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Immunization of Fish With Bacterins of *Aeromonas salmonicida*

L. R. UDEY and J. L. FRYER

ABSTRACT—Highly virulent Aeromonas salmonicida *strains were shown to have an additional layer external to the normal cell wall, whereas avirulent strains lacked the layer. Strains possessing the additional layer were also found to aggregate and to adhere to cultured fish tissue cells. Consequently, the choice of* A. salmonicida *strains may play an important role in determining the nature of vaccine preparations.*

The route of administration of bacterins was also investigated. Three types of orally administered bacterins, produced using virulent strain SS-70, were not effective in conferring protection to juvenile coho salmon naturally challenged in a hatchery environment. Parenteral immunization with a whole cell bacterin in combination with Freund's Complete Adjuvant conferred a high degree of protection under the same challenge conditions. A possible explanation for the lack of protection in orally vaccinated fish was proposed.

Bacterial furunculosis has a long history in studies of fish diseases. The causative agent, *Aeromonas salmonicida,* was first isolated by Emmerich and Weibel (1894) in Germany. It was the first really well characterized bacterial fish disease organism and was isolated in this country in 1902 (Marsh, 1902). *Aeromonas salmonicida* has probably been the most studied of all bacterial fish disease organisms. Even though we now are able to control furunculosis disease fairly well with certain antibiotics (McCraw, 1952; Herman, 1968), there have been problems with drug resistant organisms.

The organism itself is a short, gram-negative, nonsporulating, nonmotile rod. It ferments selected carbohydrates anaerogenically, is cytochrome oxidase positive, and usually produces a characteristic melanin pigment from tyrosine. The disease is a generalized septicemia which can either be acute or chronic and causes necrosis and edematous lesions (Klontz et al., 1966). The disease has been found in practically every country in the world which raises fish (except Australia and New Zealand) and economically has probably been the most important fish disease agent.

Immunization of fish with *A. salmonicida* vaccines has been tried for many years. D. C. B. Duff, in the late 1930's and early 1940's introduced the idea of oral immunization in fish following attempts at human oral immunization with the enteric pathogens (Duff, 1942). His results were promising, but unfortunately, he was not able to carry on his experiments after the war. He prepared a chloroform-killed whole cell vaccine of a known virulent isolate and was able to show what looked like protection, although on a very small scale, in the laboratory.

G. W. Klontz pursued Duff's work at a later time and subsequently patented a vaccine which is termed Furunculosis Soluble Antigen (FSA). The FSA is a sonicated extract in which the cell wall remnants are centrifuged out and the soluble portion of the organism is precipitated with aluminum hydroxide and administered in the fishes' diet. In the laboratory, Klontz showed that this vaccination procedure was effective, but on subsequent field testing, its efficacy was questionable (Klontz, 1967, 1968).

Krantz (Krantz et al., 1964a, b) injected brook trout, *Salvelinus fontinalis,* and brown trout, *Salmo trutta,* with an *A. salmonicida* bacterin and found that those fish which were injected (especially in combination with Freund's adjuvant) were provided protection against laboratory challenge. This protection persisted even when the fish are severely stressed, e.g., at the time of spawning.

Results of previous experiments pointed to two problems in evaluating and developing vaccines: 1) There probably was not sufficient standardization of the challenge, and 2) not enough was known about the bacterium itself. The literature is somewhat confusing about the types of strains of this organism. This report is a discussion of the differences between aggregating and nonaggregating strains of *A. salmonicida* and how these differences might affect vaccines prepared from them.

AGGREGATING AND NONAGGREGATING STRAINS

A culture of the nonaggregating strain is shown on the left in Figure 1. On the right is the aggregating strain, and the bacteria can be seen settled into a small bottom zone. Figures 2A and B are scanning electron micrographs depicting clumped cells of the aggregating strain. We have shown this aggregation to be totally reversible and dependent upon divalent cations. In culture, the clumping can be extensive and the number of cells per aggregate ranges from 20 to 200.

Preliminary results suggested that these clumping strains appear to be

L. R. Udey is with the Department of Microbiology, School of Medicine, University of Miami, Miami, FL 33152. J. L. Fryer is Chairman, Department of Microbiology, Oregon State University, Corvallis, OR 97331.

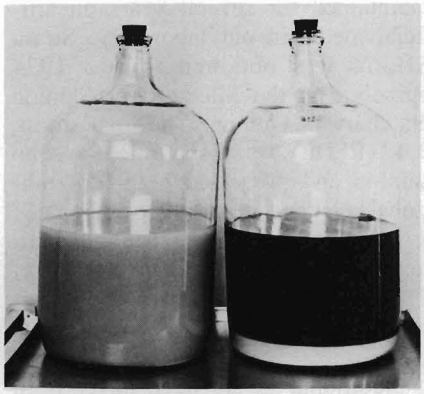

Figure 1.—Cultures of nonaggregating (left) and aggregating (right) *Aeromonas salmonicida* strains.

more virulent than the nonclumping strains. In some cases, the use of a nonaggregating strain may have accounted for lack of immunization in previous vaccination attempts. This has been difficult to ascertain because the type of strain used as antigen was seldom reported.

We felt that the differences between the aggregating and nonaggregating strains might be significant enough to warrant a detailed investigation. To determine if there were differences between these strains, the cell wall structure was examined by transmission electron microscopy.

Figure 3 depicts a cell of the nonaggregating type of *A. salmonicida* with the characteristic gram-negative cell-wall structure. Illustrated are the rigid layer (R), the plasma membrane (PM), and the outer membrane area (OM). The structures labelled (B) are thought to be lipopolysaccharide extrusions. These appendages have also been recently reported on *Neisseria meningitidis*.

In contrast, we have found that the aggregating strains of *A. salmonicida* produce an additional layer (A) as shown in Figure 4. The additional layer itself in gram-negative bacteria is nothing new. The dark staining additional layer beyond the area where one would normally find the O antigen polysaccharides is similar to those described for *Acinetobacter* (Glauert and Thornley, 1969).

In an oblique section (Figure 5A) of an aggregating cell, the additional layer exhibits a periodic staining pattern which probably stems from the regular molecular configuration of the layer as demonstrated in additional layers of other bacteria. The additional layer is tightly bound to the outer membrane even when the cell is completely lysed, as seen in Figure 5B. This picture shows the plasma membrane, rigid layer, and outer membrane with the additional layer still attached.

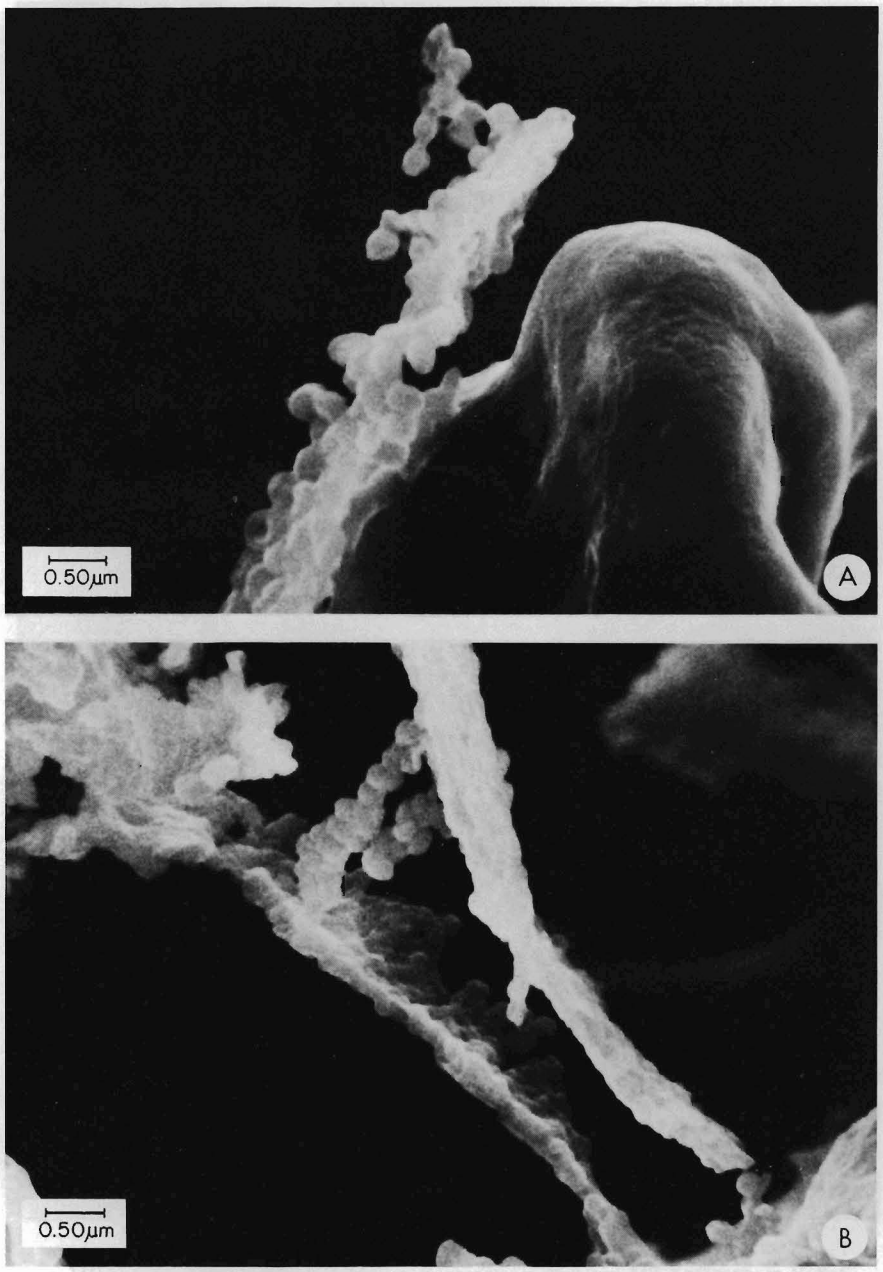

Figure 2.—Scanning electron micrographs of clumps formed by the aggregating strains of *Aeromonas salmonicida*.

VIRULENCE OF STRAINS

We found that all of the strains from recent epizootics throughout the United States and British Columbia were of the aggregating type. We also found that

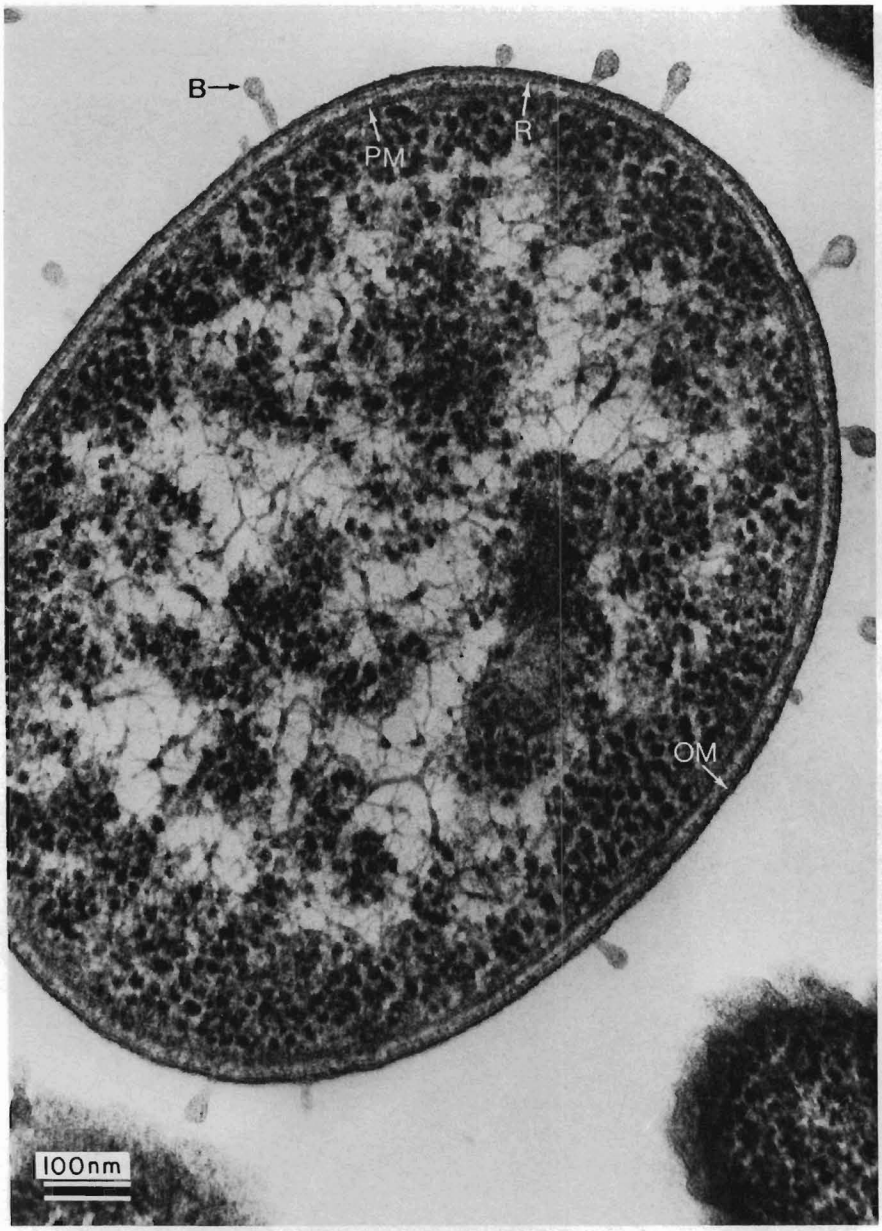

Figure 3.—Ultrathin section electron micrograph of a nonaggregating *Aeromonas salmonicida* cell. B = appendage, PM = plasma membrane, R = rigid or peptidoglycan layer, and OM = outer membrane.

strains maintained for some time in culture collections were of the nonaggregating type. We compared the virulence of the two types to see whether there really was a correlation between aggregation, cell wall structure, and virulence (Table 1).

The ATCC 14174 culture is of the nonaggregating type, lacking the additional layer. Isolate SS-70 is virulent, and aggregating, and it possesses the additional layer. Strain SS-70-Sm^d-REV is a streptomycin-sensitive revertant derived from a streptomycin-dependent mutant. It has lost its aggregating ability as well as the additional layer but otherwise appears identical to SS-70. The SIL-67 strain was maintained for several years on artificial media in our laboratory. Strain SIL-74 was obtained from a 1974 epizootic at the Siletz River Salmon Hatchery in Oregon. The final strain, 3.47 (EFDL), was also virulent in coho salmon and possessed both the additional layer and the ability to aggregate.

It is our conclusion that the additional layer is a requisite for virulence. The question still remains, however, whether this layer alone can confer virulence.

Additional layers have never been correlated with virulence before, nor have they been known to cause aggregation (Glauert, pers. commun.). The ability of bacteria to attach to tissue has been associated with virulence in infections caused by *Escherichia coli, Shigella dysenteriae,* and *Neisseria gonorrheae*. In these infections, however, the attachment of the bacterium is due to pili or to K-antigens. In preliminary experiments, the aggregating strains of *A. salmonicida* were found to adhere to human, rabbit, and fish white cells and to fish intestinal mucosa cells. This finding was interesting to us because it may be a mechanism by which the organism can deliver aggressins which it may possess. If the bacteria were attached to the tissue cell surface, aggressins could be secreted directly onto the cell without dilution. Klontz (1967, 1968) demonstrated a leukopenia in furunculosis infections, and suggested the presence of a leukocidin in *A. salmonicida*. It seems very likely that *A. salmonicida* could easily cause a leukopenia by attaching directly to leukocytes.

A model system was used to demonstrate that the aggregating strains have a marked affinity for tissue cells;

Table 1.—Correlation between aggregation, cell wall (CW) structure, and virulence in *Aeromonas salmonicida* strains.

Strain	Aggregates	Staining CW layers	Lethal dose LD_{50} (CFU) (colony forming units)
ATCC 14174 (NCMB-833)	—	3	$> 1.0 \times 10^8$
SS-Sm^d-REV	—	3	$> 1.0 \times 10^8$
SIL-67	—	3	$> 1.0 \times 10^8$
SS-70	+	4	8.8×10^0
SIL-74	+	4	6.8×10^0
3.47 (EFDL)	+	4	8.2×10^3

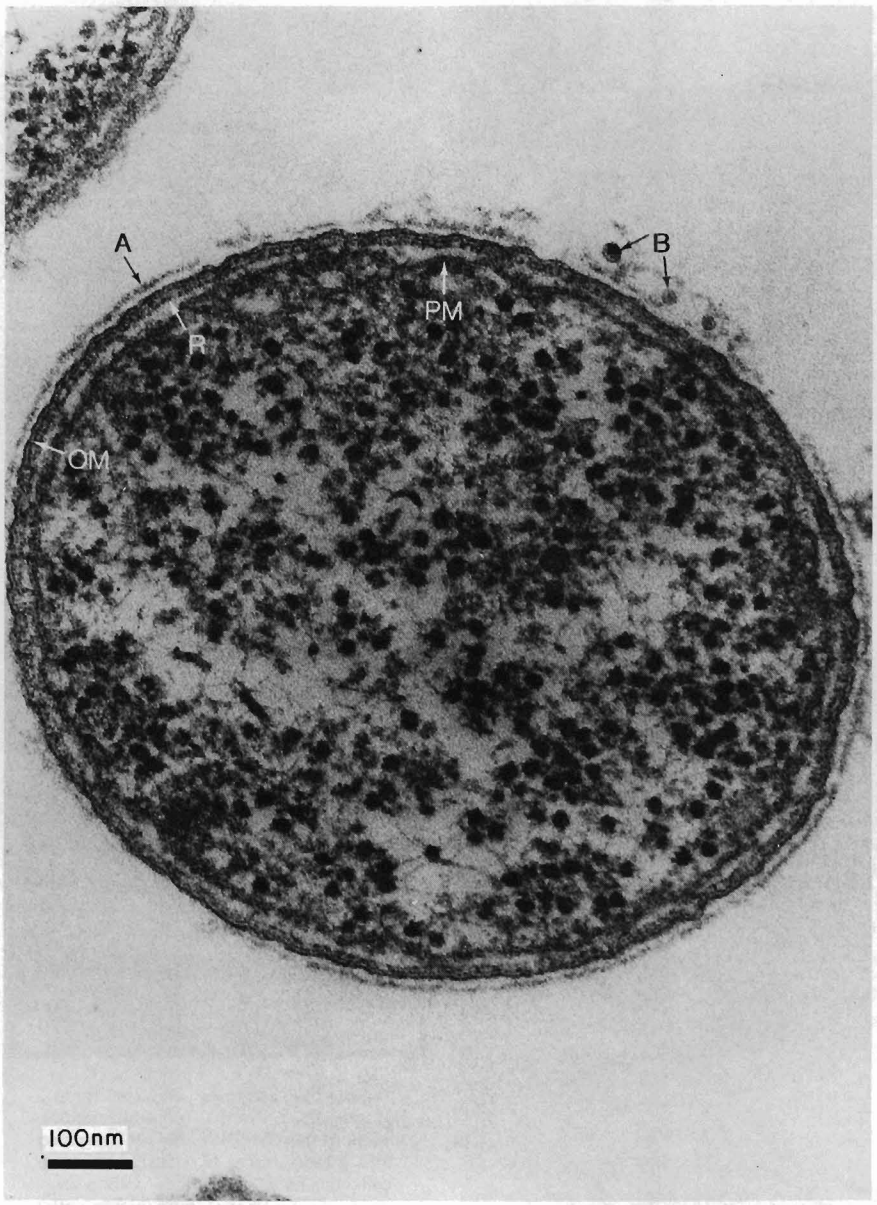

Figure 4.—Ultrathin section electron micrograph of an aggregating *Aeromonas salmonicida* cell. B = appendage, PM = plasma membrane, R = rigid layer, OM = outer membrane, A = additional layer.

the nonaggregating strains did not show this affinity. Figure 6A is a scanning electron micrograph of a chinook salmon embryo cell (CHSE-214) grown in monolayer. In the second picture in this series (Fig. 6B) the same cells were incubated with the nonaggregating strain SS-70-Sm^d-REV. It can be seen that the bacteria have little or no tendency to adhere to the CHSE cells. Strain SS-70, an aggregating strain, was incubated with CHSE cells and is shown in the third figure (Fig. 6C). The bacterial cells of this strain adhere markedly to the tissue cells; 30 times as many aggregating bacteria adhered to the tissue cells compared with the nonaggregating type.

VACCINES AND RESULTS OF VACCINATION

We felt also that the additional layer may well be an important antigen and that its presence should be considered in vaccine preparation. The effects of vaccine preparation methods on the recovery of Kjeldahl nitrogen and organic carbon from two strains of *A. salmonicida* were examined (Table 2). Strain Sil-67 is nonaggregating, and SS-70 is of the aggregating type. The cell wall of SS-70 was not as easily lysed, and the recoveries were consistently higher in the first four preparations. Since the FSA vaccine relies on obtaining soluble material, SS-70 gave a substantially lower yield (and different composition) than strain Sil-67.

Three of the vaccines listed in Table 2 were evaluated in an immunization trial. The Formalin[1]-killed whole cell, Formalin-killed whole cell + Al $(OH)_3$, and FSA vaccines were administered in the diet at 500 μg vaccine-carbon/gram of diet. Additionally the Formalin-killed whole cell vaccine was injected with Freund's Complete Adjuvant (FCA) intraperitoneally. Each vaccine was evaluated in three groups of 500 fish, and an additional three groups served as unvaccinated controls. All groups were exposed to a natural epizootic at Siletz River Salmon Hatchery using effluent water from a pond containing fish with

Table 2.—The effects of vaccine preparation on the recovery of nitrogen and carbon from two *Aeromonas salmonicida* bacterins.

Vaccine type	Vaccine strain			
	SIL-67		SS-70	
	%N	%C	%N	%C
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

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

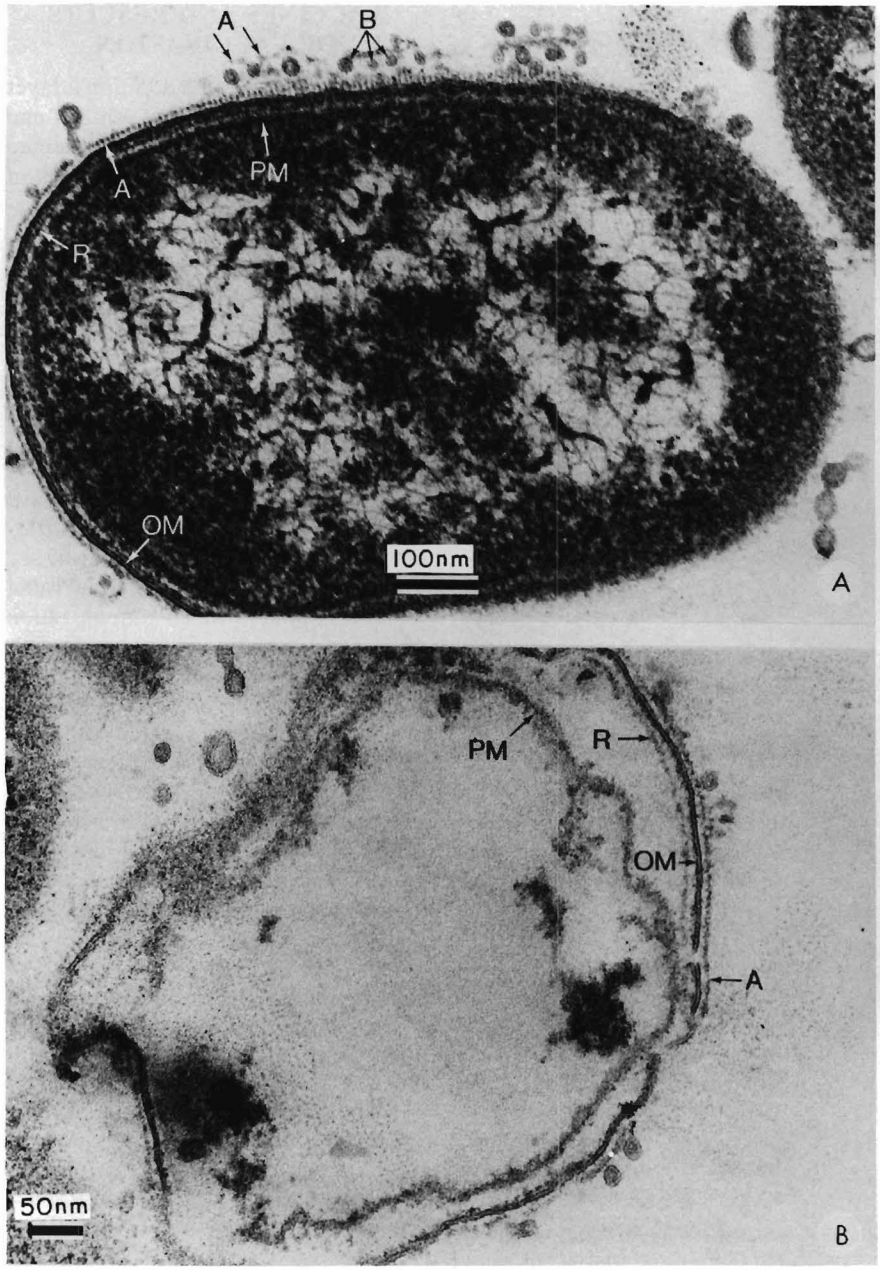

Figure 5A.—An oblique section of an aggregating cell of *Aeromonas salmonicida* showing the periodic staining pattern of the additional layer (A). 5B. An ultrathin section through a lysed *Aeromonas salmonicida* cell of the aggregating type. Note that the additional layer (A) is still firmly bound to the outer membrane (OM).

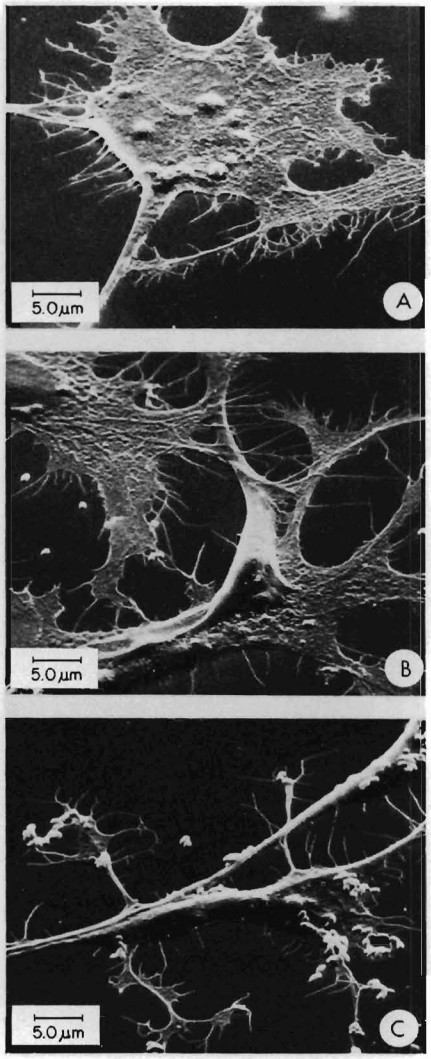

Figure 6.—Scanning electron micrographs of: A. Normal (control) chinook salmon embryo (CHSE-214) cell attached to a glass surface. B. CHSE-214 tissue cells incubated 5 minutes with nonaggregating *Aeromonas salmonicida* cells. C. CHSE-214 tissue cells incubated 5 minutes with aggregating *Aeromonas salmonicida* cells. Clumps of bacteria can be seen attached to the tissue cells.

active furunculosis. Table 3 is a summary of the results of this experiment. We were able to show that none of the oral vaccines provided any protection from furunculosis. Fish injected with the bacterin possessed a high level of protection which resulted in significantly reduced mortality ($P < 0.01$), however.

This experiment was disappointing to say the least. I would like to comment briefly on a possible explanation for the lack of success using oral vaccination with *A. salmonicida*.

An experiment was conducted in which groups of fish were: 1) Untreated, 2) fed strain SS-70, 3) injected with FCA and SS-70, and 4) fed and injected with SS-70. The injected groups were inoculated on the same day as feeding began. Vaccine was fed for

45 days, and all groups were bled 63 and 93 days after the end of vaccine feeding. Fish which received only oral vaccine exhibited no significant increase in humoral agglutinins (Table 4). The group, which only received the injection of vaccine, gave agglutinin titers of 1:2,048.

Interestingly, the group which was both injected and fed vaccine had suppressed humoral antibody response. This trend was maintained even 93 days after the end of vaccine feeding. This phenomena may be a case of immune suppression. Much additional work needs to be done in this area before an effective oral *A. salmonicida* vaccine can be expected.

Table 3.—Results of oral and parenteral vaccination of juvenile coho salmon with *Aeromonas salmonicida*.

Vaccine	Mortality (%) (± S.E.)	*A. salmonicida* mortality (%) (± S.E.)	Recovery rate (%) (± S.E.)
Formalin-killed whole cells (per. os.)	10.2±1.4	8.6±1.7	83.8± 2.2
Formalin-killed whole cells + $Al(OH)_3$ (per os.)	12.8±3.0	11.0±2.4	85.8± 1.3
FSA vaccine (per. os.)	9.4±1.4	7.5±0.9	88.4± 0.4
Formalin-killed whole cells +FCA (i.p.)	3.5±0.3	1.9±0.6**	55.4±10.7
Unvaccinated	12.4±0.8	9.9±0.2	78.7± 3.8

**Significant at $P < 0.01$.

Table 4.—Antibody response of juvenile coho salmon to *Aeromonas salmonicida* bacterins given orally and/or parenterally.

Treatment	Days after initiation	Days after end of feeding	Reciprocal agglutination titer
Untreated	0	—	16
Untreated	108	—	16
Untreated	138	—	32
Fed	0	—	16
Fed	108	63	32
Fed	138	93	64
Injected	0	—	32
Injected	108	—	2,048
Injected	138	—	1,024
Injected + fed	0	—	32
Injected + fed	108	63	128
Injected + fed	138	93	128

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