River Salmon Hatchery (Overholser 1968). Juvenile coho

salmon were fed FSA at a level of 200 mg/kg diet which resulted in experimental groups receiving between 160-380 μ g FSA per fish during the vaccination periods. A natural furunculosis epizootic challenge resulted in 37% and 22% mortalities in unvaccinated groups but only 0.7% mortality in the immunized groups.

MATERIALS AND METHODS

Bacterial Cultures

Isolation Data

Thirteen strains of Aeromonas salmonicida were examined during this study (Table 1). The majority were isolated from dead or moribund salmonid fish during epizootics of furunculosis disease occurring in the United States. Others were obtained as spontaneous mutations of existing strains.

Cultures were cloned on Brain Heart Infusion Agar (BHIA; Difco Laboratories, Detroit, Michigan). Brain Heart Infusion (BHI; Difco) broth cultures were examined for Gram reaction, motility, cytochrome oxidase and agglutination with specific rabbit anti- A. salmonicida serum. Cultures showing a negative-negative-positive-positive pattern, respectively for these four tests were transferred to BHIA slants, incubated for 48 h and the cells preserved

Table 1. Isolation data and sources of *Aeromonas salmonicida* strains used in this study

Strain	Location Isolated	Year Isolated	Original Host species	Source
ATCC 14174	Leetown, W. Va.	1959	brook trout	ATCC
SIL-67	Siletz, Ore.	1967	coho salmon	OSU collection
SS-Sm^d	Corvallis, Ore.	1972	-	Strain SS--70
SS-Sm^d 7,8,9	Corvallis, Ore.	1972	-	Strain SS-70
Sm^d-REV	Corvallis, Ore.	1974	-	Strain SS-Sm^d
WFDL-R	Leavenworth, Wa.	1961	cutthroat trout	Western Fish Disease Laboratory
WFDL-S	Seattle, Wa.	1973	coho salmon	
SS-70	South Santiam, Ore.	1970	chinook salmon	OSU collection
EFLD-3.47	Bear Creek, Md.	1970	brown trout	Eastern Fish Disease Laboratory
EFDL-3.58	Columbus, Oh.	1971	rainbow trout	
GAL-E	Corvallis, Ore.	1974	-	Strain Sm^d-REV

as indicated below. Unless otherwise specified, all cultures were incubated at 18°C.

Preservation of Isolates

Cultures of A. salmonicida were preserved either by lyophilization or storage at -60°C. The growth from BHIA slants was resuspended in an aqueous mixture of dextran 6,000 (5.0%; Pharmacia Fine Chemicals, Piscataway, N.J.), sucrose (7.0%) and monosodium glutamate (1.0%), for lyophilization or in 15% glycerol for freezing. These preservation methods were used to minimize antigenic, biochemical and structural changes of the selected strains during the course of this study.

Aeromonas salmonicida Antigen Preparations

Whole Cell Antigens

The method used in preparing stock suspensions of whole A. salmonicida cells was similar to that indicated by Cisar and Fryer (1974). Preserved cell preparations were resuspended in BHI broth and streaked onto BHIA plates. Isolated colonies, which arose within 48 to 72 h after inoculation, were resuspended in BHI broth to give a concentration of about 1 X 10^9 cells/ml. Several BHIA slants (prepared in 32 oz prescription bottles) were each inoculated with 5 ml of this bacterial suspension and incubated for 24 h. The resultant A. salmonicida cells were suspended in neutral phosphate buffered saline (PBS; Williams and Chase 1967) and washed

three times by centrifugation[2] (3,000 X g for 10 min at 4°C). The final bacterial pellet from each slant was resuspended in 50 ml of 0.3% formalin in PBS for 1 h at room temperature (RT) and stored at 4°C. Shelf life of these suspensions was approximately two weeks. Antigen for microtiter agglutination tests was prepared by diluting the stock cell suspensions (from BHIA) with PBS to an adsorbance (A) of 0.85 (λ = 520 nm).

Antigens used to immunize rabbits were obtained from A. salmonicida grown in a chemically defined medium. Colonies from BHIA plates were resuspended and washed 3X in sterile Hank's balanced salt solution (HBSS; Microbiological Associates, Walkersville, Md.) buffered to pH 7.2 with 50 mM HEPES buffer (Sigma Chemical Co., St. Louis, Mo.). Washed cells were resuspended in Eagle's Minimal Essential Medium (EMEM; Microbiological Associates) containing an additional 0.8% glucose and 50 mM HEPES instead of bicarbonate buffer. After 72-h incubation, the cells and high molecular weight extracellular products were separated from low molecular weight medium components and metabolic byproducts in a Diaflow unit equipped with a PM-10 membrane (Amicon Corp., Lexington, Mass.). This separation was achieved by continuous addition of PBS containing 0.3% formalin, until 10 volumes had been passed through the membrane (removing the low molecular weight fraction). The suspensions were concentrated

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[2]Reference to "washing by centrifugation" will be used throughout this dissertation to describe the process of: (1) centrifuging cells into a pellet; (2) removing the overlying solution; and (3) resuspending the pellet in the same amount of dilutent as previously removed.

10 fold in the unit, dispensed as aliquots into sterile vials, and frozen at -20°C until used.

To produce large volumes of A. salmonicida antigen, the bacterium was grown in a Fermacell Fermentor, Model CF50 (New Brunswick Scientific Co., Inc., New Brunswick, N.J.). Thirty-liter batch cultures were grown in BHI broth supplemented with 10 ml of silicon anti-foam Y-4988 (Union Carbide Corp., N.Y.). Two liters of 48-h broth culture were used to inoculate the fermentor.

During the growth period in the fermentor, the cultures were aerated at 5.7 ℓ/min and mixed with an impeller rotating at 200 rpm. Sterile sucrose (250 ml of a 50% wt/vol solution) was added at 18 h to supply additional nutrient. Cultures were monitored periodically for pH, A, and purity. After 36 h of incubation the cells were collected by continuous centrifugation using a Sharples Super Centrifuge Type T1P.

Disrupted Cell Preparations

Disrupted bacterial cells used for antigen were prepared either from stock cell suspensions or from fermentor cultures prior to formalin fixation. Suspensions were adjusted to 20 g wet weight cells/100 ml distilled water prior to disruption.

One preparation was made by shaking a cell suspension with glass beads (0.25 to 0.3 mm) for 4 min in a Braun shaker (The Braun Co., Melsungen, Germany). The suspensions were maintained at less than 10°C throughout the process with liquid CO_2. Glass beads

were removed by passing the suspension through a course sintered glass filter (Millipore filter base). Formalin was added to the filtrate at a final concentration of 0.3%.

A second preparation was produced by disrupting cells with a Sonifier Cell Disrupter (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). Twenty-milliliter portions of a cell suspension (as above) were continuously cooled in a -30°C bath and subjected to alternate 1-min disruption and cooling periods. Total disruption time varied from 1 to 10 min depending upon the use intended for the antigen. The sonic energy supplied with the type W horn ranged from 80 to 100 watts and was a function of viscosity. These suspensions, except those used to make FSA, were formalized as above.

Extracted Antigens

Boiven type antigen was prepared in cooperation with Dr. D.W. Paterson. Preparation of the antigen has been described by Paterson and Fryer (1974b). Briefly, A. salmonicida strains SS-70 and SIL-67 were extracted with 0.25 N trichloroacetic acid (TCA) in a manner similar to that of Staub (1967).

Degraded somatic polysaccharide, having O-antigen specificity, was prepared from A. salmonicida strains SS-70 and SIL-67 with hot acetic acid as described by Kabat and Mayer (1961). Whole bacterial cells grown in the fermentor were washed twice in PBS and lyophilized. Ten grams of cells (dry wt) were refluxed for 1.5 h at 100°C in 200 ml of 10% acetic acid. After cooling, the

mixture was centrifuged at 10,000 X g for 15 min and the cells rehydrolyzed under the same conditions. Supernatants from both extractions were concentrated to one-tenth the original volume. This crude polysaccharide was then purified. One volume ethanol precipitation of the extract removed contaminating macromolecules while an additional five volumes of ethanol precipitated the polysaccharide. Two drops of saturated sodium acetate was added prior to the six-volume precipitation to aid in floculation. The one and six-volume precipitations were repeated until no further material was removed with one volume. The final solution was dialyzed extensively against distilled water and finally lyophilized. Approximately 0.1 g of purified polysaccharide (dry wt) was obtained.

Preparation, Collection and Storage of Anti-serums

Coho Salmon Anti-Aeromonas salmonicida Serums

Coho salmon used to produce serums were obtained from the Oregon Department of Fish and Wildlife's (ODFW) Fall Creek Salmon Hatchery. The fish were maintained at the Oregon State University Fish Disease Laboratory in 12°C pathogen-free well water in three-foot circular tanks and were fed Oregon Moist Pellet (OMP; Hublou 1963) once daily.

Antiserum prepared against A. salmonicida strain SIL-67 was obtained from Dr. J.O. Cisar. This antiserum had an agglutinating antibody titer of 1:20,480. A serum to strain SS-70 was produced in a manner similar to that used by Cisar and Fryer (1974).

Disrupted cells (1.75 X 10^{10} whole-cell equivalents/ml) prepared in the Braun shaker were emulsified 1:1 in CFA (Difco). Each fish was injected i.p. with 0.1 ml of the emulsion after being anesthetized in dilute methyl pentynol (no longer manufactured) prior to injection.

Serum was collected 90 to 95 days after injection. Fish were exsanguinated by severing the caudal artery and the blood collected in Vaseline-coated small tubes as suggested by Williams and Chase (1967). These samples were left at RT for 1 h and placed at 4°C overnight to permit clot retraction. Serum was harvested after centrifugation for 10 min at 600 X g, then filtered (0.45 µm pore size membrane). A major portion of the pooled serum was distributed into small screw-cap vials and frozen at -60°C for storage; the remaining serum was stored at 4°C. The agglutinin titer of this serum (using SIL-67 cells) was 1:20,480.

Rabbit Anti-*Aeromonas salmonicida* Serums

Antiserums to *A. salmonicida* strains SS-70 and SIL-67 were produced in young female New Zealand white rabbits.

The immunogens were grown in EMEM as described previously. Immunizing suspensions, which contained 1 X 10^{10} cells/ml and a 10X concentration of extracellular products were emulsified 1:1 in CFA. Rabbits were inoculated and their serum later harvested using the following routine:

Day 1: One, 1 ml subcutaneous (s.c.) injection near each inguinal and popliteal lymph node (4 ml. total).

Day 15: Two, 0.5 ml intramuscular (i.m.) injections into each gluteal muscle.

Day 45: Approximately 40 ml of blood collected from the ear of each rabbit.

Day 49: One, 2 ml i.p. injection (without CFA).

Day 50: Two, 0.5 ml i.m. injections into each gluteal muscle.

Day 80: Approximately 40 ml blood collected from the ear of each rabbit.

Rabbit serums were processed identically to those from salmon, except that the blood was held at 37°C during the initial 1 hr clotting period. Agglutination titers for the four serums are listed in Table 2.

Table 2. Agglutination titers of rabbit anti-*A. salmonicida* serums collected at different points in the immunization regimen

Antiserum	Reciprocal agglutination titer	
	Day 45	Day 80
SIL-67	2,560	5,120
SS-70	1,280	1,280

Chemical Determinations

Total Organic Carbon

Total organic carbon (TOC) is defined as the carbon (in the form of CO_2) released from a sample when it is combusted at 600-650°C (Konrad et al. 1970). The TOC analysis is a convenient method by which to standardize vaccines because it is insensitive to variations in the biochemical and inorganic chemical composition of the sample.

For this study, TOC content of vaccines was determined using a Sargent Company (Chicago, Ill.) combustion apparatus. Thirty-milligram lyophilized samples (in triplicate) were weighed to the nearest 0.01 mg in porcelain combustion boats. Each sample was heated to 650°C for 5 min in the combustion tube. A stream of CO_2-free oxygen was passed through the tube at 30 ml/min to facilitate combustion. Water, nitrogen oxides, and halogen gases were removed from the combustion products by adsorbtion onto $MgO-Ag_2O-Ag_2WO_4$ (Konrad et al. 1970). Carbon dioxide was specifically adsorbed onto a 1:1 mixture of Ascarite and magnesium perchlorate in sealed preweighed tubes. After each combustion, the CO_2 adsorbtion tube was removed and weighed to the nearest 0.01 mg. The percent carbon in the sample was calculated according to the equation below:

$$\%C = \frac{(0.27298)\ (CO_2 \text{ sample} - CO_2 \text{ blank})}{\text{sample weight}} \times 100$$

where: 0.27298 = K determined by $\frac{\text{atomic wt C}}{\text{molecular wt } CO_2}$

CO_2 sample = wt of CO_2 from sample in milligrams

CO_2 blank = wt of CO_2 from blank in milligrams

sample weight = wt of sample in milligrams

Nitrogen Determinations

Nitrogen content of vaccines was determined by the semi-micro Kjeldahl method (Kabat and Mayer 1961; Williams and Chase 1967). Kjeldahl nitrogen (KN) like TOC is a conservative property of vaccines and can, therefore, be used to standardize and compare various vaccine preparations.

Three 5-mg samples of each vaccine were analyzed. The ammonia released during digestion and distillation was titrated with N/70 HCl secondary standard. Ammonium sulfate (Fisher No. A-938) served as the primary nitrogen standard. Percent nitrogen in the samples was calculated by the equation:

$$\% N = \frac{(0.2)\ (V_{sample} - V_{blank})}{\text{sample weight}} \times 100$$

where: 0.2 = K determined by $\frac{14 \text{ mg N/mM}}{70 \text{ mg of N/70M HCl/mM}}$ assuming 1 mg/ml of the acid

V_{sample} = ml of N/70 HCl titrant for sample

V_{blank} = ml of N/70 HCl titrant for reagent bank

sample wt = wt of sample in milligrams

Immunodiffusion

Gel immunodiffusion analyses were conducted using a macro-method employing 100 by 15 mm disposable plastic petri plates. Molten 2% agarose was mixed 1:1 with hot double-strength immuno-

diffusion buffer (NaCl, 8.5 g; Thimerosal, 0.1 g; sodium barbital, 8.0 g; distilled water, 1000 ml; pH 7.4). The resultant solution was cooled to 45°C and 17 ml were added to each plate. Using a Shandon gel cutter (Shandon Scientific Co., Inc., Seweakley, Pa.), one 9 mm diameter center well and four 7 mm diameter outer wells were cut in each plate. Spacing between wells was 7.5 mm (edge-to-edge).

Both sonically disrupted bacteria and bacterial extracts were employed as antigens. Antiserums used for this study were those obtained from rabbits. Diffusion of antigens and antibodies was allowed to proceed for three days at RT in a moist chamber, at which time the plates were viewed and photographed on a dark-field illuminator (Williams and Chase 1967).

Determination of Agglutinating Antibody Titers

The level of agglutinating antibody to A. salmonicida was determined with Cooke microtiter equipment (Cooke Engineering Co., Alexandria, Va.). This system has been widely used to detect bacterial agglutinins and was shown to be reliable using A. salmonicida antigens (Paterson and Fryer 1974a). Disposable "U" bottom plates were found to give the best end point. Initial dilutions were made by adding 50 µl of serum (or serum dilution) to 50 µl of PBS in the first well. Subsequent two-fold dilutions were made in PBS using 50 µl diluters mounted in the multiple

diluter handle. Following the dilution process, 50 µl of antigen suspension was added to each serum dilution. Plates were sealed with mylar film, incubated at RT for 2 h, and then placed at 4°C for 16 to 18 h.

Antigen produced from nonaggregating strains SIL-67 and SM^d-REV gave the most clearly defined end points (i.e.,4+ agglutination in positive wells with buttons of cells in the bottom of negative wells). Antigen from aggregating strains such as SS-70 autoagglutinated, but true agglutination was distinguishable. Rough strain (GAL-E) cells autoagglutinated so strongly that reactions produced by antibody were indistinguishable.

Estimation of Virulence of *Aeromonas salmonicida* Strains

The number of bacterial cells capable of killing 50% of inoculated hosts (LD_{50}) was determined for several aggregating and nonaggregating strains of *A. salmonicida*. Preserved cultures were inoculated onto BHIA plates. Growth from the plates was suspended in BHI broth and the cultures incubated at 18°C for 48 h. The number of cells per ml was estimated using a Petroff-Hauser counting chamber and the number of colony-forming units (CFU) per ml determined by the pour-plate method in BHIA.

Juvenile coho salmon used for the LD_{50} determinations weighed an average of 25 g each. They were placed in 18°C water two days

before inoculation. For each strain of A. salmonicida examined, six groups of 10 fish each were utilized. Fish which died during the experiment were collected once daily, necropsied and a portion of each kidney was cultured on a BHIA plate.

The LD_{50} dilution was determined by the moving-averages method of Weil as described by Meynell and Meynell (1965). The LD_{50} dilution was multiplied by the number of CFU/ml in the culture to obtain the LD_{50} in terms of CFU/fish.

Conditions Necessary for Aggregation of Aeromonas salmonicida Cells

To determine the contribution of aggregation to the virulence of A. salmonicida, it was necessary to investigate the mode by which aggregation occurs. The following experiment was designed to find which inorganic ions are involved.

Cells for this study were grown as previously described for virulence determinations. Cells were collected by centrifugation at 3,000 X g for 15 min, washed three times (3X) in PBS and finally washed 3X in 1.39% ethanol. When cells of aggregating strains were washed in the latter solution, it was found that they disaggregated into individual cells. Ethanol solutions were used in place of distilled water because A. salmonicida lysed in the latter. These solutions also have the advantage over buffers in that few ions are present. The cell concentration for these tests was adjusted to 1.0 $\underline{A}_{520}$.

Solutions of NaCl, $MgCl_2$, Na_2SO_4, and $MgSO_4$ served as sources of mono and divalent anions and cations. Concentrations of these salts were at 52.5, 42.5, 32.5, 22.5, and 12.5 mN. The osmolarity of each solution was adjusted to 0.300 $\pm$ 0.005 osmolar with ethanol. A volume of 0.5 ml of each solution was mixed with an equal volume of cell suspension in Kolmer tubes and held at RT for 12 h, at which time the amount of aggregation was determined visually (see Table 7).

Attachment of *Aeromonas salmonicida* to Fish and Human Cells

It was observed qualitatively that cells from aggregating strains attached to the epithelium of the digestive tract and gills of coho salmon and to circulating white blood cells while those from nonaggregating strains did not. In order to quantitate the differences in attachment, a model system was employed using a cell line derived from chinook salmon embryos (CHSE-214) and one derived from a human tumor (HEP-2). Cells from the CHSE-214 line were grown at 18°C on 1.0 cm diameter coverslips in EMEM, supplemented with 5% fetal calf serum. Human cells were grown in a similar manner at 37°C. The tissue cells were grown until they formed a 60% monolayer on the coverslip. Prior to use, monolayers were washed 5X with HBSS + 10 mM HEPES buffer to remove serum and spent medium.

Bacterial cells of strains SS-70 and Sm^d-REV were grown as in the previous experiment and washed 3X with HBSS + 10 mM HEPES buffer. After being washed, the bacterial suspensions were adjusted to 0.3 $\underline{A}_{520}$ with the same balanced salt solution.

The attachment assay was conducted as follows. A 5 ml volume of bacterial suspension was added to a 4 cm petri plate containing three coverslips of tissue cells. The bacteria were incubated for 5 min at RT and continuously agitated at 50 rpm around a circle of 3 cm radius. An additional 10 ml of HBSS + 10 mM HEPES was added and approximately 75% of the resulting suspension was aspirated off the tissue cells. The cells were quickly washed 3X more with buffered HBSS, then fixed for 45 min in 3% glutaraldehyde prepared in buffered HBSS. Following fixation, the samples were washed with buffered HBSS and dehydrated for 10 min at RT in the following solutions:

30% ethanol to buffered HBSS (1:1)
30% ethanol
50% ethanol
70% ethanol
95% ethanol
Absolute ethanol

One coverslip of each sample was processed for scanning electron microscopy and the others were stained at RT and mounted for quantitative counting as follows:

Giemsa stain	20 min
Rinse in distilled water	30 s
Rinse in acetone (two baths) each	30 s

Rinse in 2:1 acetone-xylol (three baths) each	30 s
Rinse in 1:2 acetone-xylol (three baths) each	30 s
Dry in xylol	10 min
Mount on slides with HSR mounting medium.	

Mounted preparations were viewed at 1,000X magnification by light microscopy. The number of bacteria adherent to each of 20 randomly chosen tissue cells was counted and the mean, plus or minus the standard error, computed.

Selection for Mutants of *Aeromonas salmonicida*

Streptomycin-Dependent Strains

Strains of *A. salmonicida* dependent on streptomycin for growth (Sm^d) were isolated because of their potential usefulness as live attenuated oral vaccines (Mel et al. 1965, 1965b, 1965c; Felsenfeld et al. 1970; Reitman 1967; Sanyal et al. 1969; and Sergeev et al. 1967). Single-step (noninduced) mutations were selected by a method similar to that used by Miller and Bohnoff (1947). The flowchart in Figure 1 depicts the methods used.

Growth of four Sm^d strains at seven different streptomycin (Sm) concentrations is shown in Table 3. Increase in mass (Figure 2) is shown for two strains grown with 1,600 µg Sm/ml and 0 µg Sm/ml.

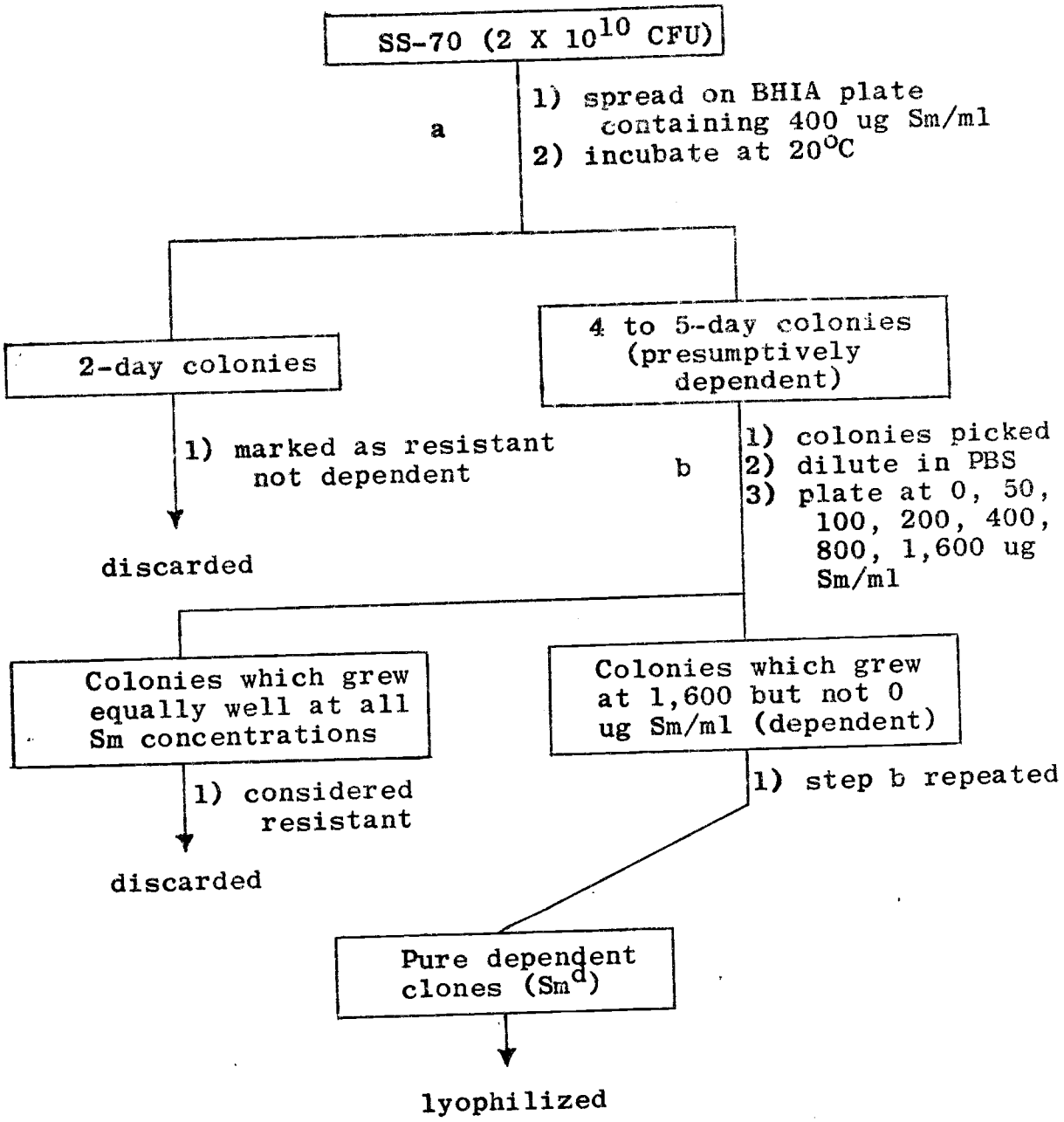


Figure 1. Selection of Aeromonas salmonicida mutations dependent upon streptomycin (Sm) for growth.

Table 3. Growth[a] of four streptomycin-dependent strains of Aeromonas salmonicida in seven selected concentrations of streptomycin

Strain	Incubation period at 20°C (days)	µg Sm/ml (100 CFU/plate)						
		0	50	100	200	400	800	1,600
SS-Sm^d	2	-	-	-	-	-	±	1
SS-Sm^d-7	2	-	-	±	1	1	1	2
SS-SM^d-8	2	-	1	1	2	2	3	3
SS-Sm^d-9	2	-	-	-	1	2	3	3
SS-Sm^d	5	-	-	-	1	3	3	4
SS-Sm^d-7	5	-	1	1	2	3	4	4
SS-Sm^d-8	5	1	2	3	3	4	4	4
SS-Sm^d-9	5	-	-	2	3	4	4	4

[a] 1 = detectable growth
2 = moderate growth but without distinct colonies
3 = colonies pinpoint to 1 mm
4 = colonies larger than 1 mm

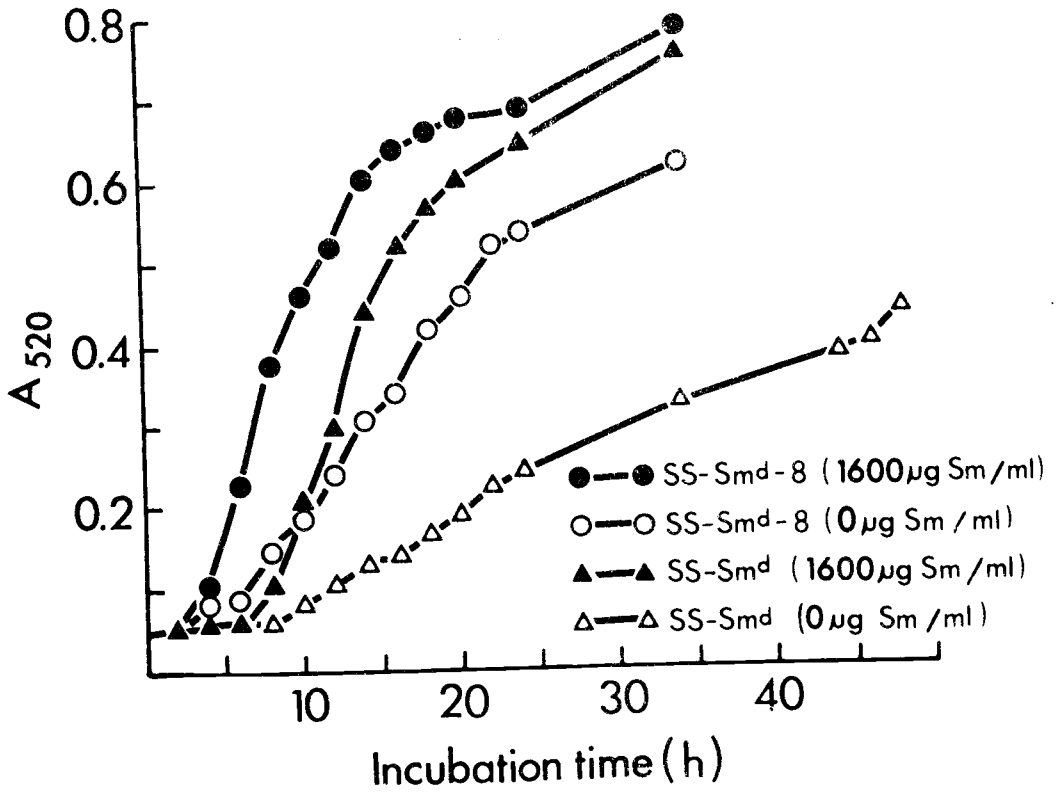


Figure 2. Growth of two streptomycin-dependent strains of Aeromonas salmonicida (SS-Sm^d and SS-Sm^d-8) in the presence and absence of exogenous streptomycin.

These growth patterns are similar to those reported by Miller and Bohnoff (1947) for meningococcus and by Spotts (1962) for E. coli. Streptomycin-dependent mutants of A. salmonicida, therefore, grew similarly to Sm^d mutants from other gram-negative organisms. However, the Sm^d mutants from aggregating strains all lost the ability to aggregate.

Streptomycin Sensitive Revertant Strain

To determine if the loss of aggregation in Sm^d strains was a function of their growth on streptomycin, a streptomycin-sensitive revertant (Sm^d-REV) was isolated. Tubes of BHI broth (without streptomycin) were inoculated with 1×10^9 SS-Sm^d cells, incubated for 24 h at 18°C and plated on BHIA. Resultant colonies were screened for their sensitivity to streptomycin and all were found to be as sensitive to streptomycin as strain SS-70.

It was necessary to confirm that SM^d-REV did not carry a suppressor mutation which would make it sensitive to streptomycin. Strains carrying suppressor mutations are inhibited from growing but not rapidly killed by streptomycin (Brownstein and Lewandowski 1967).

Strain Sm^d-REV was rapidly killed in broth containing 50 or 100 µg Sm/ml. This was determined by plating aliquots at hourly intervals for six hours. The sensitivity of Sm^d-REV to streptomycin was, therefore, considered to be the result of a back mutation and not of a suppressor mutation.

Isolation of a Rough Mutant of the Uridinediphosphogalactose 4-Epimeraseless (GAL-E) Type

A bacteriophage was isolated from water at Siletz Salmon Hatchery (ODFW) as described by Paterson et al. (1969) using strain Sm^d-REV as host. This phage designated as ϕSIL-74 produced plaques on nonaggregating strains of A. salmonicida (SIL-67 and Sm^d-REV) but not on aggregating strains (SIL-74 and SS-70).

Phage-resistant mutants of strain Sm^d-REV were obtained by plating the phage at a multiplicity of infection (m.o.i.) of 10 to produce confluent lysis of the host lawn. The colonies which arose were resistant to phage infection. It was likely that some of the mutants were resistant due to loss of O-antigen side chains (probable phage receptors) as described by Maleka and Stocker (1969). Those colonies which appeared rough were transferred by toothpick to master plates (BHIA) and incubated at RT for two days. Resultant growth was replicated onto BHIA and Tetrazolium-galactose (Miller 1972) plates. Since galactose is toxic to cells lacking UDP-galactose 4-epimerase, colonies which grew on BHIA but not on Tetrazolium-galactose were considered deficient in the enzyme. One typical colony was chosen and designated strain GAL-E.

Transmission Electron Microscopy

Eight strains of A. salmonicida were chosen for ultrastructural studies based on a combination of their aggregation properties

and their site of initial isolation. Each strain was taken from its preserved state and grown as described for virulence determinations.

The bacteria were harvested by centrifugation at 3,000 X g for 15 min and then resuspended in PBS and washed twice in PBS. The pellets obtained after the second wash were fixed for 1 to 2 h at 4°C in 5% glutaraldehyde prepared in PBS (pH 7.2). Small pieces of pellet were embedded in melted (45°C) 2% ionagar (Difco), cooled and allowed to fix for an additional 2 h. The agar-embedded pellets were washed free of excess glutaraldehyde in PBS for 18 h at 4°C. The samples were then stained and secondarily fixed in 1% osmium tetroxide (OsO_4) prepared by diluting a 2% solution 1:1 in PBS (pH 7.2).

Acetone in water at 30, 50, and 70% was applied to the samples for 10 min each at 4°C. This dehydration was followed by an 8-h staining at 4°C with 1% uranyl acetate in 70% acetone-water (Finck 1960). For the final dehydration, cold 100% acetone was applied to the pellets and changed 4X in 40 min. The plastic embedding mixture used was a modification of the Mollenhauer mixture No. 1 (Mollenhauer 1959) and contained the following:

Dodecyl succinic anhydride-DDSA HY9643	53%
Araldite 6005	35%
Epon 812	12%
Benzyl dimethylamine-BDMA	0.05 ml/ml total plastic

Prior to embedding, each sample was infiltrated with a 2:1 solution of the plastic mixture (without BDMA) in acetone for 25 to 36 h at RT. Finally the specimens were embedded in the above plastic mixture, and cured 3 to 6 h at RT and then for 18 to 24 h at 60°C.

Ultrathin sections, 5 to 50 nm thick were cut from trimmed blocks on a Porter-Blum MT1 ultramicrotome equipped with a diamond knife. Sections were mounted on formvar-coated 300 mesh grids. A third stain, lead citrate (Reynolds 1963), was applied for 5 s to the sections before viewing.

Aeromonas salmonicida strain SS-70 was also examined using the negative staining technique (Brenner and Horne 1959). This strain was grown and harvested as above and the final pellet resuspended in 0.001 M ammonium acetate (pH 7.0). A drop of 2% potassium phosphotungstate at pH 7.0 (KPT) was added to a drop of bacterial suspension on a formvar-coated grid. Excess suspension was removed with filter paper and the sample air dried.

All preparations were viewed on a Phillips EM 300 electron microscope at either 60 or 80 kV accelerating voltage. Electron micrographs were taken on Kodak Electron Image Plates (Eastman Kodak Co., Rochester, N.Y.) and developed in Kodak HRP developer.

Scanning Electron Microscopy

Two types of samples were observed by scanning electron microscopy. One consisted of clumps of bacteria reaggregated

from a dissociated state. Cells of strain SS-70 were grown and harvested as described for ultrathin sectioning except 10 mM HEPES buffer (pH 7.2) was substituted for PBS. It was found that 10 mM HEPES buffer could disaggregate clumps of cells in much the same way as 1.39% ethanol. To reaggregate the bacteria, $MgCl_2$ was added to a 0.3 $\underline{A}_{520}$ suspension of bacteria to give a final Mg^{++} concentration of 50 mM. The aggregated bacteria were frozen by placing 50 µl of the suspension on aluminum-backed adhesive tape situated on an aluminum stub; both the stub and adhesive were precooled to $-60^{\circ}C$. The sample and stub were placed in a 1-ℓ lyophilization flask which was then evacuated until the sample was dry. A gold-palladium alloy was evaporated uniformly over the sample surface for three seconds.

The other preparations examined by this technique were of fish tissue cells with and without bacteria adherent to them. The tissue culture cells on the surface of 10 mm glass coverslips were fixed and dehydrated as previously described.

Dehydrated specimens were prepared for critical point drying (Cohen et al. 1968), by passing them through the following series of intermediate fluids:

30% Trichloro-trifluoro ethane (TC-TFE) in absolute ethanol (AETOH)	10 min at RT
50% TC-TFE in AETOH	10 min at RT
70% TC-TFE in AETOH	10 min at RT
90% TC-TFE in AETOH	10 min at RT
Absolute TC-TFE	30 min at RT

Samples which were still wet with TC-TFE were placed in the critical point drying apparatus and dried using Freon 13 as the transition fluid; transition conditions for Freon 13 are 28.9°C at 32.2 atm. Each cover glass was mounted on a stub and coated with gold-palladium as described previously.

All scanning electron micrographs were taken with Polaroid type 55 film on a ISI model MSM-2 (mini-SEM) scanning electron microscope using the secondary electron mode.

The Effects of Dose and Stress on Furunculosis Mortality During Laboratory Challenge

It is desirable to employ a natural challenge to furunculosis when evaluating the effectiveness of various vaccines, but because this method is often unreliable, laboratory challenge is an important adjunct (Spence et al. 1965).

It was felt that a standardized laboratory challenge, which could be replicated at other laboratories, would be of value. The method used was a refinement of several techniques used by Duff (1937), Ehlinger (1964), and Spence et al. (1965). Common to all these techniques was the addition of bacterial broth cultures of virulent A. salmonicida to water containing experimental fish.

Bacterial growth from BHIA plates inoculated from lyophilized cultures was suspended in BHI broth to 0.65 A_{520}. Ten milliliters of this suspension were used to inoculate three, 2-liter

flasks each containing a liter of BHI broth. Cultures were incubated statically for 50 h at 18°C at which time they were pooled and chilled to 0°C. The cell concentration was determined by direct count and confirmed by plating in BHIA.

The tank used for the bulk of these experiments had the characteristics described in Table 4. In a tank of this design, different experimental groups can be exposed simultaneously to the same challenge dose and the same environmental parameters, including various stressors. In this arrangement, however, fish from one group cannot physically come into contact with fish from another group. This may be of serious consequence if contact is the major mode of transmission of the disease.

Dose Effects

To investigate the effect of dose on furunculosis mortality, two groups of 25 untreated coho salmon (approximately 10 g each) were placed in the challenge tank containing 12°C water. Warmer water (18°C) was then added to the tank at the prescribed 6.25ℓ min. rate. After 5 h, the water level was lowered from 301.5ℓ to 120.5ℓ , the water turned off, and the appropriate amount of bacterial suspension added to give the desired challenge dose. The fish were exposed at this level for 15 min after which time the water flow was resumed. The same procedure was followed for the four challenge doses of 1×10^5, 5×10^5, 1×10^6, and 5×10^6 cells/ml.

Table 4. Characteristics of the tank used to simultaneously expose several groups of fish to *Aeromonas salmonicida*

Feature	Description
Tank material	Green fiberglass
Inside diameter	76 cm
Number of compartments	1 to 8
Dividers	Aluminum frame with 1/8 in. woven aluminum screening
Normal water volume	301.5 ℓ
Water volume during static exposure	120.5 ℓ
Water temperature	18°C
Rate of water exchange	6.25 ℓ/min
Water replacement time	48 min

Fish which died during the 14-day experimental period were collected at 8-h intervals, necropsied and cultured for A. salmonicida as previously described.

Actively immunized and unimmunized juvenile coho salmon (10 g each) were subjected to a water temperature elevation of $6^{o}C$. Fish were subjected to this change either two days before, or immediately prior to, challenge with 1×10^{6} cells/ml of A. salmonicida strain SS-70. The reproductibility of this assay was determined by replicating the experiment and analyzing the data by analysis of variance. Actively immunized fish were from the large group used for collection of anti-SS-70 serum.

Relative Stability of Aeromonas salmonicida Strains SS-70 and SIL-67

Effects of Killing and Precipitation Procedures

Chloroform and formalin are two commonly used agents for killing bacterial cells used in whole-cell vaccines (Ross and Klontz 1965). In addition, these vaccines can be combined with $Al(OH)_3$ which functions as an adjuvant (Williams and Chase 1967). The killing and $Al(OH)_3$-precipitation procedures have the potential of lysing cells or differentially extracting cellular components, thereby altering the nature of vaccines made from either soluble or insoluble portions of the cells.

The effect of killing and precipitation procedures on vaccine composition was investigated by first growing and collecting 30 ℓ of each strain in the fermentor as previously described. Wet packed cells from each strain were resuspended at 20% (wt/vol) in 0.85% saline. The starting volume of the SIL-67 cell suspension was 1,931 ml and for SS-70 it was 2,000 ml. The flowchart shown in Figure 3 (page 52) indicates the subsequent treatment steps.

Portions of the lyophilized samples were analyzed for total organic carbon (TOC) and Kjeldahl nitrogen (KN) as described under "Chemical Determinations." The percent recovery of TOC and KN were computed as follows, using TOC as an example:

$$\% \text{ Recovery} = \frac{(C_{TOC})_t \quad (W)_t \quad (Vi)_t}{(C_{TOC})_u \quad (W)_u \quad (Vi)_u} \times 100$$

Where: $(C_{TOC})_t$ = TOC concentration (mg/g) of the lyophilized treated sample

$(W)_t$ = Weight in grams of the lyophilized sample

$(Vi)_t$ = Initial volume of the cell suspension. Note that the cell concentration for $(Vi)_t$ and $(Vi)_u$ are the same.

$(C_{TOC})_u$, $(W)_u$, and $(Vi)_u$ are the same as above except they apply to an untreated sample.

Resistance to Ultrasonic Disruption

To prepare the FSA vaccine patented by Klontz (1970) it was necessary to sonically disrupt the cells and to precipitate the soluble fraction with potassium alum and sodium hydroxide as previously described. The stability of the cells can greatly affect the nature of the resultant vaccine.

To demonstrate that there was a difference of the cells of strains SIL-67 and SS-70, samples of the 20% (wt/vol) cell suspension of each strain were subjected to 0, 1, 5, and 10 min of sonic disruption. Aliquots of disrupted cells were centrifuged at 37,000 X g for 60 min after disruption, and the soluble portion used as antigen for gel-immunodiffusion analysis. Rabbit anti-SIL-67 and anti-SS-70 serums collected at 80 days (Table 2) were used for these investigations. The soluble supernatant obtained from the suspension sonicated for 10 min was used to produce FSA vaccine.

Passive Immunization of Juvenile Coho Salmon

In order to transfer humoral immunity without transferring cellular protection, coho salmon hyperimmune serums were injected i.p. into unimmunized juvenile coho salmon (10 g each). Nonimmune serum was similarly administered to unimmunized and to actively immunized salmon (previously described) of the same size; the first group served as susceptible hosts while the latter acted as animals protected from furunculosis challenge.

Fish receiving hyperimmune serum (either coho salmon anti-SS-70 or anti-SIL-67) were administered 0.1 ml of the serum containing 2,000 agglutinating units;[3] animals which received normal serum got less than 2 agglutinating units in the same volume. Serums from actively immunized fish had a geometric-mean agglutinin titer of 1:20,480.

For each of the four classifications mentioned, two groups of 25 fish were injected with the appropriate serum and placed in water 6°C warmer than their holding temperature (12 to 18°C shift). After 5 h all groups were challenged with 1 X 10^6 cells/ml of strain SS-70 for 15 min as outlined earlier. Dead specimens were collected every 8 h, necropsied, and cultures taken to confirm that furunculosis was the cause of death.

Method for Evaluating Orally and Parenterally Administered Vaccines

Experimental Design

Siletz River Salmon Hatchery (ODFW) has for many years been plagued by annually recurring epizootics of furunculosis disease in coho salmon fry. This hatchery was selected as the site for evaluating several furunculosis vaccines.

In order to insure that an adequate challenge would be presented to the experimental fish, the hatchery stock was not immuni-

[3] An agglutinating unit is equal to the reciprocal agglutination titer, multiplied by the volume of the sample.

zed or treated by chemotherapy, but instead served as a reservoir of the disease agent. An experimental design was developed in cooperation with Mr. Earl Pulford (ODFW) which met four essential criteria: (1) all experimental groups had a sufficient number of fish to represent a normal hatchery population; (2) the groups were replicated adequately to yield data which lent itself to rigorous statistical treatment (i.e. analysis of variance); (3) the nature of the groups (or replicates) was not known to the workers administering the vaccine or necropsying fish; (4) all groups were adequately challenged (with A. salmonicida) so that differences could be detected between unvaccinated and vaccinated groups, but not challenged to a point where no level of immunity would suffice to protect them. These criteria were met in the following way.

Trask River juvenile coho salmon (1974 brood) hatched and reared at Trask River Salmon Hatchery (ODFW) were brought to the experimental facilities at Siletz Hatchery approximately one month prior to delivery of the hatchery's stock. The fish, having an average weight of 0.8 g each, were randomly distributed as 500-fish groups into 15 tanks, each holding approximately 350 ℓ of water. Tanks were assigned at random to three replicates of each of five treatment groups; the hatchery personnel responsible for feeding the vaccine and caring for the fish were not informed

as to the assignments. In addition, all food (OMP) whether it contained vaccine or not, was preweighed and coded by tank, to maintain the double-blind nature of the experiment. The challenge to furunculosis was provided in a manner similar to that used by Ehlinger (1964). Effluent water from three hatchery ponds was pumped through PVC piping and delivered to the tanks at a rate of 2 ℓ /min (similating hatchery water flow). Low mortality rates in the experimental fish prompted a change on June 2, 1974 to using only effluent water from the pond with highest furunculosis mortality at that time.

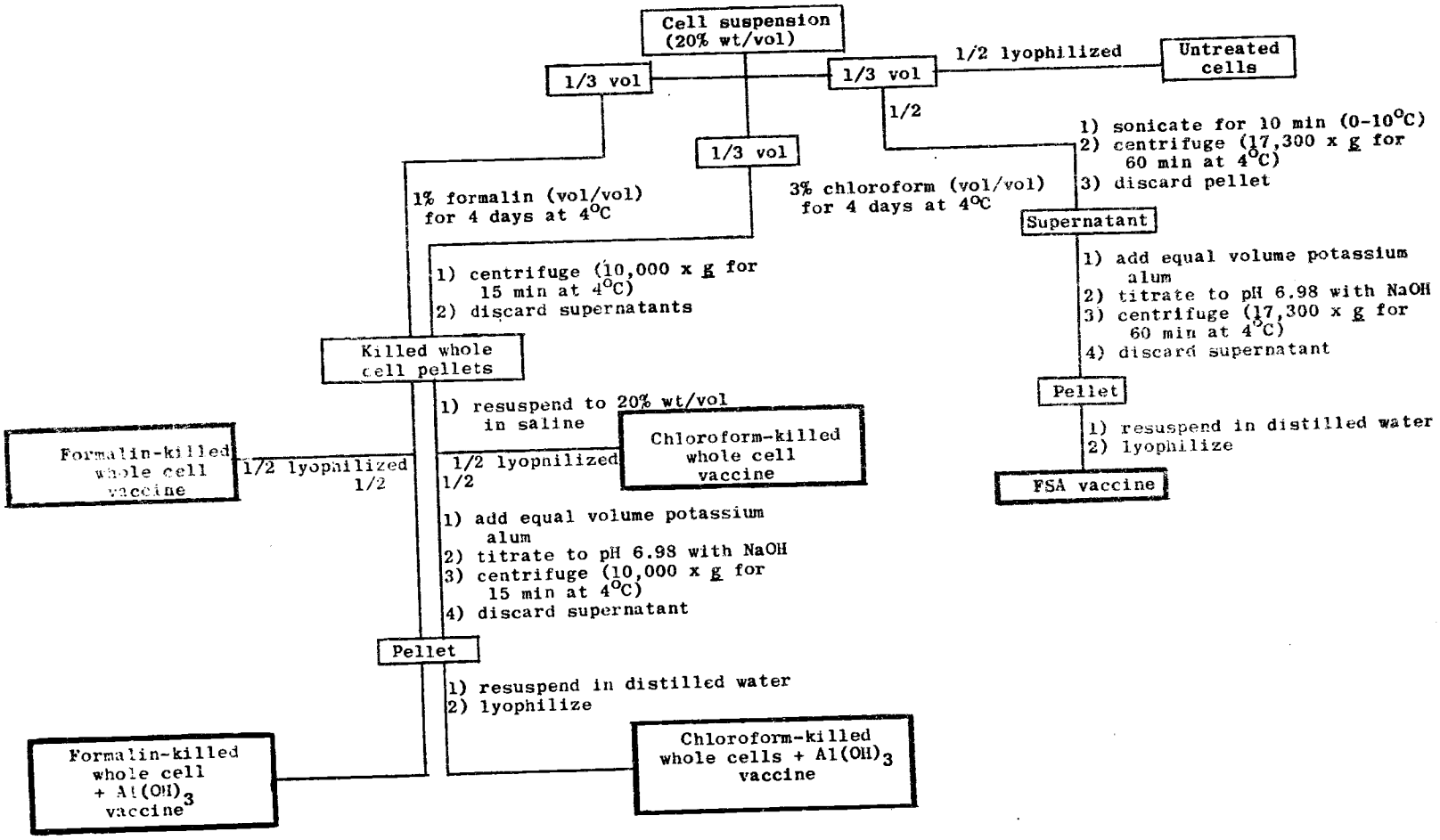
Vaccine Preparation

Three A. salmonicida (strain SS-70) bacterins were used in this study, and their preparation is outlined in Figure 3. The vaccines used were: (1) formalin-killed whole cells, (2) formalin-killed whole cells with $Al(OH)_3$ adjuvant, and (3) Furunculosis Soluble Antigen (FSA). Total organic carbon and Kjeldahl nitrogen content of the vaccines are summarized in Table 13.

Vaccine Administration

The amount of vaccine given to each group of fish was standardized on the basis of its TOC content. This parameter was chosen because it most closely represented the amount of biological material in the vaccines. This standardization was necessary due to the widely divergent nature of the vaccines.

Figure 3. Flowchart depicting the procedures used to prepare five vaccines from Aeromonas salmonicida strains SS-70 and SIL-67.


Cell suspension (20% wt/vol)
1/3 vol
1/3 vol
1/2 lyophilized
Untreated cells
1/2
1) sonicate for 10 min (0-10°C)
2) centrifuge (17,300 x g for 60 min at 4°C)
3) discard pellet
1/3 vol
1% formalin (vol/vol) for 4 days at 4°C
3% chloroform (vol/vol) for 4 days at 4°C
Supernatant
1) centrifuge (10,000 x g for 15 min at 4°C)
2) discard supernatants
1) add equal volume potassium alum
2) titrate to pH 6.98 with NaOH
3) centrifuge (17,300 x g for 60 min at 4°C)
4) discard supernatant
Killed whole cell pellets
Pellet
1) resuspend to 20% wt/vol in saline
Formalin-killed whole cell vaccine
1/2 lyophilized 1/2
1/2 lyophilized 1/2
Chloroform-killed whole cell vaccine
1) resuspend in distilled water
2) lyophilize
1) add equal volume potassium alum
2) titrate to pH 6.98 with NaOH
3) centrifuge (10,000 x g for 15 min at 4°C)
4) discard supernatant
FSA vaccine
Pellet
1) resuspend in distilled water
2) lyophilize
Formalin-killed whole cell + Al(OH)3 vaccine
Chloroform-killed whole cells + Al(OH)3 vaccine

The parenterally vaccinated treatment group received the formalin-killed whole cell vaccine at a level of 75 µg vaccine-carbon/fish. These fish were injected on April 1, 1974 with 0.1 ml of a 1:1 emulsion made with a saline-bacterin suspension and CFA.

Vaccines given orally were incorporated into OMP diet at a level of 500 µg vaccine-carbon/g of diet. The vaccine food was fed for 14 consecutive days starting April 1, 1974. The amount of food given was in accordance with the feeding schedules used at the hatchery and is listed by date in Table 5. In addition, a "booster dose" was fed once a week for two months following the initial vaccination period. Normal food (OMP) was fed to these groups during the interim periods as well as to the parenterally vaccinated and unvaccinated groups through the duration of the experiment.

The orally vaccinated groups were given a total of 94 mg vaccine-carbon/500 fish during the initial 14-day feeding and a total of 205 mg vaccine-carbon/500 fish including the booster periods.

Induction of Immune Suppression

Previous attempts to immunize fish by oral vaccination with A. salmonicida bacterins have been mostly unsuccessful. On the other hand, parenteral vaccination with similar bacterins has

Table 5. Amount of Oregon Moist Pellet (OMP) diet[a] fed experimental juvenile coho salmon at Siletz River Salmon Hatchery (ODFW) between April 1 and July 31, 1974[b]

Month	Diet per 500 fish per day (g)		
	Dates		
	1 - 10	11 - 20	21 - end
April	12.3	16.3	19.3
May	26.1	32.5	38.2
June	48.6	54.1	65.9
July	72.4	80.8	91.2

[a]Based on a feeding schedule devised by D.A. Leith, biologist, Oregon Department of Fish and Wildlife

[b]Vaccine-containing OMP was substituted for normal OMP at the same feeding rates from April 1 to April 14 and once a week thereafter until July 14, 1974

given rise to high antibody titers in both adult and juvenile salmonids (Spence et al. 1965; Paterson and Fryer 1974a, b). This study was conducted to determine if oral vaccination could suppress the humoral antibody response of parenterally vaccinated salmon.

Fish for this experiment were hatched, and reared to late yolk-sac fry stage at the Fall Creek Salmon Hatchery (ODFW). Before they were capable of external feeding they were transferred to the OSU Fish Disease Laboratory where they were held in 12°C disease-free water. These fish weighed an average of 0.45 g each. Fish received only semi-synthetic Oregon Test Diet (OTD; Lee et al. 1967) so that they would not be exposed inadvertently to A. salmonicida antigens which might be present in OMP diet.

Four experimental groups of 500 fish each were treated as follows:

(1) Unvaccinated (control)

(2) Orally vaccinated

(3) Parenterally vaccinated

(4) Parenterally and orally vaccinated

Orally vaccinated fish (groups 2 and 4) were fed OTD containing vaccine. This ration was prepared by adding formalin-killed whole cell bacterin at a level of 383 µg vaccine-carbon/g of diet. Fish which were parenterally vaccinated (groups 3 and 4) received

127 µg vaccine-carbon in 0.05 ml of an emulsion made with equal volumes of CFA and saline-bacterin suspension.

The animals which were given oral vaccine were fed OTD for the first seven days in the laboratory and were started on the vaccine containing diet on the same day as groups 3 and 4 were injected. Vaccine diet was fed for 45 consecutive days according to the feeding schedule used by the Fall Creek hatchery. At the end of the vaccine feeding period, OTD (without bacterin) was fed until the conclusion of the experiment. Pooled serum samples (thirty fish) from each group were collected at 0, 108, and 138 days after initiation of the experiment and the anti-A. salmonicida agglutinin titers determined.

RESULTS

Ultrastructure of Aggregating and Nonaggregating *Aeromonas salmonicida* Cells

Since the earliest work with *A. salmonicida* in the United States (Marsh 1902) it has been recognized that some strains of the organism grow in aggregates while others grow as single, non-aggregated cells. It has been noted that upon initial isolation most (or maybe all) strains aggregate but some strains lose this property upon repeated transfer on culture medium (Figure 4).

A study of the cell wall (CW) structure and surface appendages (B) of both aggregating and nonaggregating strains was undertaken, not for its own merit, but to provide information which might help answer the following questions:

1. Is there a difference in the CW or B of aggregating and nonaggregating strains which could account for their different physical behavior?

2. What, if any, additional CW structures or B are associated with virulent strains and not with avirulent ones (to be discussed later)?

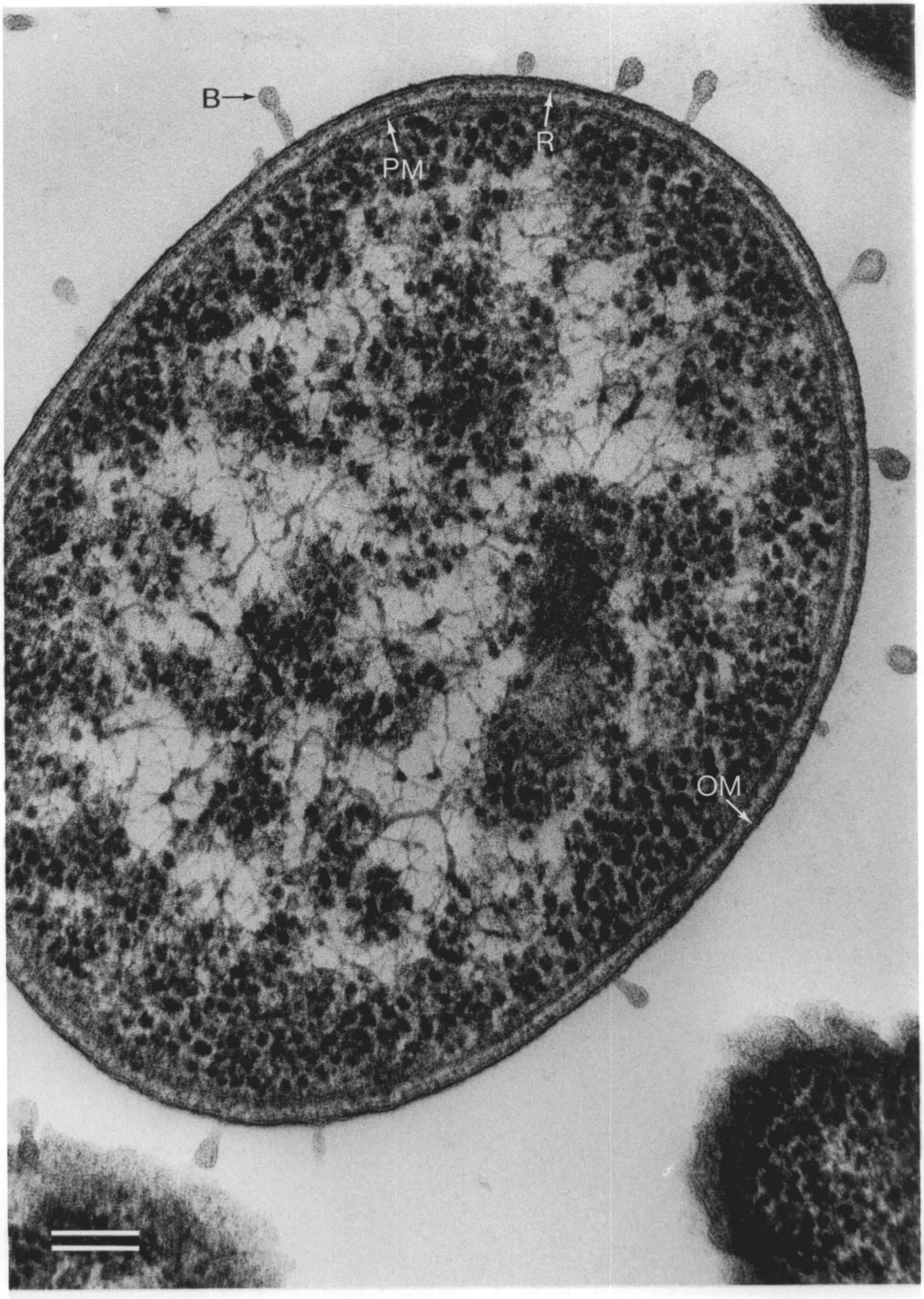
Figure 4. Cultures of Aeromonas salmonicida strains SIL-67 (left) and SS-70 (right). Growth from SIL-67 is uniformly distributed while growth from SS-70 forms a thick layer of sediment on the bottom of the vessel.

3. Are potential major antigens, such as those associated with pili (Brinton 1959; Duguoid et al. 1955) or with additional layers (Glauert and Thornley 1969), present on either aggregating or nonaggregating strains?

Ultrastructure of Nonaggregating *A. salmonicida* Cells

The cell envelope of nonaggregating strain Sm^d-REV, shown in thin secion (Figure 5), is typical of that seen in many Gram-negative bacteria (Glauert and Thornley 1969; Murray et al. 1965). It has a tripartate plasma membrane (PM) with two electron-dense layers separated by an electron translucent zone. The PM is 6-7 nm thick (Table 6) and separates the cytoplasm from the periplastic space (not labeled). The layer immediately outside of the periplastic space is thought to be the rigid layer (R) and contain peptidoglycan as described by Weidel (1960). This layer, sometimes called the dense layer (D) (Sleytr and Thornley 1973) or mucopeptide layer (M) (Beveridge and Murray 1976) is approximately 3 nm thick and lies in close apposition to the outer membrane (OM), separated from it by about 4 nm. The OM is a membrane-like structure (tripartate staining) and appears similar in appearance to the PM except that it is 7.5-8.5 nm thick and has slightly wider electron-dense layers.

Figure 5. Electron micrograph of an ultrathin section from Aeromonas salmonicida strain Sm^d-REV. Features shown: OM, outer membrane; R, rigid or peptidoglycan layer; PM, plasma membrane; B, surface appendages. Scale = 100 nm.


B
PM
R
OM

Surface appendages (B) extend from the cell surface in a near perpendicular fashion. In this view, the appendages appear as elliptical nodules attached to the cell by means of an indistinct "stalk." The elliptical portion is bounded by a tripartite membrane-like layer (approximately 5 nm wide) similar to the PM and OM layers. Thicker sections of cells from this strain (not pictured) showed no evidence of pili or other surface structures. Surface appendages, similar to but longer than those in Figure 5, were observed, however, and many took on a twisted "flattened-hose" configuration.

Nonaggregating strains SIL-67 (Figure 7D) and ATCC 14174 (Figure 7B) have an ultrastructure similar to that described for Sm^d-REV. Strain ATCC 14174 however, has fewer surface appendages than the other two nonaggregating strains (none shown in Figure 7B).

A summary of cell-wall measurements from Figure 5 and other figures is presented in Table 6. This table contains measurements from aggregating strain SS-70 for comparison.

Ultrastructure of Aggregating *A. salmonicida* Cells

Ultrastructural organization of the cell envelope of aggregating strain SS-70 is shown in Figure 6. The arrangement and dimensions (Table 6) of the PM, R, and OM layers of the envelope are similar to those of the nonaggregating strains. All three

Figure 6. Electron micrograph of an ultrathin section from Aeromonas salmonicida strain SS-70. Features shown: A, additional layer; OM, outer membrane; R, rigid or peptidoglycan layer; PM, plasma membrane; B, surface appendages. Scale = 100 nm.

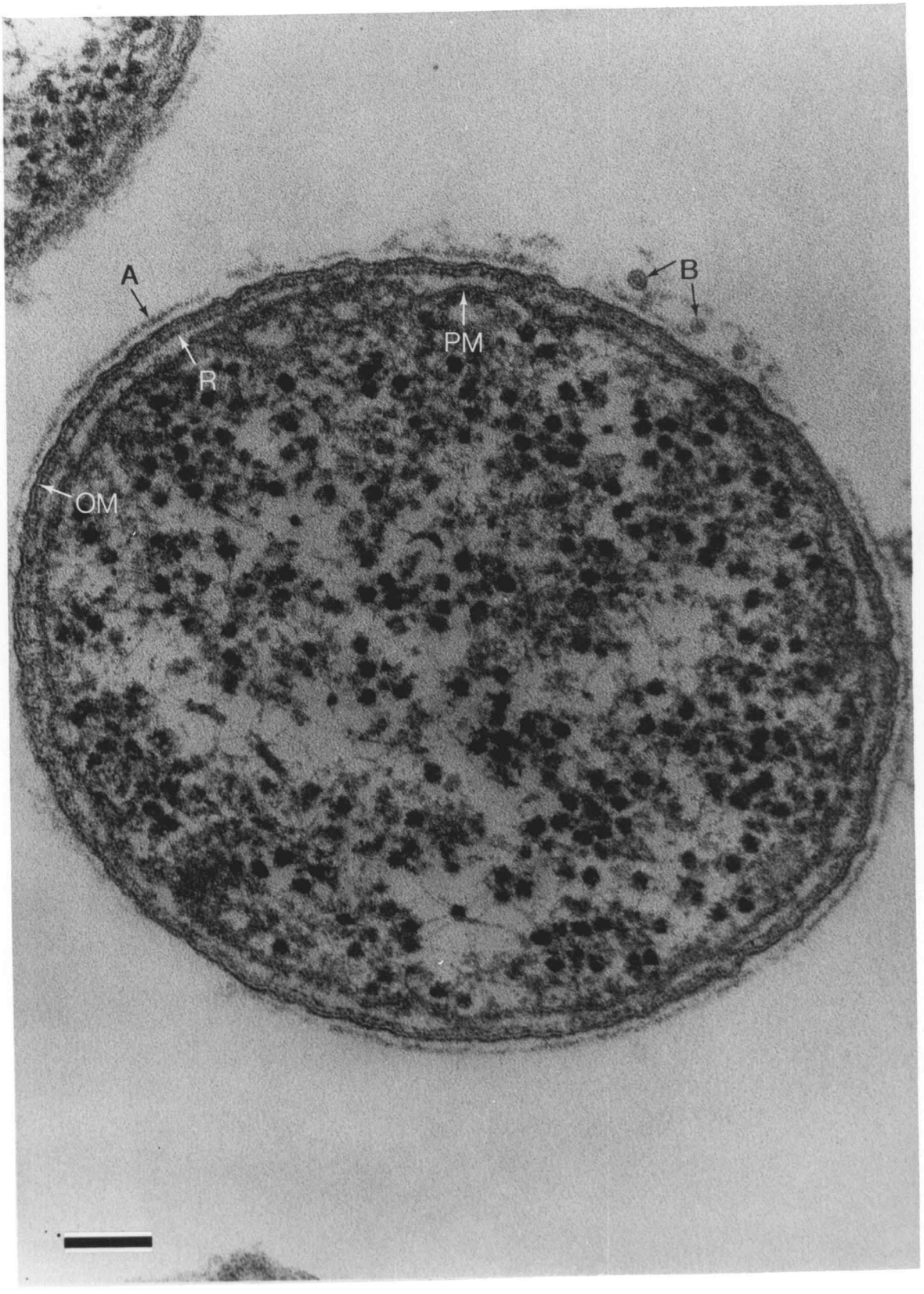

A
B
PM
R
OM

Table 6. Summary of cell wall measurements taken from ultra-thin-section electron micrographs of a nonaggregating (Sm^d-REV) and an aggregating (SS-70) strain of Aeromonas salmonicida

Feature	Average dimension[a] (in nm)	
	Sm^d-REV (nonaggregating)	SS-70 (aggregating)
OM	8	9
R-layer	3	3
PM	7	9
OM plus R-layer	15	17
Surface appendage wall	6	8
A-layer	-	9
Outside OM to inside A-layer	-	9
Outside surface appendage wall to inside A-layer	-	10

[a]Four measurements from printed electron micrographs of calibrated magnification

of these layers appear slightly more convoluted or wavy than those of the nonaggregating strain Sm^d-REV.

Strain SS-70 (Figure 6), unlike Sm^d-REV or other nonaggregating strains, has an electron-dense layer (A) external to the normal cell wall. This layer is 8-9 nm wide and is separated from the OM by an electron-translucent zone of the same dimensions. The A-layer is less distinct than other cell-wall layers and in some areas appears interrupted. This profile closely resembles that observed in Spirillum serpens (Buckmire and Murray 1970), and in Acinetobacter strain MJT/F5/5 (Sleytr et al. 1974).

Surface appendages (B) are also present on strain SS-70. In this figure (6) they are not attached to the cell surface as those shown in Figure 5. These "donut-shaped" structures look similar to the elliptical ends described previously and may represent a cross-sectional view of a tubular type appendage. These structures are also surrounded by electron-dense material similar to that of the A-layer. This material is separated from the surface of the appendage by an electron-translucent zone.

Ultrathin Sections of Additional A. salmonicida Strains

To show that the differences observed in the ultrastructure of nonaggregating (Figure 5) and aggregating (Figure 6) strains was not merely a result of the two strains examined, thin sections of other strains were examined. Other features of the A-layer are also described in this sub-section.

Aggregating strain EFDL-3.47 possesses an A-layer (Figure 7A) similar to that of strain SS-70. This A-layer surrounds many of the surface appendages in a similar manner to that shown in Figure 6. In addition, the A-layer can be seen to extend from the cell as much as 30-40 nm and enclose several appendages within an electron translucent zone (lower black arrow) exterior to the outer membrane.

In areas where EFDL-3.47 cells are adjacent to each other (white arrows) their outer membranes are separated 15-20 nm by A-layer material (Figure 7A). Cells of nonaggregating strain ATCC 14174, by contrast, do not have an A-layer and when pelleted their outer membranes lie in close apposition (Figure 7B).

Thin sections of strains SIL-74 (aggregating) and SIL-67 (nonaggregating) are shown in Figures 7C and 7D respectively. Again, the aggregating strain possesses an A-layer while the non-aggregating one does not.

The A-layer appears to be firmly attached to the cell wall. Even after a cell lyses the outer membrane and its associated A-layer can remain intact and attached to cells (white arrows, Figure 7C). In another lysed cell of strain SIL-74 (Figure 8B) the A-layer appears to be present in most all areas where the OM layer is intact.

Figure 7. Electron micrographs of ultrathin sections from four additional Aeromonas salmonicida strains. Scales = 100 nm.

A. Pelleted cells of aggregating strain EFDL-3.47. White arrows show spacing between opposing outer membranes caused by the presence of the additional layer (A). Note periodic staining of the A-layer.

B. Pelleted cells of nonaggregating strain ATCC 14174. White arrows show lack of spacing between outer membranes when no additional layer is present.

C. Strain SIL-74. Cell wall of a lysed cell attached to an intact cell (white arrows) by means of the additional layers (A).

D. Strain SIL-67. Nonaggregating strain lacking an additional layer.

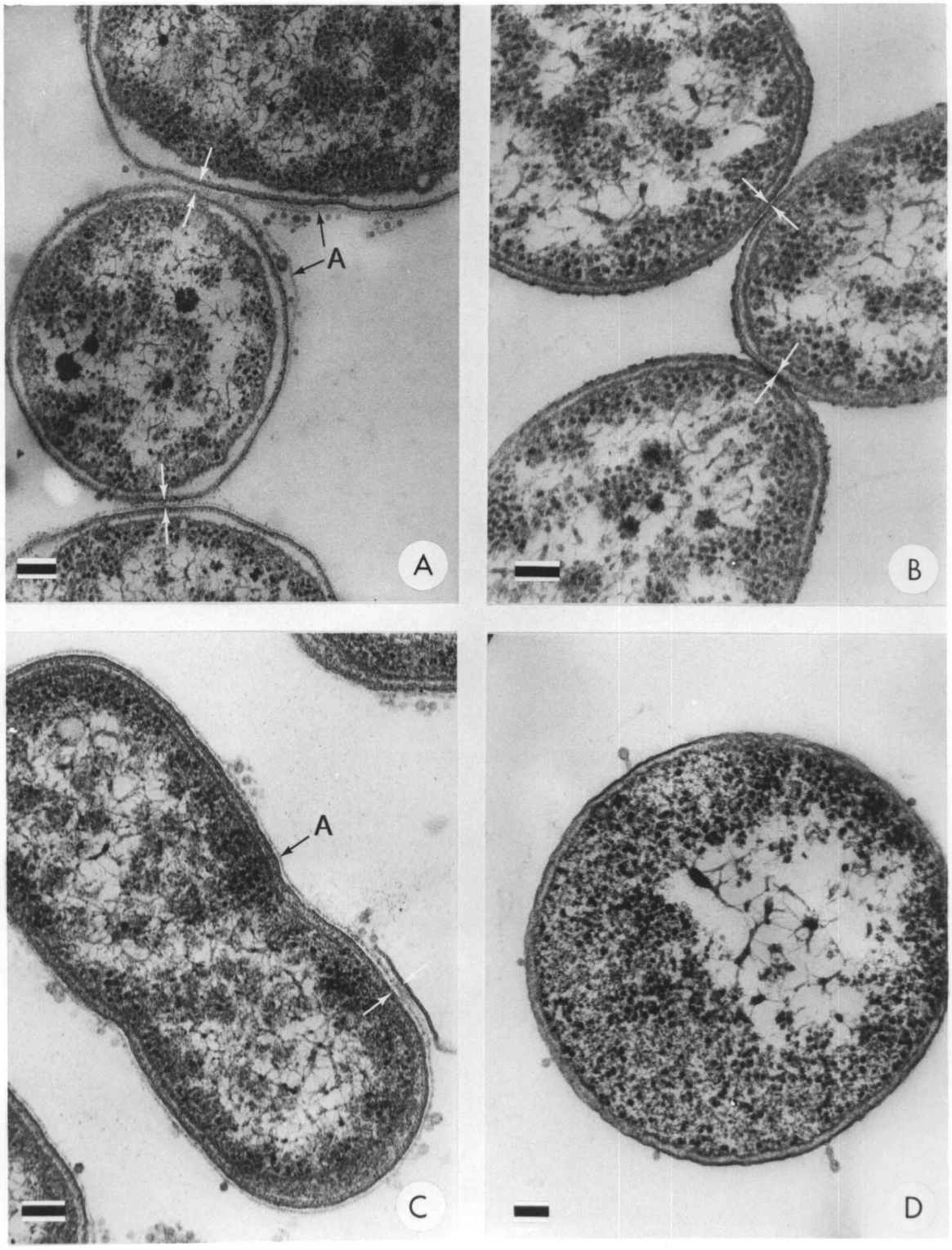
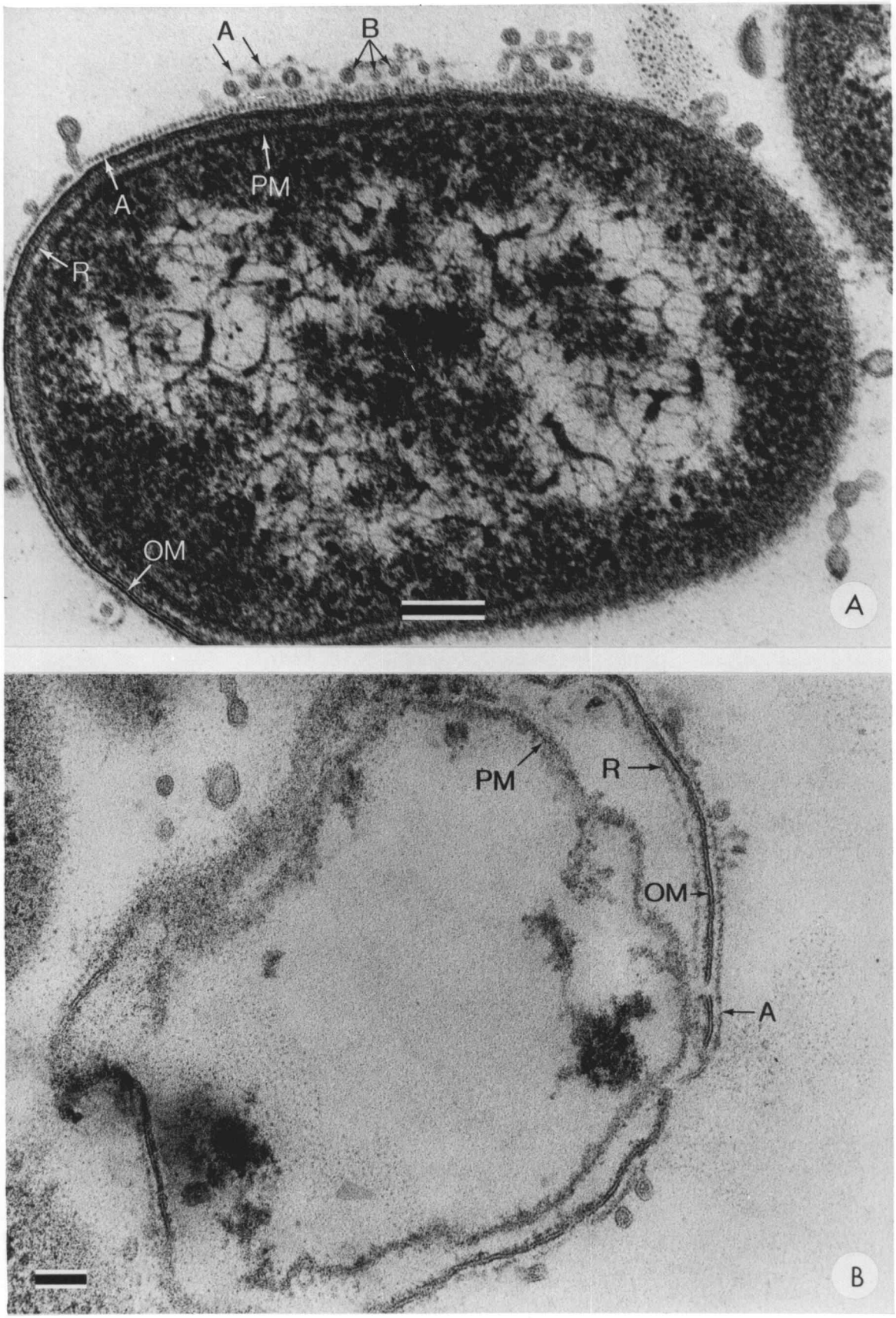

A
A
B
A
C
D

Figure 8. Two electron micrographs of ultrathin sections from Aeromonas salmonicida strain SIL-74.

A. Oblique section showing periodic staining (white A) of the additional layer. Other features: A(black), additional layer surrounding surface appendages; OM, outer membrane; R, rigid or peptidoglycan layer; PM, plasma membrane; B, surface appendages. Scale = 100 nm.

B. Section of a lysed cell or ghost with additional layer (A) still attached. Other features: OM, outer membrane; R, rigid layer; PM, plasma membrane. Scale = 50 nm.


A
B
A
PM
R
OM
A
PM
R
OM
A
B

When the A-layer is cut at a proper angle, it is interrupted and displays a periodic pattern. From an enlarged view of Figure 7A, the center-to-center spacing of this pattern for strain EFDL-3.47 measures 11-12 nm. In the obliquely cut cell of strain SIL-74 (Figure 8A) the periodic nature of the A-layer is more easily seen. In this view, the center-to-center spacing measures only 7 nm.

Negatively Stained Aggregating *A. salmonicida* Cells

Using the technique of negative staining, Buckmire and Murray (1970) showed for *Spirilum serpens* that the periodicity of the additional layer seen in thin sections was due to a hexagonal array of subunits. Following this example, cells of strain SS-70 were examined in a similar fashion (Figures 9, 10A, 10B).

The surfaces of the cells appear highly laced with surface appendages which can also be seen extending away from the cells in the form of small tubes. These structures are particularly evident in Figure 10B.

The arrows in Figure 9 point to numerous areas having a definite pattern. It is believed that these areas represent portions of the A-layer which have been detached from the cell during staining and air drying. The predominant pattern is a lattice with elements which lie in a single plane in close apposition. The center-to-center distance of the fibers is approximately 7nm

Figure 9. Electron micrograph of four Aeromonas salmonicida strain SS-70 cells negatively stained with potassium phosphotungstate (KPT). White arrows point to areas believed to be portions of additional layer; note the lattice-like periodic staining pattern.

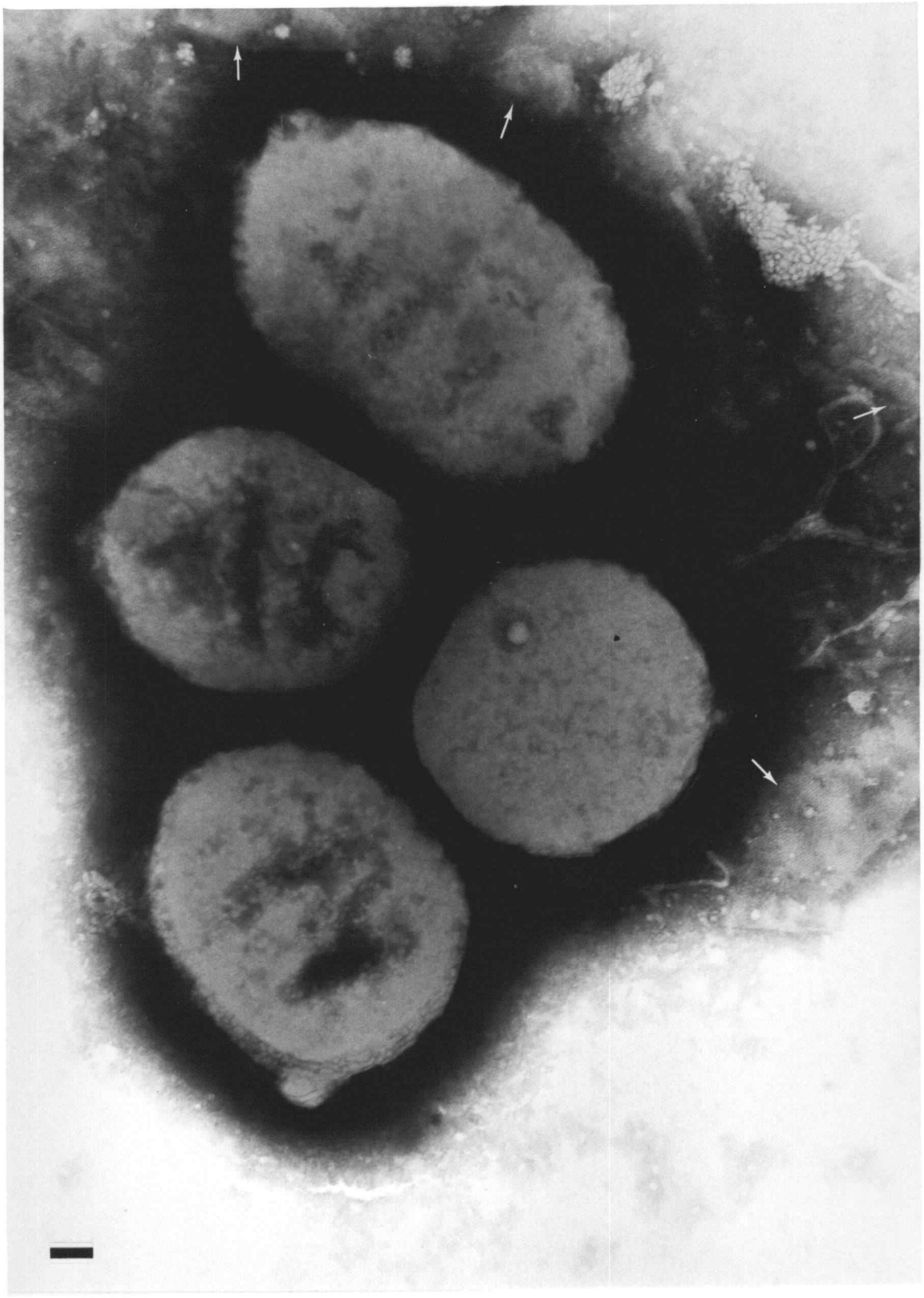
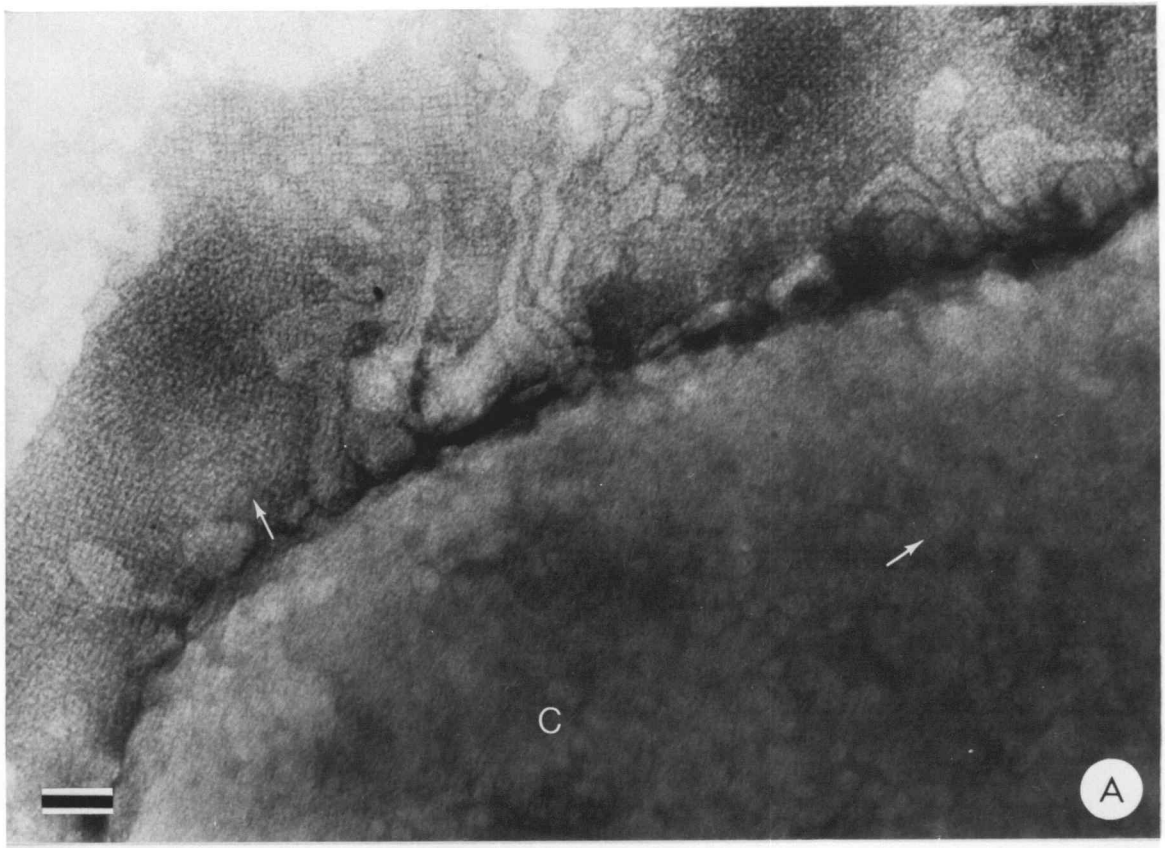
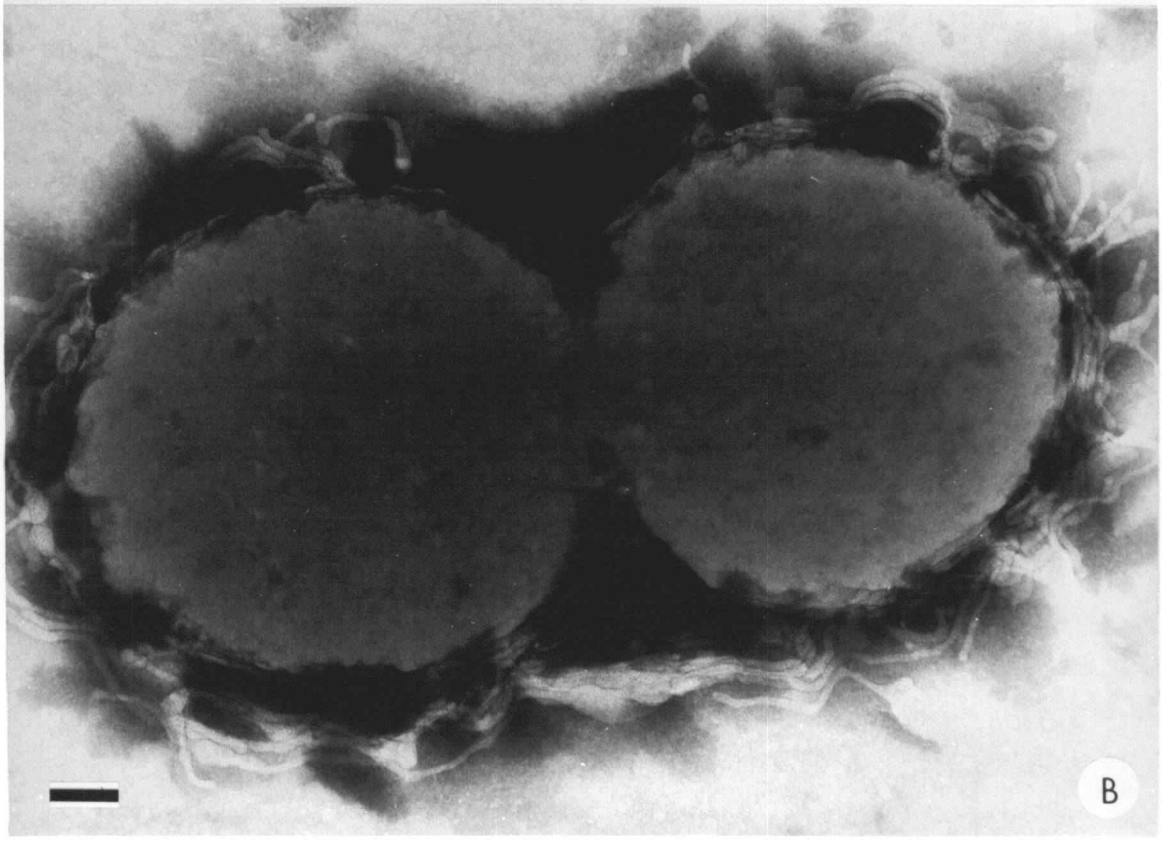


Figure 10. Two electron micrographs of negatively stained Aeromonas salmonicida strain SS-70 cells.

A. Tetragonal pattern (white arrows) both at the edge of and covering the cell surface (white C). Scale = 50 nm

B. Two adjoining cells wrapped with numerous tubular surface appendages. Scale = 100 nm .


C
A


B

(taken over 24 units). The elements have a width of 6 nm and have a 1 nm space separating them.

An additional pattern was observed in one instances. The regular design pictured in Figure 10A (white arrow) is a nearly square tetragonal array. The center-to-center spacing of 7 nm is the same as observed in the fibrous array. No patterned material was observed in negatively stained preparations of nonaggregating cells.

Mechanisms Involved in Aggregation Of the Organism

Originally, the aggregation of some strains of A. salmonicida was thought to be due to the presence of pili as was demonstrated in Neisseria gonorrhoeae studies by Swanson (1972). The results of ultrastructural studies, however, indicated that the A-layer is probably responsible for aggregation.

At least four possible mechanisms can be listed which might account for the observed cell-to-cell adhesion.

(1) Incomplete separation of cell wall following division (as with Staphylococcus aureus; Davis et al. 1973).

(2) Formation of "hydrophobic patches" on the cell surface by mutual dehydration (Harris and Mitchell 1973).

(3) Neutralization of repulsive surface charges (Matijevic and Allen 1969).

(4) Specific chemical interactions between the surfaces, such as complex formation or hydrogen bonding.

It was observed in earlier stages of this work that aggregates could be separated into single cells by washing three or more times with 1.39% ethanol (physiologic osmolarity) in distilled water; or in 10 mM HEPES-NH_4OH buffer. This observation eliminated the first mechanism listed. Further studies (below) suggested that the aggregation was freely reversible and was probably dependent upon the ionic nature of the suspending medium.

Four salt solutions at five concentrations were tested for their ability to promote aggregation of ethanol-washed (1.39%) cells of strains SS-70 and SIL-67 (Table 7). Solutions of NaCl and Na_2SO_4 were chosen because they represented a monovalent cation with a monovalent and divalent anion respectively. Similarly, $MgCl_2$ and $MgSO_4$ solutions were picked to represent a divalent cation combined with a monovalent and divalent anion.

Cell suspensions of the aggregating strain SS-70 exhibited macroscopic signs of aggregation in the presence of both magnesium salts at Mg^{++} levels as low as 10 mN. Heavy floculation occurred at levels of 30 mN in $MgSO_4$ solutions and 20 mN in those containing $MgCl_2$. No aggregation was observed in NaCl solutions at con-

Table 7. The effects of monovalent and divalent cations and anions on aggregation[a] of Aeromonas salmonicida strains[b] SS-70 and SIL-67

Salt[c]	Strain	Concentration (mN) 52.5	42.5	32.5	22.5	12.5
NaCl	SS-70	-	-	-	-	-
	SIL-67	-	-	-	-	-
$MgCl_2$	SS-70	4	4	4	4	2
	SIL-67	-	-	-	-	-
Na_2SO_4	SS-70	3	2	-	-	-
	SIL-67	-	-	-	-	-
$MgSO_4$	SS-70	4	4	4	2	1
	SIL-67	-	-	-	-	-

[a]Degree of aggregation rated 1 through 4:
1 = slight bottom floc, no clearing in tube
2 = moderate bottom floc, moderate clearing
3 = heavy bottom floc, slight remaining turbidity
4 = heavy bottom floc, clear supernatant

[b]Prewashed in isotonic (1.39%) ethanol to dissociate natural aggregates

[c]Salt solutions made isotonic (0.300 ± 0.005 osmolar) with ethanol

centrations up to 50 mN (highest tested). A solution of 40 mN KCl also failed to induce aggregation. Some aggregation was observed in 50 and 40 nM solutions of Na_2SO_4; however, the manufacturer's (Allied Chemical Co.) analysis of this salt indicated that contaminating multivalent cations were present in excess of 10 mN in 50 mN solutions. No macroscopic or microscopic aggregation was observed for cells of the nonaggregating strain SIL-67 in any of the solutions used.

Since mechanisms 2 and 3 are affected primarily by ionic strength and not by the nature of the charged species it appears doubtful that these are the primary mechanisms involved in aggregation. Solutions of NaCl or Na_2SO_4 should have been about as effective as the magnesium salts. These mechanisms might be more important in stabilizing the aggregates once they are formed.

To demonstrate that Mg^{++} was not the only multivalent cation which could provoke aggregation, disassociated cells were suspended in 40 mN solutions (chloride salts) of: divalent cations; Ca^{++}, Co^{++}, Cu^{++}, Cd^{++}: trivalent cations; Al^{+++}, La^{+++}: and the quatravalent cation Sn^{++++}. All of these cations were found to induce aggregation comparable to that obtained with Mg^{++}. From these results, it is inferred that multivalent cations form chemical complexes with sites on the A-layer of two cells. This, in turn, results in extensive cross-bridging and the observed aggregation.

Ethylenediaminetetracetic acid (EDTA), adjusted to pH 7.4 (NaOH) and added to aggregates at levels from 0.003 to 0.015 M, was ineffective in reducing the degree of clumping. This suggests that the divalent cations are sequestered in areas of cell-to-cell contact.

Topology of Cell Aggregates

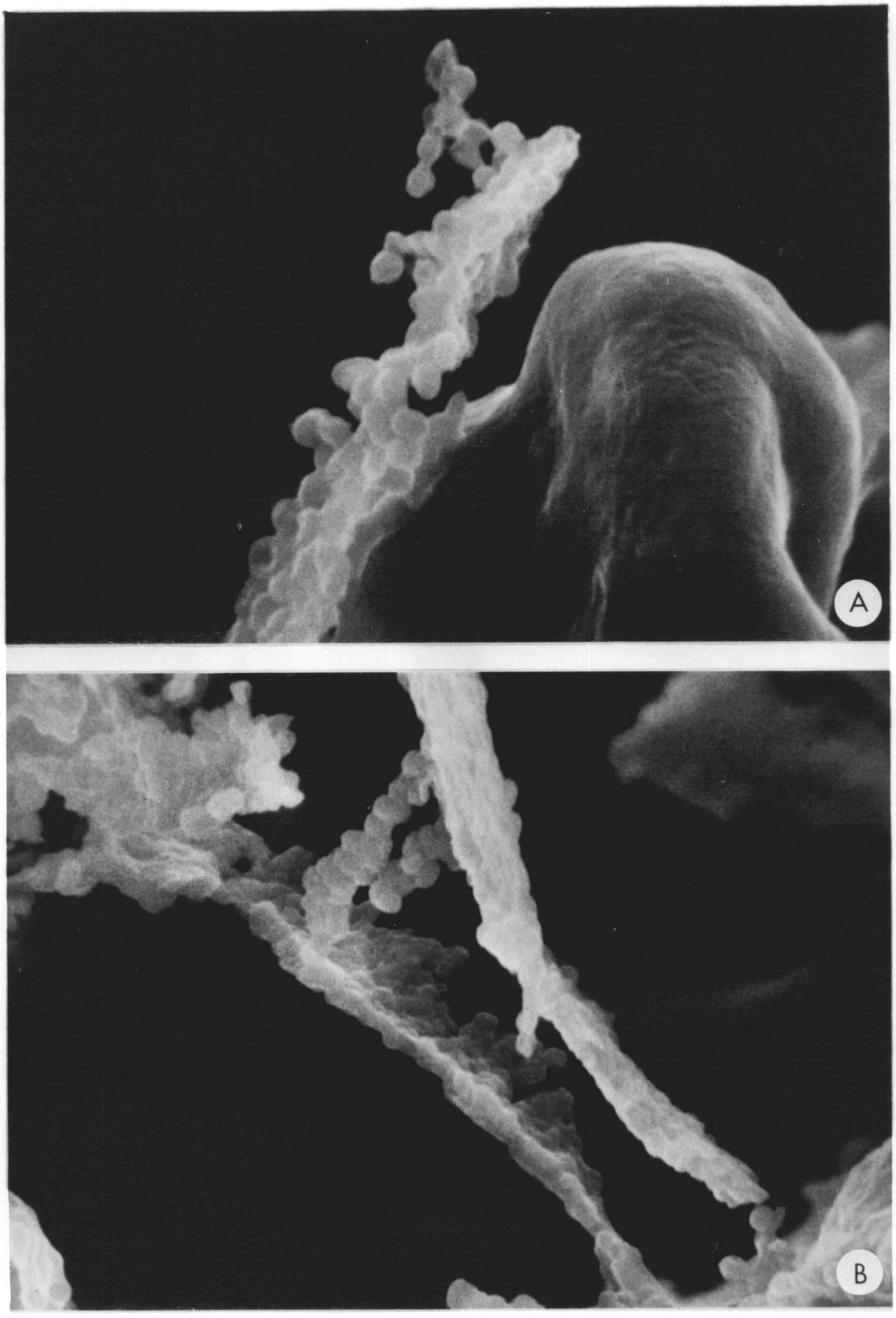
It was observed that aggregating cells in broth cultures tended to associate in irregular chains, in planar arrays, and in random clusters. For this study, large aggregates of cells were formed by adding 50 mM Mg^{++} to single-cell suspensions (dis-aggregated suspensions) of strain SS-70 prepared in 10 mM HEPES buffer.

Aggregates formed in this latter manner were examined by scanning electron microscopy (SEM). Some aggregates were composed of randomly associated cells and cells in short chains (Figure 11A). More commonly, the cells were found in chains and planar arrays (Figure 11B), and characteristically contained several hundred cells. These arrays were most commonly one or two cells thick and appeared to extend from a chain cross-bridge at one side of the structure (Figure 11B). In these SEM photographs most of the cells appear tightly packed and adherent to several adjacent cells. This suggests that a substantial portion of the cell surface can be involved in the formation of aggregates as shown previously in ultrathin sections (Figure 7A).

Figure 11. Two scanning electron micrographs detailing the topology of Aeromonas salmonicida strain SS-70 cell aggregates formed in the presence of 50 mM Mg^{++}.

A. Bacteria randomly arranged or in short chains (14,700X).

B. Planar arrays of cells connected by rigid chains (10,280X).


A
B

Virulence of *Aeromonas salmonicida* Strains: Correlation with Ultrastructure and Aggregation

Paterson (1972) first observed that strains of *A. salmonicida* which aggregated were more pathogenic than those which had lost this characteristic. In subsequent studies, 12 strains obtained from recent epizootics in Oregon, Washington, Maryland, West Virginia and Ohio were all found to form aggregates. Four strains from this group were picked for virulence testing: (1) SS-70, (2) WFDL-S, (3) EFDL-3.47, and (4) EFDL-3.58. In addition, four nonaggregating strains were also assessed for their pathogenic potential. Three of these were isolates which had been carried on culture medium for several years (strains ATCC 14174, WFDL-R, and SIL-67) but one, Sm^d-REV, was obtained from the aggregating strain SS-70 as a result of two natural mutations (see Materials and Methods). In addition, EM thin sections of cells from all eight isolates were prepared and examined for the presence of the additional layer.

It was found that, without exception, strains which aggregated possessed the additional layer, while those which did not aggregate were missing it (Table 8). The nonaggregating strains, including the mutant of strain SS-70, all had LD_{50} values in excess of 10^8 CFU/fish and were considered avirulent.

Table 8. Correlation of aggregation, additional layer, and virulence (LD_{50}) in selected Aeromonas salmonicida strains

Strain	Aggregation	Additional layer	LD_{50} (CFU/fish)
ATCC 14174	-	-	$> 1.0 \times 10^8$
WFDL-R	-	-	$> 1.0 \times 10^8$
SIL-67	-	-	$> 1.0 \times 10^8$
Sm^d-REV	-	-	$> 1.0 \times 10^8$
SS-70	+	+	8.8×10^0
WFDL-S	+	+	6.4×10^1
EFDL-3.47	+	+	8.2×10^3
EFDL-3.58	+	+	$> 1.0 \times 10^7$

Three of the four aggregating strains proved to be highly virulent with LD_{50} values (in CFU/fish) of 8.8×10^{0} (SS-70), 6.4×10^{1} (WFDL-S) and 8.2×10^{3} (EFDL-3.47). The fourth strain, EFDL-3.58, proved to be the exception. This isolate was considered avirulent because it had an LD_{50} in excess of 10^{7} CFU/fish.

Attachment of *Aeromonas salmonicida* to Fish and Other Animal Cells

Because of the high degree of correlation between aggregation and virulence, it was of interest to determine if aggregating cells attached to tissue cells as they adhered to each other.

Ellen and Gibbons (1972) suggest that M-protein-mediated adherence of *Streptococcus pyogenes* to epithelial surfaces is a prerequisite for virulence. Pili mediated attachment of *Shigella spp.*, *E. coli* and *N. gonorrhoeae* to epithelial cells has also been strongly correlated with pathogenicity of these organisms (Duguoid and Gillies 1957; Jones and Rutter 1972; Swanson 1972).

Preliminary experiments indicated that nonaggregating strains showed no particular adhesion to human, rabbit, chicken or fish leukocytes or erythrocytes, nor did they attach to the mucosal epithelium of fish intestine. Aggregating cells, however, demonstrably adhered to all the cell types except the erythrocytes.

A model system employing chinook salmon embryo derived tissue culture cells (CHSE-214) was adopted for further investigations of bacterial adhesion to tissue cells. The ability of the bacterium to attach to human cancer cells (HEP-2) was also determined.

The predilection of the aggregating cells (SS-70) as compared to nonaggregating cells (Sm^d-REV) for both CHSE-214 and HEP-2 cells is clearly evident from the data presented in Table 9. An average of 109 $\pm$ 36 SS-70 cells attached per CHSE-214 cell while only 3 $\pm$ 3 strain Sm^d-REV cells adhered. In this assay, 36 times as many aggregating as nonaggregating bacteria attached to the fish cells. Similar results were obtained with HEP-2 cells where the binding ratio was 34:1 (34 $\pm$ 22 to 1 $\pm$ 2). Less bacteria attached to HEP-2 cells than to fish cells presumably due to the smaller cell size of the HEP-2 line. No difference, however, was observed in the bacterial binding ratios for HEP-2 and CHSE-214, indicating that the bacteria have similar adhesive affinities for both human and fish cells.

Although light microscopy was adequate for quantitation of the bacterial adherence, scanning electron microscopy provided a detailed view of how and where the bacteria attached. Unexposed control CHSE-214 cells appeared firmly attached to the glass surface and did not exhibit noticable distortion as a result of critical-point drying. Numerous filipodia as small as 100 nm can easily be seen extending from the cell body to the glass coverslip.

Table 9. Attachment of Aeromonas salmonicida SS-70 and Sm^d-REV to chinook salmon embryo (CHSE-214) and human cancer (HEP-2) tissue culture cells

Strain	Mean (± S.E.) number of bacteria per tissue cell[a]	
	CHSE-214	HEP-2
SS-70	109 ± 36	34 ± 22
Sm^d-REV	3 ± 3	1 ± 2

[a]As determined from Giemsa-stained preparations

Monolayer cells (CHSE-214) exposed to suspensions of cells of nonaggregating strain Sm^d-REV appear similar to those in control preparations (Figure 12B). Only one or two bacteria can be seen adhering to tissue cells or their filipodia. A few bacteria are seen adhering to the glass between the tissue cells.

In sharp contrast, Figure 12C depicts how the aggregating bacteria (SS-70) adhere in large numbers to both the body and filipodia of the cells. Only one clump of two bacteria is associated with the glass background, while all other bacteria are adherent to the cell surfaces. At higher magnification, the bacteria in the lower right of Figure 12C can more clearly be seen in close association with the tissue cell (Figure 13A).

In some instances (Figures 13B, C) indistinct bacterial-sized raised areas can be seen. It can not be determined from these SEM pictures whether these are bacteria. Further study using transmission electron microscopy would be required to determine if these are bacteria which have penetrated the plasma membrane (or are embedded in glycocalyx-like material), whether they are artifacts of preparation, or whether they are mitochondria or inclusion bodies.

Figure 12. Scanning electron micrographs of chinook salmon embryo derived tissue culture cells (CHSE-214) growing on a glass surface. (2,930X).

A. Untreated cell with many filipodia.

B. Cells exposed to nonaggregating Aeromonas salmonicida (Sm^d-REV). Only a few bacteria can be seen and are primarily attached to the glass background.

C. Cells exposed to aggregating Aeromonas salmonicida (SS-70). Numerous bacteria (greater than 100/cell) of this strain attach to the cells and their filipodia while few attach to the glass.

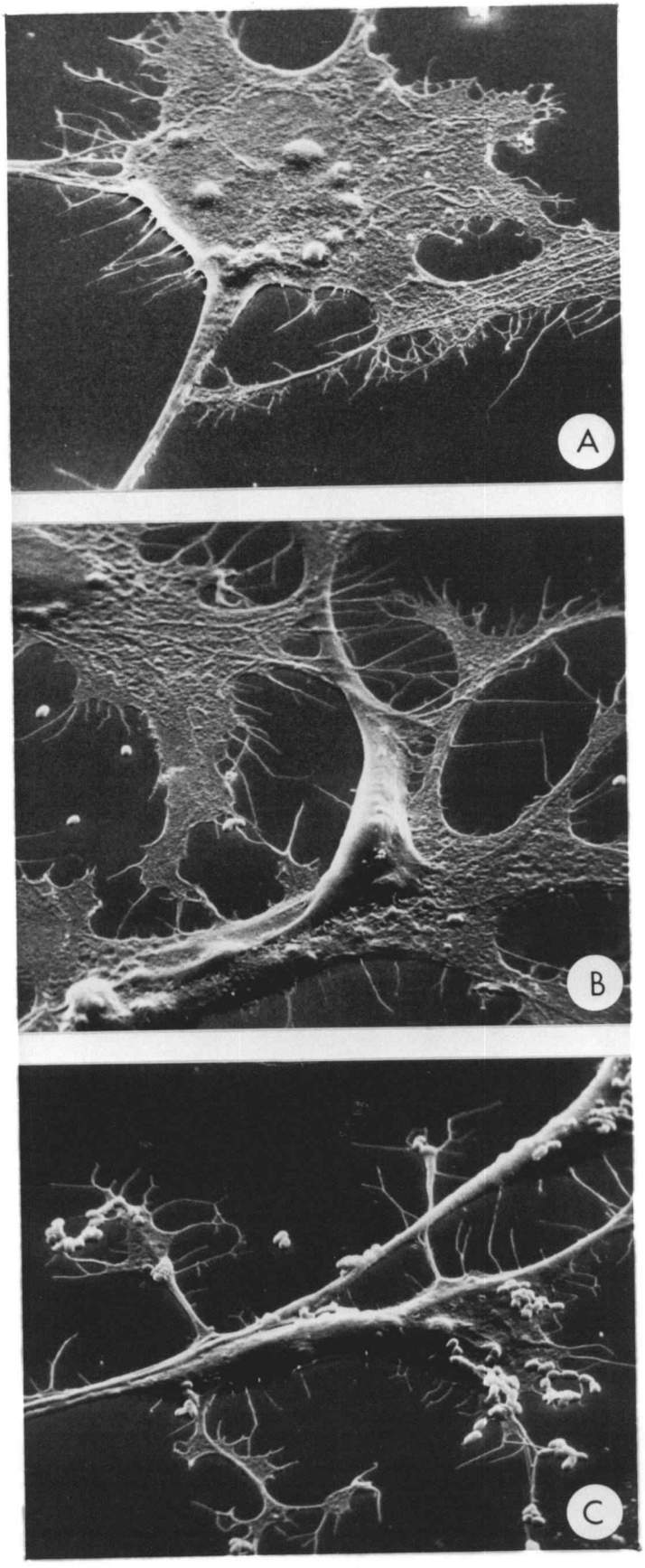
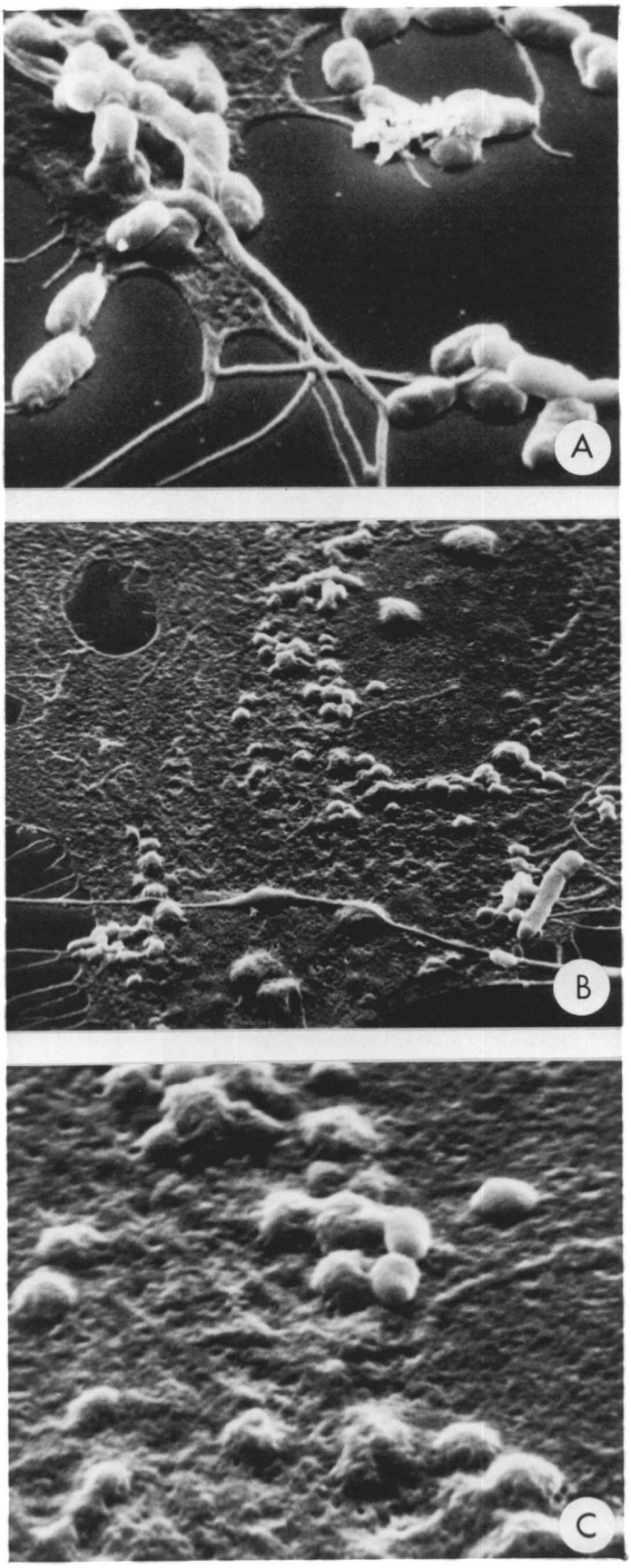

A
B
C

Figure 13. Scanning electron micrographs of CHSE-214 with strain SS-70 cells attached.

A. An enlarged view of the lower right-hand corner of Figure 12C. Bacteria are attached to the fine (100 nm) cell filipodia (14,650X).

B. Nuclear and perinuclear region of a flat cell with numerous bacterial sized "bumps" in the perinuclear region. (2,930X).

C. Enlarged view of the center section of Figure 13B (above). Bacterial sized "bumps" appear in the perinuclear region. (14,650X).


A
B
C

The Effect of Thermal and Hypoxial Stress

The role of environmental stressors in lowering a fish's resistance to disease has been often discussed but little studied (Sneiszko 1974). At Siletz Salmon Hatchery where furunculosis is a yearly occurrence, water temperatures in early summer can rise rapidly and fluctuate diurnally as much as 8°C. Temperature change provides a direct stress and also causes a large fluctuation in the concentration of dissolved oxygen (D.O.).

In preliminary laboratory exposures of juvenile coho salmon to virulent A. salmonicida, it was shown that little or no experimental furunculosis could be induced without some form of stress. The effects of hypoxial (D.O. = 4.0 for 4 min) and thermal (Δ T = 6°C) stress on A. salmonicida induced mortality in immunized and nonimmunized fish is shown in Table 10. Groups 1 and 2 were held at 18°C for 48 h before being hypoxially stressed and exposed to the disease organism. Fish in groups 3 and 4 were moved from 12° to 18°C water just prior to hypoxial stress and exposure. These types of stress are similar to what might be expected in a number of coho salmon hatcheries.

Unimmunized coho salmon experienced a mean mortality of 14% (group 2) when lack of oxygen was the only stress. They suffered a significantly higher ($p = 0.05$) mean mortality of 40% (group 4) when this stress was combined with a 6°C change in water temperature just prior to exposure. In contrast, those fish which

Table 10. Effect of thermal (T) and hypoxial (H) stress on furunculosis mortality[a] in actively immunized[b] and unimmunized juvenile coho salmon challenged in the laboratory

Group	Stressor(s)	Fractional mortality due to furunculosis		Mortality (%)		Mean mortality of two replicates[d] (%) (Arcsine means)
		Replicate 1	Replicate 2	Replicate 1	Replicate 2	
Immunized	H	1/25	2/25	4	8	6 (14)
Unimmunized	H	2/25	5/25	8	20	14 (22)
Immunized	H + T	2/25	2/25	8	8	8 (16)
Unimmunized	H + T	8/25	12/25	32	48	40 (39)

[a]Percent mortality was arcsine transformed before use in oneway analysis of variance. Least significant differences (LSD) were computed to be: $LSD_{(0.95,\ 4df)} = 15$; $LSD_{(0.99,\ 4df)} = 27$

[b]Injected i.p. with strain SS-70 in CFA

[c]Strain SS-70 at 1.0×10^6 cells/ml

[d]Groups 1, 2, and 3 are significantly different from group 4 at $p = 0.05$ but not at $p = 0.01$

were actively immunized and possessed high anti- A. salmonicida agglutinin titers (groups 1 and 3) had only a 6 to 8% mortality under both types of stress. This would indicate that even under conditions of severe stress, actively immunized juvenile salmon can resist or overcome furunculosis. A reduction from 40 to 8% mortality represents a significant protection ($p = 0.05$) due to active immunization.

Relationship Between Challenge Dose and Response Time

Characteristically, when warm-blooded animals are injected with different concentrations of a pathogenic bacterium, they die (or respond with symptoms) as a function of the dose (Meynell and Meynell 1965). It has been observed that the cumulative percent mortality (probit scale) is directly proportional to response time (logarithmic scale) if the population is statistically normal. It had not been demonstrated that these relationships hold when fish are exposed to different concentrations of virulent A. salmonicida in water.

The relationship between cumulative mortality (probit scale) and the time from exposure to death (logarithmic scale) for juvenile coho salmon exposed to four different concentrations of A. salmonicida can be seen in Figure 14. The response curves show a linear trend at all doses. Any deviation from linearity can probably be explained by the fact that it is the internal dosage of the bacterium which

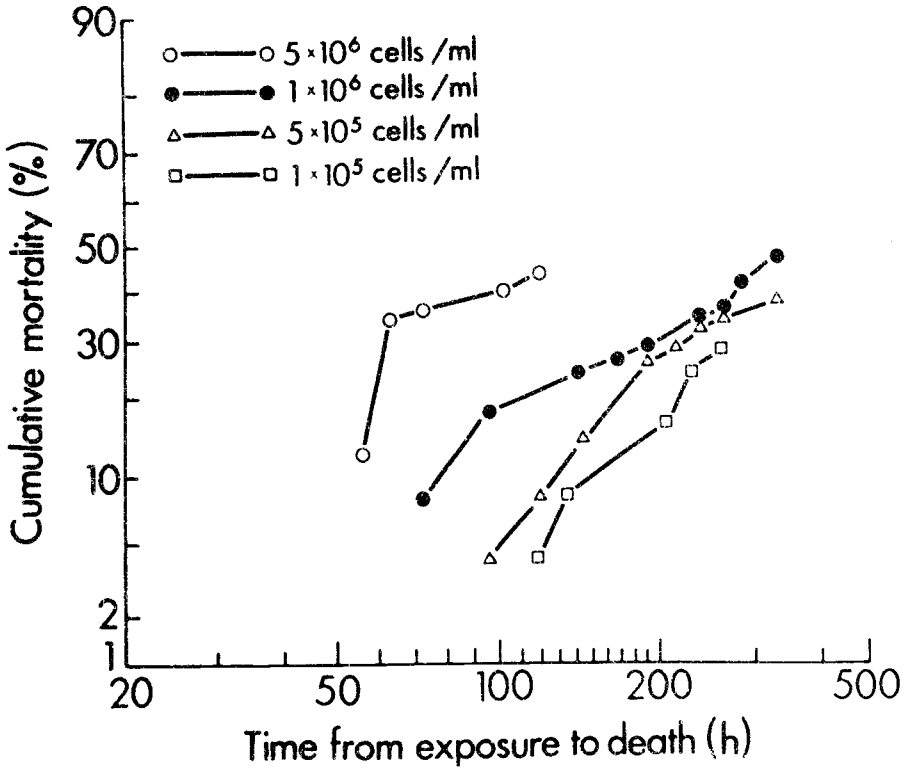


Figure 14. Effect of dose on the death rate of juvenile coho salmon exposed to Aeromonas salmonicida strain SS-70.

is critical, not the external concentration. Parameters such as the fish's susceptibility to stress, its surface area and the integrity of its mucous membranes may play a role in the number of bacteria which are able to invade the host. The time at which the first death occurs ranges from 56 h at the highest dose (5.0 X 10^6 cells/ml) to 118 h at the lowest dose (1.0 X 10^5 cells/ml).

From this data, the RT_{50} was calculated and expressed as a function of the log-dose (Figure 15). The RT_{50} is defined as the time when the median percent mortality is achieved. At the three highest dosages shown in this figure, the relationship is linear. At the lowest concentration, the curve becomes truncated, presumably because only the most susceptible fish are infected (Meynell and Meynell 1965).

In this study, the fish were transferred from 12 to 18°C water 5 h before they were exposed to A. salmonicida. According to Wedymeyer (1973) maximum physiological changes in fish occur 5 h after they had been subjected to a rise in temperature (thermal stress). Therefore, the dose-response curves reflect the susceptibility of stressed fish and do not apply to fish under nonstressed conditions.

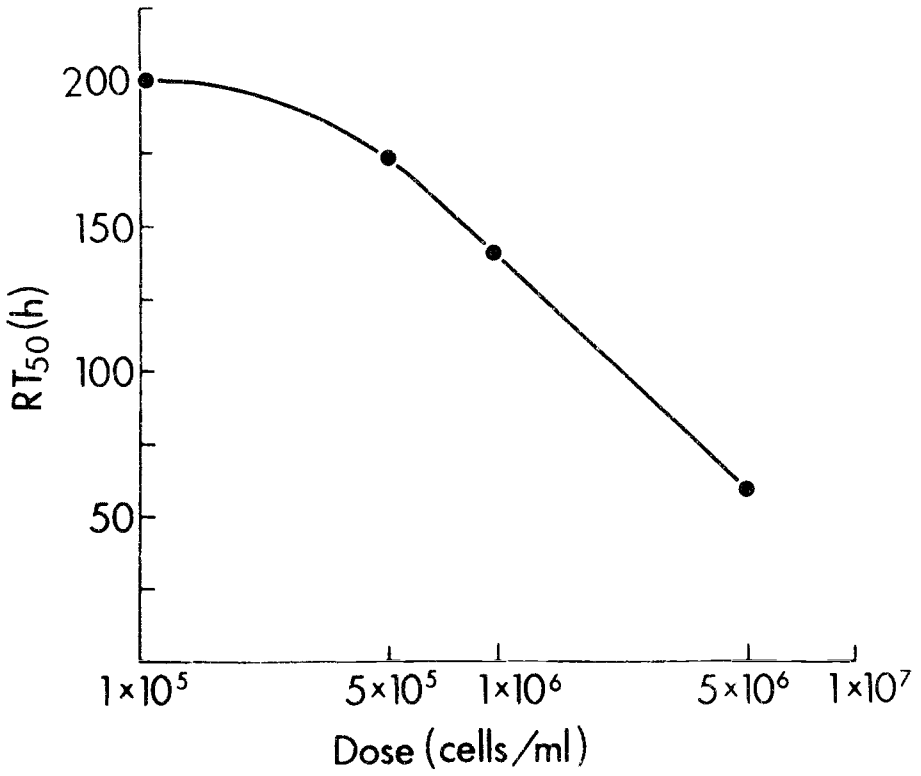


Figure 15. Effect of dose on the median time from exposure to death (RT_{50}) of juvenile coho salmon exposed to Aeromonas salmonicida strain SS-70.

Active and Passive Immunization of Juvenile Coho Salmon

Spence et al. (1965) showed that juvenile coho salmon passively immunized with rainbow trout anti- A. salmonicida serum, were significantly protected when challenged with the virulent strain used for the initial immunization. It has not been demonstrated that hyperimmune serum from juvenile fish is protective when administered to juvenile fish of the same species. Additionally, there has been no information on the protection afforded by antibodies produced to a nonaggregating (avirulent) strain of A. salmonicida.

Using the laboratory challenge procedure, several experimental groups of juvenile salmon were exposed 1.0×10^6 A. salmonicida cells (SS-70) for 15 min (Table 11). Juvenile coho salmon which received only normal juvenile coho salmon serum experienced a mortality averaging 42% (group 4). Actively immunized juveniles (group 3) administered the same serum had only a 4% mortality. These results were similar to those observed in previous challenge exposures and again pointed out the significant ($p = 0.01$) protective effect of active immunization.

Hyperimmune juvenile coho salmon serum produced against the challenge (virulent) strain reduced the mortality to 16% (significant at $p = 0.05$) even though only 2,040 agglutinating units were given intraperitoneally (group 1). A similar number of agglutinating units of the serum produced against strain SIL-67 (avirulent)

Table 11. Reduction of furunculosis mortality[a] by active and passive immunization of juvenile coho salmon challenged[b] in the laboratory

Group	Serum (agglutinating units)	Fractional mortality due to furunculosis		Mortality (%)		Mean mortality of two replicates[d] (%) (Arcsine means)
		Replicate 1	Replicate 2	Replicate 1	Replicate 2	
Control	Normal (< 4)	10/25	11/25	40	44	42 (40)
Active[c]	Normal (< 4)	1/25	1/25	4	4	4 (12)
Passive	Anti-SS-70 (2,040)	2/25	6/25	8	24	16 (23)
Passive	Anti-SIL-67 (2,040)	9/25	10/25	36	40	38 (38)

[a] Percent mortality was arcsine transformed before use in one-way analysis of variance. Least significant differences (LSD) were computed to be: $LSD_{(0.95,\ 4df)} = 13$; $LSD_{(0.99,\ 4df)} = 23$. Differences between arcsine means are contrasted to LSD values

[b] Strain SS-70 at 1.0×10^6 cells/ml

[c] This group was actively immunized with strain SS-70 in CFA and had a mean reciprocal agglutinin titer of 20,480

[d] Groups 2 and 3 are significantly different than control group 1 at $p = 0.01$ and $p = 0.05$, respectively

afforded no protection (group 2). It was not determined whether active immunization with this avirulent strain confers protection from furunculosis.

Antigenic Comparison of *Aeromonas salmonicida* Strains

Immunodiffusion Analysis

Results of the passive immunization experiments indicated that antibodies produced in fish against strain SS-70 were more protective than those produced against strain SIL-67. In an attempt to delineate the differences in these two strains which would account for the observed difference in protection, an antigenic comparison of the strains was made. Rabbit antiserums against the two strains were used in these immunodiffusion analyses. In all cases, the antiserum was placed in the larger center well and antigens were placed in each of two opposing wells to assure that the results were reproducible. The coding used is as follows:

Antiserums:

A - Rabbit anti-SS-70 (aggregating)
N - Rabbit anti-SIL-67 (nonaggregating)

Antigens:

A - SS-70 cells sonically disrupted for 5 min
N - SIL-67 cells sonically disrupted for 5 min
AT - Trichloroacetic acid extract of SS-70 (10 mg/ml)
NT - Trichloroacetic acid extract of SIL-67 (10 mg/ml)

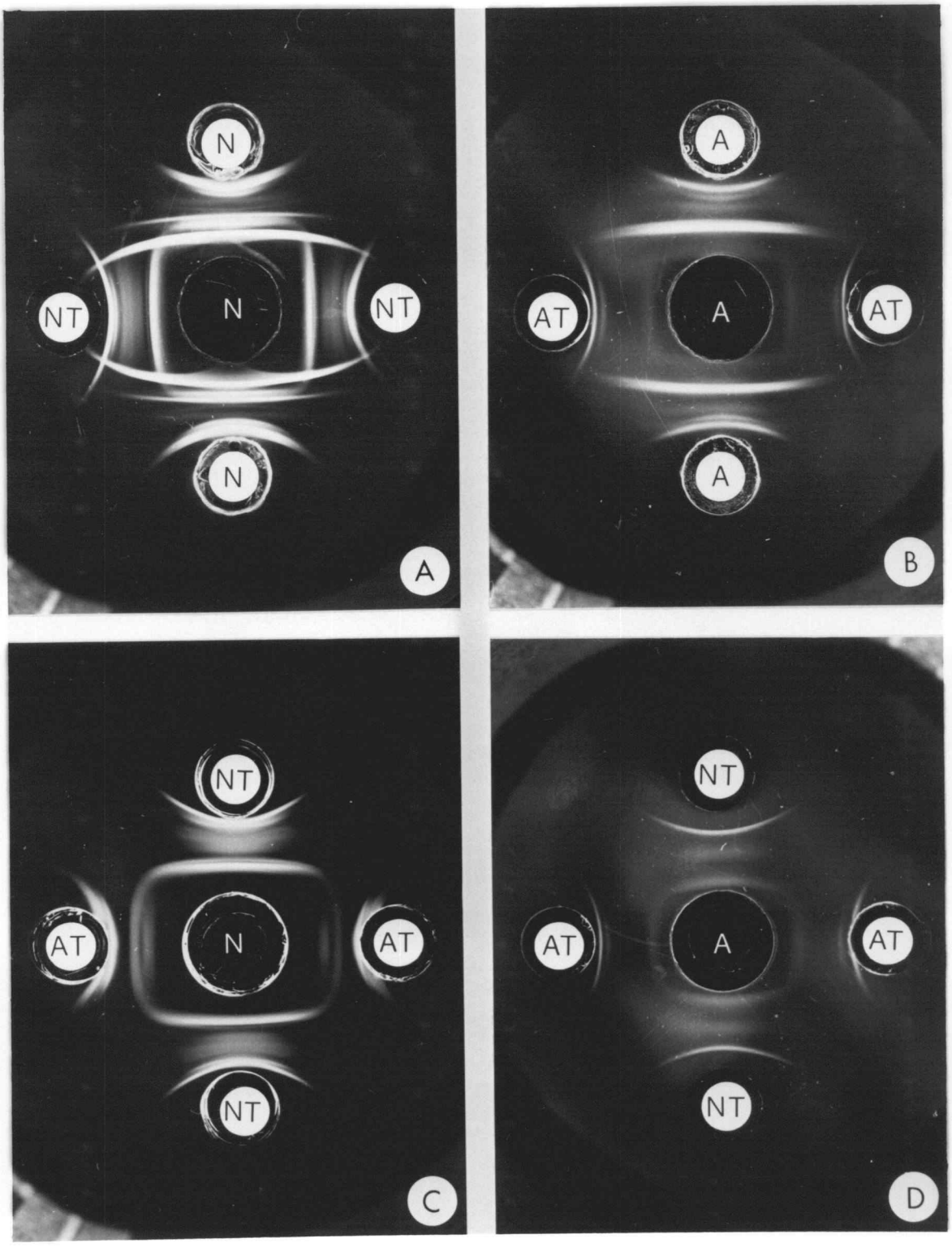
AP - Acetic acid degraded O-antigen polysaccharide from SS-70 (2 mg/ml)
NP - Acetic acid degraded O-antigen polysaccharide from SIL-67 (2 mg/ml)

A comparison of the TCA extract and the disrupted whole cell antigen from strain SIL-67 was made using the homologous antiserum (Figure 16A). The innermost TCA band merged with a band(s) from the "sonicate." It is likely that the outermost TCA bands are similar or identical to the outermost bands of the sonicate. They do not coalesce, however, due to their strongly convexted nature. At least two major bands were derived from the disrupted cell antigen that were not present in the TCA antigen. One of these bands is the major band seen in this comparison and probably represents an antigen with a molecular weight somewhat less than that of IgG (as determined by the concave nature of the band).

A similar comparison using the SS-70 antigens and antiserum was also made (Figure 16B). This antiserum was weaker, and yielded less distinct and intense bands. It again appears that the sonic treatment of the whole cells released substantial amounts of antigens similar to those extracted by TCA. A major band(s) not present in the TCA extract was also observed in this comparison.

The TCA extracted lipopolysaccharide-protein complexes from both SS-70 and SIL-67 were contrasted using antiserums to both

Figure 16. Gel immunodiffusion analyses. Rabbit antiserums in the center wells, Aeromonas salmonicida antigens in the outer wells: N(white), rabbit anti-SIL-67 serum; A(white), rabbit anti-SS-70 serum; N, 5 min sonicate of SIL-67; A, 5 min sonicate of SS-70; NT, TCA extract of SIL-67; AT, TCA extract of SS-70.

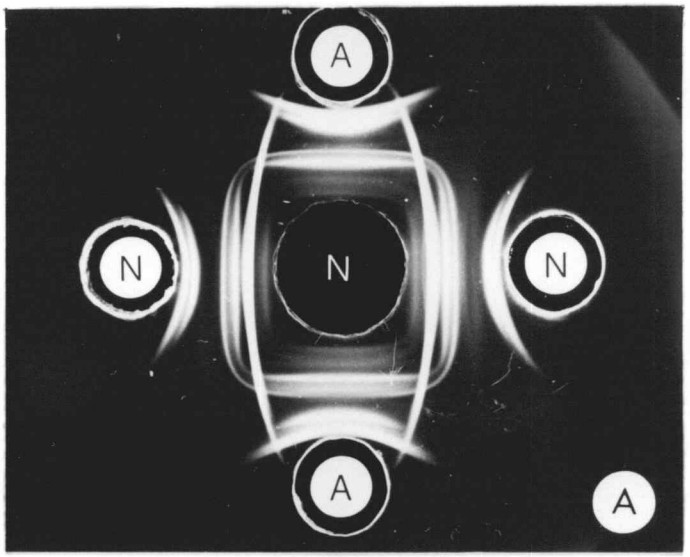

N
NT
N
NT
N
A
A
AT
A
AT
A
B
NT
AT
N
AT
NT
C
NT
AT
A
AT
NT
D

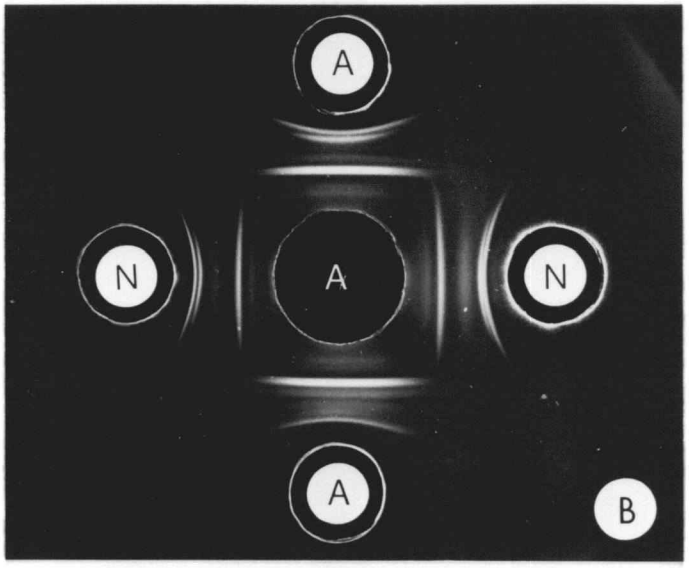
strains (Figure 16C, D). Antigen NT has numerous small diffuse bands which lie between the outer and inner sharp bands with the homologous antiserum (Figure 16C). With antiserum to SS-70 this area appears as a diffuse zone. These bands are probably degradation products of the higher molecular weight complex. Antigen AT has two separate inner bands (with its heterologous antiserum) which coalesced with the two inner bands of NT. Although minor differences are observable between the TCA extracts, no major dissimilarities were found.

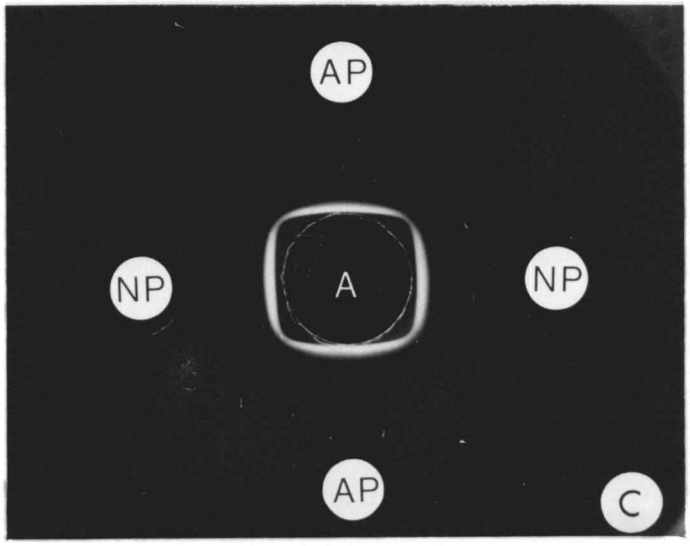
The broken-cell antigens were analysed for their differences as well, using both antiserums (Figure 17A, B). Instead of finding an extra band from SS-70 which might have corresponded to the additional layer substance, an additional band was found in the SIL-67 antigen. This antigen did not merge in any way with any of the SS-70 antigens (using anti-SIL-67 serum). No comparable band was present when the SIL-67 antigen was reacted with the heterologous antiserum, further indicating that the antigen was not present in strain SS-70.

In most gram negative bacteria, the O-antigen side chains are the predominant cell wall antigens and the major antigenic component of various lipopolysaccharide extracts (Nowotny et al. 1963). To determine if SIL-67 and SS-70 had the same O-antigen polysaccharide, an "acid degraded polysaccharide" was prepared

Figure 17. Gel immunodiffusion analyses. Rabbit antiserums in the center wells, Aeromonas salmonicida antigens in the outer wells: N(white), rabbit anti-SIL-67 serum; A(white), rabbit anti-SS-70 serum; N, 5 min sonicate of SIL-67; A, 5 min sonicate of SS-70; NP, acid degraded O-antigen polysaccharide from SIL-67; AP, acid degraded O-antigen polysaccharide from SS-70.


A
N
N
N
A
A


A
N
A
N
A
B


AP
NP
A
NP
AP
C

as described by Kabat and Mayer (1961). This type of preparation typically yields a uniform low molecular weight (20,000 to 25,000 daltons) polysaccharide essentially free of protein and lipid. It can be seen (Figure 17C) that the O-antigen polysaccharide from both the aggregating and nonaggregating strains are identical. The band shows no signs of spurring with either anti-SS-70 or with anti-SIL-67 (not pictured) and forms one continuous band of identity.

Cross Agglutination and Agglutinin Adsorption

It was felt that supplementary information could be gained about the role of the additional layer as an immunogen and/or antigen by using agglutination assays. In this study, three A. salmonicida strains were employed: (1) the wild type, virulent (aggregating) strain SS-70, (2) strain Sm^d-REV, the mutant of SS-70 lacking virulence and the additional layer (nonagglutinating), and (3) a true rough mutant lacking the additional layer and presumably the O-antigen side chains, strain GAL-E. Both juvenile coho salmon and rabbit serums produced against strain SS-70 were adsorbed extensively with all three strains.

Unadsorbed rabbit antiserum had a reciprocal agglutination titer of 1,280 with both SS-70 and Sm^d-REV antigens (Table 12). Coho salmon unadsorbed antiserum exhibited a four-fold higher titer with the nonaggregating mutant (Sm^d-REV) than it did with the wild type strain (10,240 vs 2,560) to which the antiserum

had been prepared. This difference may be due to a low ability of fish macroglobulin to penetrate the additional layer to the O-antigen side chains. This is substantiated by the fact that SS-70 could not adsorb out all anti-Sm^d-REV agglutinins (1,280 vs 40).

Strain Sm^d-REV on the other hand, removed all the agglutinins to both strains as shown in row 2 (Table 12). If the fish had produced antibodies to the additional layer, SS-70 should have been agglutinated to some degree with the Sm^d-REV-adsorbed antiserum. In contrast, rabbit antiserum had a titer of 640 following adsorption with Sm^d-REV. This suggests that rabbits can produce agglutinins to the additional layer while juvenile coho salmon cannot.

Strain SS-70 removes all the agglutinins to Sm^d-REV from rabbit serum but not from fish serum, as stated previously. The rabbit O-antigen agglutinins appear to readily penetrate the additional layer whereas the fish antibodies are less able to do so.

The rough GAL-E mutant, which should have only the core O-antigen polysaccharide and not the side chain polysaccharide, removed a significant amount of agglutinins in only one case. Rabbit serum adsorbed with GAL-E exhibited a four-fold reduction in titer to Sm^d-REV. Since the latter strain had no additional layer, the "anti-core" antibodies, which apparently aid in the agglutination reaction, were adsorbed out by GAL-E. Titers to

Table 12. Agglutination titers[a] of coho salmon and rabbit antiserums against Aeromonas salmonicida strains SS-70 and Sm^d-REV following adsorption with strains SS-70, Sm^d-REV or GAL-E

Adsorbing strain	Coho salmon anti-SS-70 serum		Rabbit anti-SS-70 serum	
	SS-70	Sm^d-REV	SS-70	Sm^d-REV
SS-70	< 40	1,280	< 40	< 40
Sm^d-REV	< 40	< 40	640	< 40
GAL-E	1,280 - 2,560	10,240	1,280	320
None	2,560	10,240	1,280	1,280

[a]Expressed as reciprocals

SS-70 were not diminished by adsorption with GAL-E because antibodies to the additional layer and to the side chains were both still present to affect agglutination.

Relative Structural Stability of *Aeromonas salmonicida* Strains

The structural stability of bacterial strains can greatly affect the nature and yield of vaccines made from them. A technique for vaccine preparation which is totally effective with one strain may produce a product of no value using another. This consideration is particularly important in *A. salmonicida* vaccine production.

To demonstrate this point, five methods were used to prepare vaccines from strains SS-70 and SIL-67. A chloroform-killed whole-cell vaccine was prepared by the method which Ross and Klontz (1965) used to make Enteric Redmouth (ERM) vaccine. Similarly killed cells were also co-precipitated with $Al(OH)_3$ for the second vaccine.

Formalin-killed whole cells have been found to be an effective bacterin in previous studies with *V. anguillarum* (Fryer et al. 1972, 1976); therefore, this type bacterin was included for evaluation. A vaccine was also prepared from formalin-killed cells using the $Al(OH)_3$ co-precipitation.

The FSA vaccine was prepared according to the patented procedure of Klontz (1970). This vaccine was comprised of the soluble portion of sonically disrupted cells, co-precipitated with $Al(OH)_3$. Untreated whole cells acted as a reference by which to gauge the effects of the preparation procedures.

Formalin killing resulted in a loss of only about 5% of the TOC and KN from the two vaccine strains (Table 13). However, when the cells of SIL-67 were further precipitated with $Al(OH)_3$, approximately 15% of the TOC and KN was lost; no such loss was observed with strain SS-70.

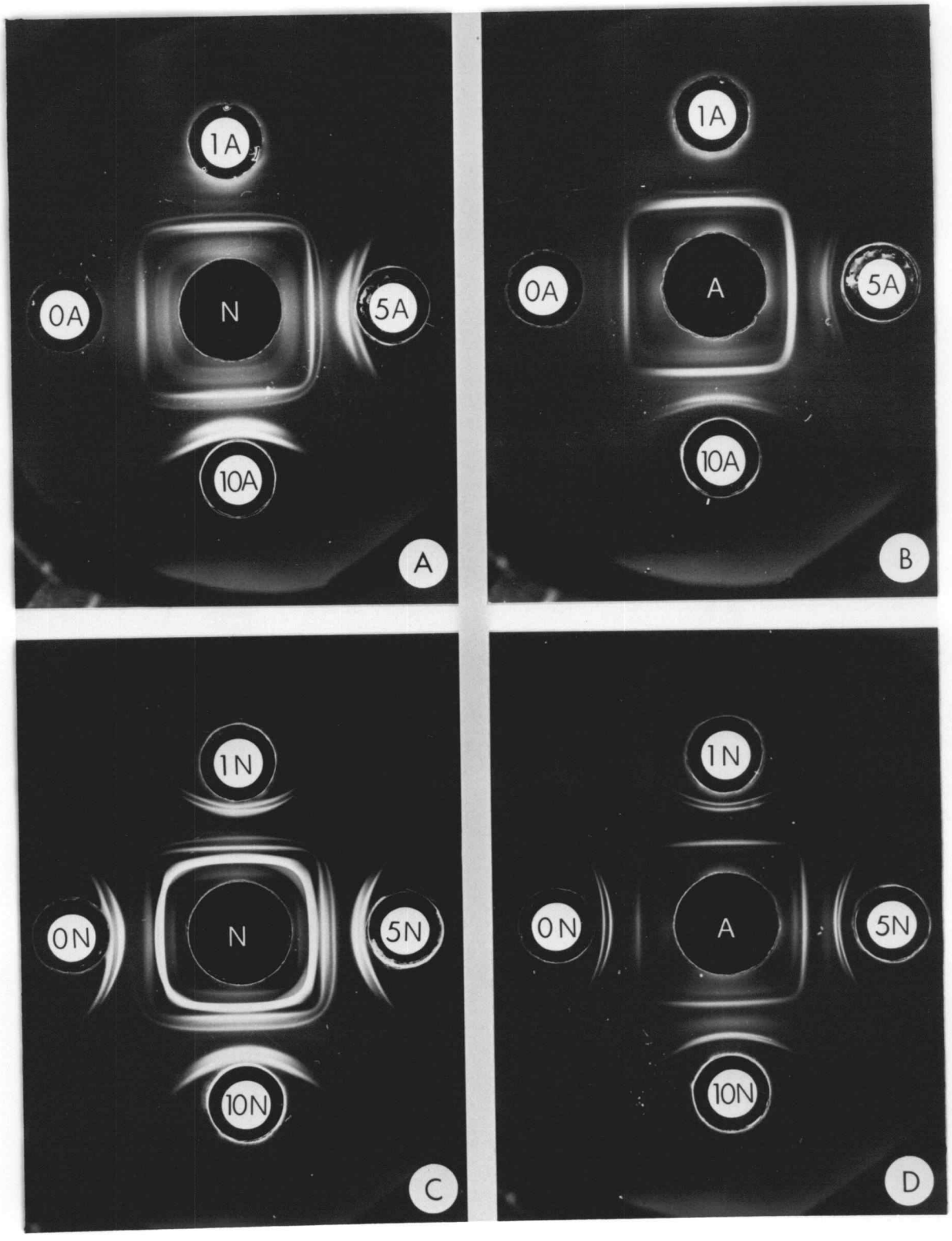
Treatment of the cells with chloroform induced more "leakage" than formalin treatment in both strains. The effect was more pronounced with strain SIL-67. Cells from this strain lost about 42% of their mass while those from strain SS-70 lost only about 20%. Aluminum hydroxide precipitation of SIL-67 cells resulted in no further loss of the insoluble fraction, but similar treatment of SS-70 cells resulted in an additional 7% loss of TOC and a 15% loss of KN.

In the preparation of FSA vaccine, the cell's structural stability is of utmost importance because only the portion of the cell solubilized during sonic disruption is available for precipitation with $Al(OH)_3$. The yield of $Al(OH)_3$ precipitable material from cells of strain SIL-67 (60%) was approximately twice

Table 13. Recovery of Kjeldahl nitrogen (KN) and total organic carbon (TOC) after preparation of five vaccines from bacterial suspensions of two Aeromonas salmonicida strains

Vaccine type	Vaccine strain			
	SIL-67		SS-70	
	percent		percent	
	KN	TOC	KN	TOC
Chloroform-killed whole cells	58.0	58.6	83.1	79.7
Chloroform-killed whole cells + $Al(OH)_3$	56.6	60.2	66.8	72.6
Formalin-killed whole cells	94.6	93.8	95.1	96.9
Formalin-killed whole cells + $Al(OH)_3$	77.6	78.0	96.8	99.1
FSA vaccine	60.1	65.3	33.0	31.4

Figure 18. Effect of sonic disruption of Aeromonas salmonicida cells upon solubilizing antigenic components. Rabbit antiserums in the center wells, Aeromonas salmonicida antigens in the outer wells: N(white), rabbit anti-SIL-67 serum; A(white), rabbit anti-SS-70 serum; 0, 1, 5, and 10N, 0, 1, 5, and 10 min sonicates of SIL-67; 0, 1, 5, and 10A, 0, 1, 5, and 10 min sonicates of SS-70.


1A
0A
N
5A
10A
A
1A
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A
5A
10A
B
1N
0N
N
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10N
C
1N
0N
A
5N
10N
D

that from SS-70 (30%). It was also shown by gel diffusion analysis that appreciable lipopolysaccharide (LPS) was released from SS-70 cells only after 5 min of sonic disruption (Figure 18 A, B), whereas considerable LPS was released from SIL-67 cells in distilled water prior to any disruption (Figure 18C, D). In both cases, the LPS is presumed to be the two heavy bands nearest the antigen wells.

Efficacy of Orally and Parenterally Administered Vaccines in Preventing Furunculosis

Many of the previous results were obtained in order to provide the information necessary for making a judicious choice of vaccine strains. Aeromonas salmonicida strain SS-70 was chosen because it was highly virulent, possessed the additional cell wall layer, adhered to fish cells, and because serum produced to it was highly protective when administered passively to coho salmon fry. Formalin killing was chosen over chloroform killing because it yielded essentially whole cells of the chosen strain.

The FSA vaccine provided essentially the internal elements of the bacterial cells with relatively little cell wall material. Aluminum hydroxide present in both the FSA and whole-cell vaccine provided a means of precipitating soluble material, possessed potential adjuvant properties (Williams and Chase 1967), and acted as an antacid to protect antigens in the stomach.

Formalin-killed whole cell bacterin administered i.p. with CFA was shown previously to afford protection from furunculosis, at least in laboratory challenge experiments. It was expected that juvenile coho given this vaccine would be protected even under hatchery conditions and would provide a positive control. Groups that received parenteral vaccination had a mean mortality of 1.9%. This mortality level was significantly lower ($p < 0.01$) than the mortality level of the unvaccinated controls (Table 14).

Unlike the injected vaccine, the three orally administered vaccines provided no protection. Fish that received whole cells with $Al(OH)_3$ had a mean mortality of 11.0%. Groups which were fed whole cells or FSA experienced mortalities of 8.6 and 7.5%, respectively. Although these percentages are lower than those for controls, they do not represent a significant difference (at $p = 0.05$).

Rates of A. salmonicida recovery from the kidneys of fish that died during the study ranged from 80-90% in the orally vaccinated and unvaccinated groups. Only a 55% recovery was obtained from parenterally vaccinated groups. From these recovery rates it can be inferred that 1.5-2.0% of the total population in each group died of causes other than furunculosis.

All mortality figures are based upon the number of fish that died between June 14, 1974 (first furunculosis mortality) and August 1, 1974. The sample size of each experimental group was determined by the number of fish alive on June 14.

Table 14. Efficacy of three oral vaccines and one parenteral vaccine in reducing furunculosis mortality in juvenile coho salmon challenged under natural conditions at Siletz River Salmon Hatchery (ODFW)

Vaccine (route)	Fractional total mortality (3 replicates)	Fractional furunculosis mortality (3 replicates)	Mean total mortality (%)	Mean furunculosis mortality (%) (Arcsine means)[a]	*A. salmonicida* recovery rate (%)
Formalin-killed whole cells (per os.)	42/491 65/495 44/491	34/491 57/495 37/491	10.2 ± 1.4	8.6 ± 1.7 (16.8)	83.8 ± 2.2
Formalin-killed whole cells + $Al(OH)_3$ (per os.)	56/492 92/496 42/488	47/492 78/496 37/488	12.8 ± 3.0	11.0 ± 2.4 (18.8)	85.8 ± 1.3
FSA vaccine[c] (per os.)	57/499 36/487	42/499 32/487	9.4 ± 1.4	7.5 ± 0.9 (15.0)	88.4 ± 0.4
Formalin-killed whole cells + CFA (i.p.)	18/448 12/385 14/398	10/448 9/385 5/398	3.5 ± 0.3	1.9 ± 0.6[b] (7.9)	55.4 ± 10.7
Unvaccinated	64/490 52/477 65/489	49/490 45/477 48/489	12.4 ± 0.8	9.9 ± 0.2 (17.9)	78.7 ± 3.8

[a]Percent furunculosis mortality was arcsine transformed before use in one-way analysis of variance. Least significant differences (LSD) were computed to be: $LSD_{(0.95,\ 10df)} = 3.9$, $LSD_{(0.99,\ 10df)} = 5.9$

[b]Parenterally vaccinated groups were the only groups to show a significant reduction in mortality compared to unvaccinated controls (significant at $p = 0.01$)

[c]One replicate lost due to tank failure

Induction of Immune Suppression

Tolerance and suppression are well known and accepted phenomena in higher animals. Neither state has been demonstrated in bony or cartilaginous fishes, although it has been suggested by Lopez et al. (1974) that sharks may have a class of suppressor lymphocytes.

The object of this experiment was to determine whether the humoral antibody response of parenterally vaccinated coho salmon fry could be suppressed (diminished) by concurrent feeding of vaccine-containing diet. Fish that received only an i.p. injection of bacterin and CFA developed a reciprocal agglutinin titer of 2,048 at 108 days which waned to 1,024 at 138 days (Table 15). This titer is similar to that reported by Paterson and Fryer (1974a) for coho salmon fry.

In contrast, fish that were orally as well as parenterally vaccinated had a markedly reduced antibody titer of 128 at 108 days. This titer did not increase even at 138 days. The reduced agglutinin titer could not have been due to the oral vaccine binding the antibody (pseudo-suppression). At 108 days, fish had been fed plain OTD for 63 days after vaccine feeding. The 16-fold reduction in agglutinin titers strongly suggest that feeding this bacterin may suppress the normal humoral response to A. salmonicida antigens.

Table 15. Agglutinating antibody response of coho salmon fry[a] administered Aeromonas salmonicida (SS-70) vaccine: (1) orally, (2) parenterally, and (3) parenterally as well as orally

Method of vaccine administration	Number of fish per group	Days after experiment initiation	Days after vaccine feeding	Reciprocal agglutination titer[d]
Unvaccinated	500	0	-	16
		108	-	16
		138	-	32
Fed orally[b]	500	0	-	16
		108	63	32
		138	93	64
Injected intraperitoneally[c]	500	0	-	32
		108	-	2,048
		138	-	1,024
Injected intraperitoneally and fed orally[b,c]	500	0	-	32
		108	63	128
		138	93	128

[a]Fry weighed an average of 0.45 g each at the start of the experiment

[b]Fed Oregon Test Diet (OTD) containing 383 ug vaccine-carbon/g diet (formalin-killed whole cells of strain SS-70) for 45 days

[c]One 0.05 ml injection of 127 ug vaccine-carbon/fish (formalin-killed whole cells of strain SS-70) emulsified with CFA

[d]Titers determined on pooled serum samples from 30 fish

DISCUSSION

Furunculosis disease of fish remains one of the most economically important diseases affecting hatchery reared salmonids. Control of this disease is almost entirely dependent upon the use of chemotherapeutic agents, particularly oxytetracycline (Terramycin). This has been necessitated by the fact that no suitable vaccine has yet been developed for control of the disease. The studies described here on the pathogenicity, antigenicity and immunogenicity of Aeromonas salmonicida point out some of the parameters which should be considered in developing an adequate vaccine.

It has been shown in this study that two distinct types of A. salmonicida strains occur. Cells from some strains form large aggregates in the presence of divalent cations while others do not. Early in the study, it was observed that all of the 40 strains (not listed in Table 1) recently isolated from fish during epizootics in the United States and Canada were of the aggregating type. In contrast, the only nonaggregating strains available were obtained from culture collections and all had been carried on bacteriological medium for at least five years. The neotype strain ATCC 14174 (Hugh and Sneiszko 1961) is included in this nonaggregating group. Because aggregating A. salmonicida appears to be the natural form in fish it is suggested that a representative from this group be considered as the neotype strain. It should be noted that the strains reported by Anderson (1972) were not truly of the rough (WFDL-R) and smooth (WFDL-S) varieties. Strain

WFDL-S was virulent, aggregated, and was similar to other recent isolates from fish. Strain WFDL-R did not appear rough, particularly when compared to strain GAL-E, and was similar in every respect to other nonaggregating strains.

A more extensive examination of A. salmonicida isolates is needed to determine if nonaggregating cells occur in the aquatic environment. It seems likely that they do occur since the bacteriophage isolated from hatchery water and used in this study (ϕ SIL-74) was specific for nonaggregating strains and would not produce plaques on aggregating ones. Both types may be common in the environment; the aggregating strains being pathogenic for fish.

It has been possible to convert aggregating strains to nonaggregating ones but not vice versa. It was found that all streptomycin-dependent mutants of the aggregating strain SS-70 did not aggregate. Furthermore, revertants did not regain the ability to aggregate when they regained sensitivity to the antibiotic. Additional study into the genetics associated with aggregation would be desirable and should be facilitated by the use of strain Sm^d-REV.

Aurstad and Dahle (1972) have reported that the cell-wall structure of neotype strain ATCC 14174 was similar to that of most Gram-negative bacteria. In the present study, the ultrastructure of three additional nonaggregating strains were examined and found to have a similar cell-wall morphology. Surface appendages were not observed by Aurstad and Dahle (1972) possibly because they examined cells from the surface of blood agar plates and not from

liquid medium (BHI).

The profile of aggregating strains seen in ultrathin section most closely resembles that reported for Acinetobacter MJT/F5/5 (Sleytr et al. 1974; Thornley 1975). These A. salmonicida strains and the Acinetobacter strain all possess an additional stained layer (A-layer) separated from the exterior of the outer membrane by an electron translucent zone. If the models for the cell envelope proposed by Shands et al. (1967) and by Schniatman (1971) are correct, the electron translucent zone should contain the O-antigen side chains. It would be of particular interest to know if the O antigen is entirely contained within the A-layer and perhaps partially sequestered.

The A-layer was shown to have an interrupted, periodic staining pattern in oblique section. In negatively stained preparations of aggregating cells areas of pattern, appearing as parallel fibrils, were seen adjacent to the cells. In both cases the spacing of pattern units was approximately 7 nm (center-to-center). An additional pattern, a square tetragonal array, was also observed and found to have a 7 nm periodicity.

Several detailed studies (Sleytr and Thornley 1973; Thorne et al. 1975; Thornley 1975) have shown that the surface of Acinetobacter strain MJT/F5/199A is covered with a patterned layer having features similar to that described for A. salmonicida. The pattern associated with this Acinetobacter strain has the appearance of fibers composed of separate regularly arranged subunits. These subunits measure 8 by 6 nm and are therefore

approximately the same size as the pattern elements for A. salmonicida. Beverage and Murray (1976) have elucidated the pattern associated with the "inner structural layer" or ISL of Spirillum "Ordal." They found this layer to possess a set of tetragonally arranged 5 by 5 nm subunits which also appear as an array of parallel fibrils. Sleytr and Thornley (1973) and Beveridge and Murray (1976) used computer enhanced optical diffraction patterns from shadowed replicas to fully detail the size and structure of the subunits of their respective organisms. These techniques, if applied to A. salmonicida, could provide considerably more information about the subunit structure in the A-layer patterns.

Neither A-layers nor surface subunits have been associated with aggregation or adhesion to surfaces (Audrey M. Glauert and Margret J. Thornley, personal communication[4]). The A-layer dependent aggregation and adhesion to tissue cells in A. salmonicida seem to be unique, although analogous mechanisms have been reported (Swanson et al. 1975a). Swanson et al. (1971) demonstrated that cells of type-2 (virulent) gonococci attach to each other by means of extensive "zones of adhesion." Initial attachment of type-2 cells to eukaryotic cells was facilitated by pili; however, permanent attachment was mediated by adhesion of their respective external membranes. Although pili are necessary for virulence of

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[4]Audrey M. Glauert; Strangeways Research Laboratories, Cambridge, England.
Margret J. Thornley; Immunology Division, Department of Pathology, University of Cambridge, Cambridge, England.

N. gonorrhoeae, they are not required for type-2 cells to attach to leukocytes (Swanson et al. 1975b).

It seems reasonable to assume that adhesion of aggregating A. salmonicida to tissue cells is by means of the same mechanism as bacterial-bacterial binding. Adhesion of eukaryotic cells to each other and to solid substrate is at least partially dependent upon divalent cation bridging (Weiss 1970). Since both tissue cells and A-layer of A. salmonicida have divalent cation binding sites, adhesion by means of cross-bridging is likely. Hydrogen bonding and hydrophobic bonding may also play roles in stabilizing the adhesive junctions.

Most importantly, the presence of the A-layer, aggregation, and presumably tissue cell adhesion are correlated with virulence in A. salmonicida. In numerous host-pathogen models it has been shown that attachment of the bacterium to a mucosal surface is a requisite for virulence (Jones 1975). Freter (1969) demonstrated that specific IgA to V. cholera did not kill the organism. Instead, it prevented the bacterium from causing disease by inhibiting its attachment to intestinal mucosal surfaces. Studies by Duguoid and Gillies (1957) showed that Shigella which bear pili adhere to intestinal epithelial cells in vitro. They found that adhesion of Shigella organisms to epithelial cells correlated positively with their virulence in experimental hosts. Takeuchi (1975) has shown using electron microscopy that Salmonella typhimurium must come within 35 nm of the brush border of intestinal epithelial cells before it can effect any damage to the cells. Only those

bacteria which adhere and maintain this proximity later penetrate the epithelium.

The two best studied models for the role of attachment in promoting virulence are those of N. gonorrhoeae and enteropathogenic E. coli. Swanson (1975) reviewed the evidence showing that attachment of gonococci to epithelial cells is a major virulence mechanism. He listed five ways in which attachment might promote virulence; these could also apply directly to A. salmonicida. He states that attachment could influence virulence by serving to promote any of the following:

> "(1) Multiplication of the bacterium by anchoring the organisms at infecting sites and precluding 'flushing' of the organism from those sites by secrections, excretions, etc.
>
> "(2) Damage to epithelial cells mediated by a relatively 'weak' exotoxin elaborated by the bacterium, in general, but effective only in instances in which close proximity between prokaryotic and eukaryotic cells was also present.
>
> "(3) Damage to epithelial cells mediated by some sort of surface-surface interactions between bacterial cell wall and eukaryotic cell plasma membrane.
>
> "(4) Penetration between epithelial cells.
>
> "(5) Penetration into epithelial cells."

None of these mechanisms have been directly demonstrated in gonococci infections, however, nor have they been implicated in A. salmonicida infections.

Smith and Linggood (1971) obtained direct evidence that adhesion of enteropathogenic E. coli to intestinal epithelium was a major virulence factor. They found that strains which produced enterotoxin but were lacking K88 antigen (adhesive factor) were avirulent as were strains possessing only K88 antigen. Only enterotoxigenic strains which could adhere to cell surfaces produced enteritis. A similar combination of virulence factors may be involved in A. salmonicida pathogenicity. Even though A-layer mediated attachment might be an essential virulence mechanism, other factors might also be required to produce infections. Strains such as EFDL-3.58 possess an A-layer and the ability to aggregate but are still not virulent. The leukocidin of A. salmonicida described by Fuller (1974) and other toxic substances may play a role in virulence and deserve further attention.

It is quite clear that avirulent A. salmonicida cannot survive the juvenile coho salmon's defense mechanism, even when administered in doses exceeding 10^8 cells/fish. As few as ten bacteria from virulent strains, however, can survive the defenses, multiply and produce symptoms of furunculosis most often leading to death. This suggests that very few virulent organisms from the fish's water environment are required to penetrate the host and initiate an infection. Several factors may determine the number of organisms which can gain entry into the host from the water environment.

In this study, the dose of virulent A. salmonicida cells added to the water was shown to influence the percent mortality,

survival time, and clinical form of the disease. Stress also greatly affected the influences of dose. When juvenile coho salmon were exposed to doses as high as 5×10^6 cells/ml for 15 min (followed by lesser dosages during dilution) without previously being subjected to thermal stress, little (14%) mortality occurred. If, however, similar fish were subjected to a thermal stress (change in water temperature from 12 to 18°C), they suffered as much as a 40 to 50% mortality at the higher doses. The number of fish dying diminished with dose (48 to 28%) in the range 1×10^6 to 1×10^5 cells/ml. In this range all fish showed typical signs of subacute furunculosis. At the highest dose (5×10^6 cells/ml) no higher mortality was observed, but the majority of fish died of the acute form of furunculosis.

The median time from exposure to death (RT_{50}) was a linear function of the log-dose at concentrations from 5×10^5 to 5×10^6 cells/ml. This indicates that higher external exposure doses result in larger internal doses (assuming death is the result of a bacteremia). In the lowest exposure dose, the RT_{50} curve is truncated presumably because at this concentration, susceptible hosts are infected with the minimum lethal internal dose (Meynell and Meynell 1965). These observations suggest that the furunculosis mortality at salmon hatcheries could be reduced significantly by controlling the level of A. salmonicida in hatchery ponds and water supplies.

Although thermal stress significantly increased the susceptibility of normal juvenile coho salmon to furunculosis, it

had little or no effect on fish actively immunized with strain SS-70. Mortality in actively immunized groups never reached over 8% in laboratory challenge experiments even when 42% of control fish died. Krantz et al. (1963, 1964a) have shown that brook and brown trout parenterally immunized could withstand a substantial challenge with virulent cells. They reported that this protection was not lost even when the fish were stressed during spawning. Paterson and Fryer (1974a) also reported that juvenile coho salmon immunized with strain SS-70 cells in CFA, developed a protective immunity (significantly increased the LD_{50} of virulent cells). This body of research provides strong evidence that parenteral vaccination with virulent strain A. salmonicida cells in CFA would be a useful measure in controlling furunculosis. Further evidence to this effect will be presented later in this discussion. Unfortunately, CFA cannot be used in fish destined for human consumption. This does not preclude its use, however, for trout brood stock or in returning adult salmon used for brood purposes. Other adjuvants might also be effective and more suitable for edible fish.

Spence et al. (1965) demonstrated that hyperimmune serum (to A. salmonicida) from adult rainbow trout, when administered i.p. to juvenile coho salmon, conferred protection from laboratory challenge with vaccine strains. Similar results were obtained in this study using hyperimmune juvenile coho salmon serum. A single injection of 0.1 ml of serum (2,040 agglutinating units) 5 h before challenge resulted in significant reduction in mortality.

Considering that the volume of serum in each juvenile fish is approximately 0.1 ml, the theoretical maximum titer attainable in the bloodstream was 1:2,040. It is more likely that actual titers were considerably lower, at least toward the end of the experiment, since antibodies are known to have a finite half-life in the circulatory system.

Agglutinin titers may not correlate with protection, however. When juvenile coho salmon were passively immunized with an equal number of agglutinating units (2,040 in 0.1 ml) produced to strain SIL-67, they were not protected from infection with strain SS-70. This suggests that fish immunized with strain SS-70 produced a protective serum component (i.e., antibody or other serum factor) which fish immunized with SIL-67 did not. Additional passive immunization experiments using serum fractions might serve to identify which component(s) is involved.

Results of the passive immunization could also be accounted for by different specificity of the agglutinins. Agglutination reactions are generally the result of specific antibodies for several surface antigens. Antiserums to two strains can have the same titer but a considerably different distribution of antibody specificities. Therefore, some of the agglutinating anti-SS-70 antibody could have been directed to an antigen associated with the A-layer and thereby provided protection in the passive immunization experiments. This hypothesis can probably be discarded since strain Sm^d-REV, lacking the A-layer, removed all detectable agglutinins from anti-SS-70 serum. This observation is of particular

interest because it implies that juvenile coho salmon do not produce agglutinating antibody to the A-layer. Similar experiments with rabbit serum suggest that rabbits do produce A-layer specific agglutinins.

Precipitating antibodies to the A-layer could not be detected by double immunodiffusion procedures using rabbit serums. Because the A-layer is associated with cell aggregation, the material composing the layer may aggregate in the immunodiffusion well and not diffuse through the agar. Alternatively, the rabbit agglutinating antibodies may not be good precipitins (e.g., IgM).

Immunodiffusion analyses did produce some interesting data, however. Trichloroacetic acid extracts of an aggregating (SS-70) and nonaggregating strain (SIL-67) were essentially the same antigenically. Acetic acid degraded O-antigen polysaccharide preparations from both strains were also identical. Therefore, differences in protection conferred by the two coho salmon serums used in the passive immunization experiments may have been due to serum components directed at other than these major cell wall antigens.

Immunodiffusion of sonically disrupted cells also revealed that SIL-67 possesses a major antigen which is not present in SS-70. This antigen was also shown to be distinct from antigens in TCA extracts. No one has reported any major differences in the soluble antigen makeup of A. salmonicida cells (Karlsson 1964). This may have implications regarding the choice of vaccine strains and warrants additional research.

Vaccine preparation procedures were found to substantially affect the percent recovery of Kjeldahl nitrogen and total organic carbon in bacterial suspensions. In comparison to strain SIL-67, SS-70 was less easily lysed by treatment with chloroform, by formalin killing followed by $Al(OH)_3$ precipitation, and by sonication. Resultant vaccines prepared from these two strains differed markedly from each other. For this reason, all vaccine doses were standardized on the basis of TOC. In this way, doses of vaccines containing varying amounts of $Al(OH)_3$ and other salts were administered based only upon the amount of organic matter which they contained.

The evaluation of vaccines for the control of furunculosis has been greatly hampered by the lack of a consistent natural challenge. This was clearly demonstrated in the field trials of Frost (1968), Klontz and Anderson (1970), and Paterson (1972). Siletz River Salmon Hatchery (ODFW) was chosen for the present study (as it was by Frost and by Paterson) because it is a site of frequent epizootics. The major factor controlling the level of furunculosis mortality at this hatchery is the water temperature. As in previous trials, unseasonable cold water temperature in the spring and summer of 1974 limited the mortality in the hatchery ponds and in the experimental tanks. Although a 30 to 40% mortality in control groups was desired, the 10% mortality which occurred was adequate.

As in laboratory challenge experiments, groups of fish injected with A. salmonicida bacterin in CFA had a significantly

lower mortality ($p = 0.01$) than unimmunized groups. These results support past findings that this method of immunization can effectively control furunculosis without the use of antibiotics. Other means of parenteral administration of vaccine such as hyperosmotic infiltration (Amend and Fender 1976) may make it feasible to immunize a large population of fish. However, with A. salmonicida this may require high-potency vaccines and the use of adjuvants.

None of the oral vaccines tested in this experiment afforded protection. The dose of vaccine in the diet (500 µg vaccine carbon/g of diet) was equivalent to 15 mg wet wt cells/g of diet. A similar concentration of V. anguillarum vaccine was found to be highly effective in the control of vibriosis (Rohovec et al. 1975). If an oral furunculosis vaccine is going to be developed, a number of variables must be examined. These include the effect of: (1) vaccine dose; (2) duration of vaccine administration; (3) temperature; and (4) the level of challenge upon protection. These parameters have been studied in detail for oral vaccination to V. anguillarum (Fryer et al. 1972, 1976; Rohovec et al. 1975) but have not been the subject of laboratory studies with A. salmonicida.

The need for additional studies on immune suppression in fish is also evident. Preliminary studies suggest that A. salmonicida vaccines co-administered by the parenteral and oral routes suppress the humoral response in coho salmon fry. A number of studies in mammals (Gershon 1975) have shown that the route of administration of an antigen can greatly affect its ability to suppress antibody production. This phenomenon is particularly evident with antigens

of low immunogenicity. If antigens of low immunogenicity in fish (e.g., antigens associated with the A-layer) are fed orally, they may actually inhibit the formation of protective factors in the serum or secretions. Further investigation into this area might help to explain why little success has been achieved with oral vaccination to furunculosis.

Control of furunculosis in fish is still a desirable goal. Parenteral vaccination, whether by injection or by less time-consuming methods, could provide a means to fulfill this aim. Research on adjuvants which will be compatible with parenteral vaccination methods, acceptable by the U.S. Food and Drug Administration, and effective in increasing the level and duration of the immune response should have high priority. High potency vaccines containing all antigens necessary to evoke a protective response should also be the subject of future development.

SUMMARY AND CONCLUSIONS

1. Strains of Aeromonas salmonicida can be divided into two types; those which form aggregates of cells and those which do not.
2. Nonaggregating strains have a cell envelope similar to that of most Gram-negative bacteria, consisting of a plasma membrane, rigid layer, and outer membrane layers.
3. The cell envelope of aggregating strains has an additional electron dense layer exterior to the outer membrane. This layer (A-layer) is separated from the outer membrane by an electron translucent zone.
4. Strains of A. salmonicida lacking the A-layer were shown to be avirulent (LD_{50} in excess of 1.0×10^8 cells/fish), whereas three of four strains possessing the A-layer were virulent (LD_{50} less than 1.0×10^4 cells/fish). The remaining strain was avirulent indicating that other virulence factors may be required for a strain to be highly virulent.
5. The A-layer has a periodic stain pattern which appears as parallel lattice in electron micrographs of negatively stained cells. These lattices may be composed of 7 X 7 nm subunits lying in a tetragonal array .
6. Aggregating cells not only adhere to each other but also to eukaryotic cells (excluding erythrocytes) from fish and human origin. Aggregating strain SS-70 was shown to have

a 30-fold greater propensity to attach to chinook salmon embryo (CHSE-214) and to human cancer (HEP-2) tissue cells than the nonaggregating strain Sm^d-REV.

7. Aggregation was shown to be reversible and dependent upon multivalent cations. Reaggregation of cells (in the presence of 50 mM Mg^{++}) from the disaggregated state resulted in aggregates composed mainly of chains and planar sheets of cells.

8. Thermal stress was found to make juvenile coho salmon more susceptible to A. salmonicida infection.

9. Increasing the dose of virulent A. salmonicida in the water tended to increase the mortality and decrease the survival time of exposed hosts. At 5.0×10^6 cells/ml the acute form of furunculosis was most prevalent while at lower doses the subacute form of disease predominated.

10. Juvenile coho salmon actively immunized with virulent strain A. salmonicida cells in CFA were significantly protected against subsequent furunculosis challenge both in the laboratory and in the hatchery environment. This protection was retained even when the fish were stressed.

11. Hyperimmune juvenile coho salmon serum produced to the virulent challenge strain, SS-70, provided protective immunity to A. salmonicida infection whereas a similar serum to the avirulent strain SIL-67 did not. This result is indicative of immunogenic differences in these two strains.

12. Juvenile coho salmon failed to produce specific agglutinins to the A-layer as determined by agglutinin adsorption. Rabbits, on the other hand, did produce A-layer agglutinins. Coho salmon may produce other serum components (e.g., other types of antibody) which could account for the immunizing capacity of anti-SS-70 serum.

13. Antigens associated with the A-layer could not be detected by double immunodiffusion. Strain SIL-67 was found to have a major soluble antigen not present in strain SS-70, however. This should be considered in vaccine strain selection. Boivin-type antigens and acetic acid degraded polysaccharide were essentially the same for both strains.

14. Total organic carbon (TOC) and Kjeldahl nitrogen proved to be suitable methods for standardizing vaccines of different composition.

15. Cells of strain SS-70 were much more structurally stable than cells of strain SIL-67. It is possible that the A-layer of SS-70 adds structural stability to this strain.

16. Three orally administered vaccines failed to effectively immunize juvenile coho salmon against a natural furunculosis challenge. Formalin-killed whole cells, formalin-killed whole cells plus $Al(OH)_3$, and FSA vaccines administered at a level of 500 ug vaccine carbon/g of diet were tested.

17. A preliminary experiment suggested that orally fed A. salmonicida bacterins may suppress the humoral immune response to the same antigens.

18. Parenteral vaccination with A. salmonicida vaccines appears to be the only feasible method to immunize against furunculosis at this time.

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