# The Development of a Live Attenuated Vaccine for the Control of Salmonid Furunculosis

by

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## ABSTRACT

*Aeromonas salmonicida,* the causative agent of salmonid furunculosis, was examined with respect to D-glucose catabolism. The major pathway for glucose catabolism of 'typical' strains of *A. salmonicida* was the Entner-Doudoroff pathway, whereas 'atypical' strains and other members of the Vibrionaceae family, utilized the Embden-Meyerhoff-Parnas pathway. The tricarboxylic acid cycle of *A. salmonicida* was apparently expressed only when cells were cultured with excess glucose. However, during glucose limitation, the cycle became an unusual branched pathway.

During attempts to isolate potential live vaccine strains, a slow growing, aminoglycoside resistant mutant (A450-10S) and a normaly growing pseudorevertant (A450-10SR) were isolated from *A. salmonicida* strain A450. These mutants continued to elicit a variety of classical virulence factors associated with *A. salmonicida* pathogenesis. Although both mutants were similar to wild-type with respect to cell surface composition, they were altered in the architecture of the A-layer, and displayed pleiotropic effects in many aspects of cellular physiology.

The slow-growing, antibiotic-resistant mutant A450-10S, differed significantly from the wild-type in an apparent loss of virtually all aerobic metabolism; the pseudorevertant had partially recovered the ability to aerobically metabolize certain carbon sources. Difference spectra of the cytochromes of the parent strain, A450, demonstrated the presence of cytochrome *c* , cytochrome *b*, cytochrome *o* oxidase and cytochrome *d* oxidase. A450-10S was demonstrated to be devoid of cytochromes. The partially compensating mutation in A450-10SR apparently restored all cytochrome types, but A450-10SR remained defective in $FADH_2$ coupling through complex II.

A450-10S and A450-10SR were both defective in their ability to generate a normal electrochemical gradient of protons, $\Delta p$, and were apparently more sensitive than

the wild-type strain to acidification of the cytoplasm. A450-10S and A450-10SR were also disabled with respect to their ability to survive in different aqueous environments, suggesting that the mutations used for the construction of these strains are suitable for inclusion in vaccine strains that will be released into the environment.

*Aeromonas salmonicida* strains grown inside intraperitoneal implants in Rainbow trout (*Oncorhynchus mykiss*) were examined for unique antigen expression. Western immunoblots using immune rabbit serum raised against in vivo grown cells revealed several novel antigens. The majority of these antigens were proteins, and were not induced in vitro in response to either iron limitation or anaerobiosis. Electron microscopy demonstrated the presence of a putative capsule on in vivo grown cells. Purification and fractionation of a carbohydrate material from cells grown in carbon rich synthetic media resulted in the isolation of an antigenically distinct LPS not seen with cells grown in standard media. Antisera directed against in vivo grown cells was demonstrated to be 10 times more sensitive than sera directed against in vitro grown cells for detection of *A. salmonicida* in infected fish kidney.

The mutant A450-10SR, and other mutants of *A. salmonicida* strains lacking either the A-protein, O-antigen, or both of these major surface antigens, were tested in Rainbow trout (*Oncorhynchus mykiss*, Walbaum) for virulence and their potential as live vaccine candidates. All mutants were shown to be attenuated as fish receiving ~5 x $10^7$ cells of the respective strains showed no clinical signs of furunculosis.

The mutants were subsequently tested in Rainbow trout (*Oncorhynchus mykiss*, Walbaum) for their suitability as live vaccines. Immersion vaccination of fish with these strains with an identical immersion dose fourteen days later, resulted in significant protection by all strains from challenge with a heterologous virulent strain of *A. salmonicida*. The levels of protection conferred were all greater than or equal to that provided by an injected bacterin using the same vaccination schedule. When antibody

responses of vaccinated fish were compared, it was found that only injection vaccination with a bacterin gave rise to a measurable agglutinating titer. Western immunoblots using the immune fish sera failed to reveal any major differences in antigen recognition in fish that received any of the vaccines tested.

These data suggest that the immune response generated by the use of live vaccine strains is different from that generated by a bacterin, and that any, or all, of the mutations described may be used for the construction of live vaccine strains for the prevention of furunculosis.

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# Chapter I

## Introduction

Fish represent the largest group of vertebrates, whose diversity of species in excess of 25,000 surpasses that of all vertebrates combined (Powers, 1989). Fish have been utilized by mankind as a source of protein, but the wild catch has unfortunately been unable to sustain the ever increasing demand. The global consumption of all seafood was estimated at 101 million tonnes for 1990 and is expected to increase to over 120 million tonnes by the year 2000 (Chaimberlain, 1993). Therefore, with the ever-increasing world population, alternatives to the world's capture fisheries are required to meet the need for the increasing need for protein from seafood. Fish farming represents an acceptable alternative for this demand for protein. The global production of finfish by aquaculture in 1990 was approximately 7.5 million tonnes, with salmonids (all salmon and trout species) representing approximately 550,000 tonnes (Chaimberlain, 1993). The production of farmed salmon now accounts for about 33% of the world salmon supply.

Next to feed costs, diseases are widely considered to be one of the major economic factors that affect profitability of finfish farming, thus it has been increasingly necessary to understand more about the relationship between bacterial pathogens and fish hosts in order to develop the needed fish health products.

### A Brief Outline of Finfish Immunology.

Fish, like other vertebrates, will mount both specific and non-specific responses to eliminate an infectious agent (for reviews see Anderson, 1974; Corbel, 1975; Ingram,

1980; Lamers, 1985; Stolen et al., 1986; Vallejo et al., 1992; and Newman, 1993). Non-specific defense mechanisms include the presence of physical barriers (e.g. skin, scales, and mucus), bacteriolytic activities (e.g. complement, lysosomal cytolysins, lysozyme, etc.), non-opsonic phagocytosis by macrophages followed by killing (oxidative or non-oxidative), and sequestration of essential micronutrients (e.g.. iron). The specific immune response of fish, like in other animals, is mediated by lymphocytes and antibodies acting against foreign antigens.

The major lymphoid organs in teleost fish are the thymus, spleen, and kidney. The thymus contains mainly developing lymphocytes, and apparently plays little or no active role in the immune response other than the eventual supply of mature lymphocytes. The spleen of fish is composed, in part, of specialized capillaries that contain a network of reticulin fibres and macrophages. These networks appear to be involved in trapping immune complexes, and are possibly involved in the development of immune memory (Ellis, 1980). The kidney of fish is the major antibody producing organ that contains most of the hemopoietic tissue (Ellis, 1988a). The hemopoietic tissue of the kidney contains high levels of plasma cells. Also, as the kidney is involved in filtration, there are high levels of macrophages involved in antigen uptake and subsequent processing.

The lymphocyte population of teleost fish consists of both T cells that originate in the thymus, and B lymphocytes that are thought to originate in the kidney of the fish (Tatner and Manning, 1985; Tatner, 1986). The lymphoid organ and lymphocyte maturation in salmonids begins almost immediately after hatching, with the most rapid growth of the organs occurring during the first 2 months (Tatner and Manning, 1983). The development and maturation of lymphoid organs correlates well with the weight of the fish, thus early rapid growth of fish plays an important role in the development of immune responsiveness. The lymphocytes of immunoresponsive adult fish all carry a surface marker, termed surface immunoglobulin, that is cross reactive with antibodies

directed to fish serum immunoglobulin (Ellis, 1977). Using antisera directed against this marker, the maturation, and distribution of lymphocytes has been examined in Atlantic salmon (*Salmo salar*) (Ellis, 1977). It was demonstrated that most lymphocytes possess this marker by 48 days post hatch. Curiously, it is at this time (>48 days post hatch) that the fry are dependent on external food sources. Thus, the oral uptake of a pathogen during feeding, will only occur when the majority of lymphocytes are mature and capable of mounting an immune response.

Like other vertebrates, fish possess a specific humoral immune response mediated by immunoglobulins (Ig) secreted from B lymphocytes (Acton et al., 1971; Corbel, 1975; Kobayashi et al., 1982). The Ig of salmonid fish has been purified and characterized (Dorson, 1981; Kobayashi et al., 1982), and it has been demonstrated to be a tetrameric, IgM-like molecule, characterized by the absence of a joining polypeptide, or J chain (Lamers, 1985). During a humoral immune response in salmonid fish there is no switching of Ig to a lower molecular weight class as is observed in mammals (Ambrosius et al., 1982). It has been demonstrated that there are both T cell independent and T cell dependent antibody responses in fish, the latter of which includes the temperature dependent participation of T suppressor and T helper cells (Lamers, 1985; Ellis, 1988a; Arkoosh and Kaattari, 1991). The humoral response of salmonid fish has been demonstrated to have a memory component (Arkoosh and Kaattari, 1991)

The cell mediated immunity in fish not well understood in comparison to that of mammals. The cellular immune response of salmonid fish has been assessed using both mixed lymphocyte response (Ellis, 1977), and allograft rejection (Tatner and Manning, 1983). Both of these assays demonstrated a memory component (Botham et al., 1980).

To date, no specific markers have been identified to separate the T-cells into specific subclasses, thus the existence of T helper, T suppressor, cytotoxic T lymphocytes, and other subclasses have only been proposed due to the fish humoral and

cellular immune responses having features analogous to mammalian responses that require the activity of any or all T cell subclasses. Using monoclonal mouse anti-trout IgM antibodies, DeLuca et al. (1983) found that trout lymphocyte populations that were depleted in IgM expressing cells had lost the ability to respond to the B-cell mitogen LPS, while still being responsive to the T-cell mitogen concanavalin A (con A). This con A responsive population was determined to be T-cell like.

**Diseases of Cultured Salmonid Fish.**

Fish, like all living organisms, are subject to numerous infectious diseases caused by fungi, protozoa, bacteria and viruses as well as other foreign organisms. The aim of this introduction is to highlight only the bacterial diseases that are of major importance to the salmonid farming industry. The major bacterial diseases of the salmonids are bacterial kidney disease, caused by *Renibacterium salmoninarum*, vibriosis, caused by the species *Vibrio anguillarum* and *V. ordalii*, cold water vibriosis (Hitra's disease) caused by *V. salmonicida*, enteric redmouth disease, caused by *Yersinia ruckeri*, and furunculosis, caused by *Aeromonas salmonicida*. Pathogens of the *Pseudorickettsia* spp., although not a problem in most of the world salmon farming, are of growing importance to the Chilean salmon farming industry. These diseases all have the potential to severely impact the profitability of salmon farming operations. The increasing interest in these economically important fish pathogens has allowed a deeper study of some of their virulence mechanisms.

**Bacterial kidney disease (BKD).** This disease, caused by the Gram positive bacterium *Renibacterium salmoninarum*, is enzootic throughout the wild salmonid populations of the world, and many farm stocks are infected with the bacterium (Fryer and Sanders, 1981; Austin and Austin, 1987; Newman, 1993). As no commercial vaccines exist, and antibiotic therapy is only weakly effective (Newman, 1993), serious

mortalities can occur when an infected population is stressed. *R. salmoninarum* exists both intra- and extracellularly during an infection. The intracellular pathogens are located mainly in monocytes and macrophages. Initially, the infection is localized in the kidney of the fish, but soon spreads to become a systemic infection. Unlike other diseases of salmonids that are more prevalent when water temperatures rise, fish carrying *R. salmoninarum* will commonly succumb to BKD when the water temperature drops in winter. This is thought to be due to the lack of responsiveness of the cell mediated immune system of salmonids at reduced temperatures (Munro and Bruno, 1988).

*R. salmoninarum* has the ability to produce intra-ovum infections in salmonid eggs, and reside in the egg's yolk (Evelyn et al., 1986a), assuring a vertical transmission to the progeny of infected female hosts. The characteristic of causing chronic infections together with the ability of being perpetuated by vertical transmission, make *R. salmoninarum* a well adapted pathogen of salmonids. The molecular mechanisms by which *R. salmoninarum* enter developed hosts are unknown, as are those mechanisms by which this pathogen penetrates the egg. However, experimental evidence has shown that *R. salmoninarum* is able to enter the developing oogonia, within the ovarian tissue of experimentally infected juvenile rainbow trout (Bruno and Munro, 1986). Also, there is experimental evidence suggesting that intra-ovum infections may occur after ovulation, by contact with infected coelomic fluid (Evelyn et al., 1986b).

Independently from the mode of transmission, virulence in *R. salmoninarum* has been associated with the presence of a 57,000 MW surface protein, also known as the F-antigen (Getchell et al., 1985). This protein is a versatile agglutinin capable of interacting with the surface of a variety of eukaryotic cells: rabbit and other mammalian erythrocytes (Daly and Stevenson, 1987); salmonid spermatozoa (Daly and Stevenson, 1989); and salmonid leukocytes (Wiens and Kaattari, 1991). Interestingly, this putative adhesin of *R. salmoninarum* does not agglutinate salmonid erythrocytes. The F-antigen has been

cloned, sequenced (Chien et al., 1992), further characterized, and shown to be structurally and immunologically conserved among different isolates, susceptible to proteolysis, and capable of self association with the surface of putative F-antigen negative cells (Daly and Stevenson, 1990). Moreover, by using a pool of monoclonal antibodies specific for different regions of the molecule, it was shown that the N-terminus domain of F-antigen carries the agglutination sites and the C-terminus domain contains a binding site involved in attachment to the *R. salmoninarum* cell surface. Different regions within the N-terminus are thought to be involved in the agglutination of different host cells, since a specific monoclonal was able to block hemagglutination but not leukoagglutination (Wiens and Kaattari, 1991).

Although the significance of the agglutinating properties of F-antigen remains to be clarified, it is evident that this molecule constitutes an important adhesin, perhaps implicated in tissue tropism and intra-ovum infections by *R. salmoninarum.*

**Enteric Redmouth Disease (ERM).** This disease is caused by the Gram negative bacterium *Yersinia ruckeri.* ERM is primarily a fresh water disease, with potential to affect trout farming operations, and other salmonid hatcheries (Ellis, 1988b). The name of the disease results from the characteristic subcutaneous hemorrhages seen on the mouth and opercula of infected fish. During the 1970's ERM was the major disease in the trout industry, but the advent of simple, but effective, bacterin-type vaccines, outbreaks of ERM are dramatically reduced.

Although at least 5 serotypes of *Y. ruckeri* exist, the vaccines based on just one serotype are apparently cross protective (Stevenson and Airdrie, 1984). Likely, the success of the vaccines has resulted in little progress on the virulence factors of this organism, as most research on bacterial diseases of fish is carried out with the aim of improving either current vaccination regimes or chemotherapy. The major immunogen is

apparently LPS, but the nature of the immune response is unclear, other than it is specific and has a memory component (Johnson et al., 1982; Ellis, 1988b).

**Vibriosis.** This is a general name for the various diseases caused by pathogenic Gram negative species of the genus *Vibrio*, including *V. anguillarum*, *V. ordalii*, and *V. salmonicida*, among others. *V. ordalii*, which was originally classified as a biotype of *V. anguillarum*, although a serious pathogen of salmon, is less frequently associated with vibriosis outbreaks than *V. anguillarum* (Shiewe et al., 1981). The variation of vibriosis caused by *V. salmonicida* is commonly referred to as cold water vibriosis or 'Hitra disease' (Egidius et al., 1981). This disease seems to occur more frequently in the winter months, possibly due to the psycrophilic nature of the pathogen (Egidius et al., 1981; Newman, 1993). As these bacteria are a normal part of the aquatic environment, most have a global distribution, although *V. salmonicida* is thought to be confined to the northern Atlantic. The most predominant form of the disease in cultured salmon is caused by *V. anguillarum*. Although one serotype predominates, there are at least 10 O-polysaccharide based serotypes of *V. anguillarum* (Kitao et al., 1983; Sørensen and Larsen, 1986).

The most thoroughly studied virulence factor of this pathogen is the plasmid encoded iron sequestering system that is required for virulence (Crosa, 1980). Although at least three different siderophore systems for iron sequestration exist in *V. anguillarum*, the plasmid encoded system of strain 775 is the best characterized (Actis *et al.*, 1986; Lemos *et al.*, 1988; Biosca and Amaro, 1991; Mackie and Birkbeck, 1992). Extensive research into the virulence factors of *V. anguillarum* has demonstrated that strains harbouring the plasmid pJM1 (or a related plasmid), are all of a highly virulent phenotype and if cured of this plasmid they become much less virulent (Crosa *et al.*, 1977). It was also demonstrated that if these plasmid cured strains are injected into fish in the presence of excess iron, their virulence is increased by approximately 300 fold (Crosa, 1980).

These plasmid cured strains of *V. anguillarum* were also shown to be unable to grow well in iron restricted conditions, but if the growth medium was supplemented with iron, growth rates returned to normal (Crosa, 1980). The siderophore from *V. anguillarum* that is a product of the pJM1-like plasmids (hereafter referred to as pJM1) is called anguibactin. It was determined to be of the phenolate-catechol type, (Actis et al., 1986; Jalal et al., 1989), and it appears that anguibactin represents a novel siderophore most closely related to pyochelin of *Pseudomonas aeruginosa*. It has been demonstrated to be ω-*N*-hydroxy-ω-[[2'-(2'',3''-dihydroxyphenyl)thiazolin-4'-yl]-carboxy]histamine that binds iron with a 1:1 stoichiometry, and has a higher affinity for iron than other *V. anguillarum* siderophores (Mackie and Birkbeck, 1992).

The levels of siderophores in *V. anguillarum* infected fish tissues are sufficient that the production of siderophores *in vivo* has recently been detected (Mackie and Birkbeck, 1992). These authors found that strains harbouring pJM plasmids did produce anguibactin in vivo, but this was not the only siderophore released by these strains. In all cases in which anguibactin was produced, a second phenolate siderophore common to all *V. anguillarum* strains was also produced. The finding that redundant systems are coexpressed probably underscores the importance of iron sequestration for organisms that choose a different organism as a niche to secure.

Other virulence factors of *V. anguillarum* include several extracellular toxins (Kodama et al., 1984). Recently, Norqvist et al. (1990) demonstrated that a zinc metalloprotease seems to be involved in host invasion by *V. anguillarum*. This apparent link was based on the discovery of a mutant that was restricted in its ability to infect rainbow trout by immersion, but was as virulent as the wild type strain when injected intraperitoneally. The only apparent defect detected at the molecular level consisted of a reduced level of an elastolytic enzyme (M.W. 36,000), which required $Zn^{2+}$ for activity and $Ca^{2+}$ for stability. The precise nature of the attenuating mutation has not been

elucidated, and thus a true cause and effect relationship cannot be made. However, the N-terminal amino acid sequence of this zinc metalloprotease revealed homology to the elastase of *Pseudomonas aeruginosa* and the protease of *Legionella pneumophila*, both of which are recognized as virulence factors (Norqvist et al., 1990).

***Aeromonas salmonicida* and Furunculosis.** *Aeromonas salmonicida* is the causative agent of the salmonid disease furunculosis. *A. salmonicida* is a non-motile, facultatively anaerobic, gram-negative rod-shaped bacterium of the Vibrionaceae family. Fish with furunculosis can display any, or all, of the following signs: darkening of the skin; loss of appetite; the appearance of furuncules that contain large numbers of the organism in serosanguinous fluid filled, raised liquefactive muscle lesions; hemorrhaging of the abdominal walls, the heart, and the liver; and a general inflammation of the spleen and lower intestine (Hastings, 1988). The disease usually occurs during autumn or spring, likely due to marked changes in temperature and/or salinity. Outbreaks are also associated with stress inducing factors such as shipping or handling. The prevalence of furunculosis is increasing on a global basis due to the increased amount of Atlantic salmon farming, as this species of fish is extremely susceptible to the disease (Hastings, 1988). *A. salmonicida* possesses several features that are likely to be important in the pathology of the disease, and may play a role in developing different strategies of immune prevention.

**The cell wall of *A. salmonicida*.** The most thoroughly studied aspect of this pathogen is the cell wall, and more specifically the regular surface array that coats the cell, the A-layer. The A-layer constitutes the outermost continuous surface layer of *A. salmonicida*, and is essential in virulence (Kay *et al.*, 1981; Ishiguro *et al.*, 1981; Munn *et al.*, 1982; Trust *et al.*, 1983; Garduño et al., 1992; and recently reviewed in Kay *et al.*, 1988, and Kay and Trust, 1991).

One of the first recognized virulence associated functions of the A-layer was protection against the lytic activity of normal serum (Munn et al., 1982). A-layer possessing strains of *A. salmonicida* were demonstrated to be far more serum resistant than A-layer negative strains. It has been assumed that enhanced serum resistance result as a consequence of the physical barrier effect of the A-layer.

The assembled A-layer is highly resistant to proteolytic attack (Chu et al., 1991). Since proteases are a main component of lysosomes, they constitute one of the major non-oxidative killing mechanisms of phagocytes. Therefore, the ability to resist proteolysis could be regarded as an important protection mechanism against host defenses.

Finally, the unique abilities of the A-layer to bind immunoglobulins (Phipps and Kay, 1988) and fibronectin (Doig et al., 1992) possibly reflects another A-layer-mediated mechanism of host defense avoidance. The non-immune binding of immunoglobulins may overthrow the purpose of the humoral immune response, and further shield the bacterium from other defense mechanisms like opsonic phagocytosis by neutrophils. In the case of soluble fibronectin binding to the A-layer, this may shield underlying bacterial surface antigens from normal immunological recognition, thus avoiding the humoral and cellular immune responses against cellular antigens. Soluble fibronectin has been identified in rainbow trout plasma and in the supernatant of rainbow trout gonad, RTG-2, cell line cultures (Lee and Bols, 1991).

A-layer mediated resistance to the bactericidal activity of reduced oxygen species has also been documented (Karczewski et al., 1991). A correlation was observed between levels of killing by superoxide anion and the presence or absence of A-layer in 11 strains of *A. salmonicida*. This correlation was coupled with the ability to produce proteases, since A-layer positive strains capable of producing proteases were the most resistant to superoxide killing. Also it was demonstrated that the enzyme superoxide

dismutase was involved, since specific inhibition of this enzymatic activity resulted in enhanced killing. It has recently been demonstrated that the A-layer provides initial protection against killing by superoxide and peroxide radicals; the main protection being mediated by an inducible protective response that required *de novo* protein synthesis (R.A. Garduño, personal communication). The mechanism by which the A-layer protects against reduced oxygen radicals is not clear.

**Extracellular virulence factors of *A. salmonicida*.** Early studies (Ellis et al., 1981) suggested that the pathology associated with *A. salmonicida* infections could be reproduced by injecting a preparation of the extracellular products produced by *A. salmonicida*. *A. salmonicida* excretes many extracellular virulence associated toxins and enzymes. There appears to be at least three different proteases (Ellis et al., 1981; Sakai, 1985; Austin and Austin, 1987; Ellis, 1991). The purified proteases from *A. salmonicida* have been demonstrated to be toxic to salmonid fingerlings by injection (Ellis *et al.*, 1988b), and to correlate with certain types of lesions formed during infection. Other studies on the extracellular products of *A. salmonicida* revealed that a complex of glycerophospholipid: cholesterol-acyltransferase (GCAT, McIntyre *et al.*, 1979), LPS, and the 70 kDa protease is lethal to Atlantic salmon, and that this complex is capable of inducing the extensive muscle liquefaction commonly associated with furunculosis (Lee and Ellis, 1989, 1990, 1991). Also, this complex has been demonstrated to act in thrombus formation (Salte et al., 1991, 1992). The 70 kDa protease has been reported to act as an activated "factor X", and the other components (GCAT and LPS) release thromboplastic material to the bloodstream via haemolysis. Intravascular injection of either the complex, or its components can lead to consumptive coagulopathy in fish.

Although not identified at the molecular level, it is known that *A. salmonicida* possesses a macrophage cytotoxin that appears to be different from the known extracellular products of *A. salmonicida* (Ellis, 1991). Destruction of the macrophage, a

key cell in the process of antigen presentation and an important effector cell of the immune response, is certainly a virulence mechanism of *A. salmonicida* involved in avoiding the defense system of the host.

**Disease Treatment: Vaccination versus Chemotherapy.**

There are only a few basic options that are practical and/or available for the control of disease in finfish aquaculture. The first and most obvious is only viable to the farmer when the disease problem is not severe or of any major economic importance. This is to do nothing and let the animals immune system do the work. This would be an acceptable practice if the fish were in an ideal environment. Unfortunately, the culture of finfish in an enclosure is not an ideal situation. It can be safely assumed that in the wild an infected fish (that is showing disease symptoms) would be culled by the natural methods of predation, starvation, or a combination of the two. This would effectively remove the infected individual from the immediate population, thus preventing excessive horizontal and/or vertical spread of the disease through that population. In aquaculture an artificial situation has been created where the entire population is always fed, and is virtually free from natural predators. This leads to the ability of infected fish to survive and slough pathogenic bacteria within a population for a greater period of time, thus amplifying any disease problem to a much higher order of magnitude.

Methods of disease control continue to be developed to deal with these problems. These methods include improved husbandry techniques, the selection of disease resistant strains of fish (domestication), antibiotic therapy (chemotherapy), and vaccination. This section will deal with the treatment of bacterial disease by chemotherapy and vaccination.

**Chemotherapy.** The treatment of bacterial diseases in cultured finfish by chemotherapeutants has played an important role in aquaculture on a worldwide level, but there are several reasons why this is a dangerous method to rely on. First, the use of

antibiotics to treat disease has lead to several drug resistant strains of all pathogens (Newman, 1983; Aoki, 1988). This resistance renders antibiotic therapy useless unless costly, and time consuming, sensitivity testing is done with the infectious agent prior to antibiotic administration. This type of regime leads to delays, and possibly leads to unnecessarily high mortalities in a given outbreak of disease. In areas where the risk of farmed stock becoming infected with a bacterial pathogen is high, a "disinfection" policy such as antibiotic administration can be expensive or ineffective. It is a recognized risk that excessive use of antibiotics can result in a selection of a bacterial population that is resistant to the antibiotic used.

In the development of resistance, a bacterium often acquires an extrachromosomal genetic element (plasmid) that encodes a system that in some way inactivates the drug. These drug resistance related genetic elements are known as R-plasmids. Most R-plasmids also encode for a mechanism that allows for inter and intra genus/species transfer of itself. Thus not only are the problems of resistance restricted to the initial disease causing agent, but the resistance marker can be transferred to the normal microflora of the animal, and subsequently to other future resident bacteria within, or near, that host. As antibiotics persist in tissues for relatively long periods of time after ingestion, tissue persistence of antibiotics has been reviewed extensively, and government guidelines have been developed to eliminate antibiotic contaminated foodstuff from the marketplace (Jacobsen, 1989). The persistence of antibiotics is not limited to the fish as recent data suggests that the antibiotics also persist in sediments from fish farms (Jacobsen and Berglind 1988).

Currently, the antibiotics commonly used in salmonid culture are oxytetracycline, potentiated sulfonamide, i.e. Romet 30™ (a mix of sulfadimethoxine and ormetoprim), and the quinolone antibiotic oxalinic acid (Aoki et al., 1983; Stamm, 1989). Unfortunately, in the case of *A. salmonicida*, strains have been isolated from disease

outbreaks that are resistant to all of the commonly used drugs (Stamm, 1989). The resistance mechanisms have been both R-plasmid encoded and mutationally derived (Torazano et al., 1983; Aoki et al., 1986; Belland and Trust, 1989). As many R-plasmids can be transferred, the development of resistance can potentially spread from one pathogen to another. In these situations vaccination becomes the most attractive alternative for disease control.

**Vaccination.** Ideally, vaccination is the process of inducing a protective immunological response, that possesses a memory component, in an organism against a foreign substance. Thus, if the correct antigen (foreign molecule) is presented, in the correct manner, to the appropriate branch of the immune system (either cell mediated, humoral, or both) an immune response may be elicited that will display memory. Unfortunately it is not always evident which of the bacterial antigens are required for protective immunity, nor is the correct method of presentation of the antigens to the cells of the immune system always clear. Also, a factor that contributes to the usefulness of a vaccine is the method with which it may be administered. Some vaccines for aquacultural use must be injected, while others can be administered orally, or by direct contact (bath, immersion, dip, or spraying)(Ellis, 1988a; Ellis, 1988b; Dunn et al., 1990). The problems associated with the use of injection vaccines include physiological and psychological stress for the fish, and a costly requirement for specialized equipment and skilled technicians to administer the vaccine. For these reasons, immersion and orally delivered vaccines have received the greatest attention. Bearing these factors in mind, vaccines designed for use in aquaculture must be at least as inexpensive as antibiotic therapy or pure economics will prohibit their use (for review see Ellis, 1988a).

The fact that immersion type vaccines can, and in some cases do work, suggests that there is a site at which the vaccine components are taken up by the fish. Experiments using vaccine preparations (Smith, 1982; Zapata et al., 1987; Tatner, 1987), and inert clay

suspensions (Goldes et al., 1986), have demonstrated that there is a substantial amount of phagocytosis by the brachial and gut epithelia of fish. The precise requirements for optimal antigen uptake by the gill tissue is at present not known, nor is it known whether uptake by these cells rather than gut epithelia, is involved in stimulating general immunity in fish.

Despite these obvious obstacles, effective vaccines have been developed for some of the diseases that affect cultured salmonids such as vibriosis and enteric redmouth disease (Johnson and Amend, 1983; Smith, 1988). Partially effective vaccines have been developed for preventing the diseases caused by *Aeromonas hydrophila* and *A. salmonicida* and used with varying results (Post, 1966; Stevenson, 1988; Hastings, 1988; Newman, 1993). The latter commonly rely on administration by injection for optimal protection. Due to the potential for vaccines as inexpensive prophylactic medicine, a substantial amount of research is being done on the development of truly effective vaccines for all finfish diseases (Ellis, 1988a and 1988b; Newman, 1993). The following sections will briefly describe the major diseases that affect salmonid culture with emphasis on two of the diseases that have had both successful, and not so successful vaccines developed. These are vibriosis and furunculosis respectively.

**Vibriosis Vaccines: A Success Story.** Possibly the most important success story in the field of salmonid culture is the development of effective vibriosis vaccines. As mentioned above, vibriosis is a general term for disease caused mainly by the two vibrio species: *V. anguillarum* and *V. ordalii* (Evelyn, 1971; Smith, 1988). A second disease, caused by *V. salmonicida*, is referred to as cold water vibriosis, or 'Hitra disease' (Holm et al., 1985). Effective immersion and/or injection vaccines have been developed for the control of all vibrio diseases, and oral vaccines for these diseases have been used with limited success (Holm and Jorgensen, 1987; Smith, 1988; for review see Newman, 1993).

Based on vaccine trials and immunological analysis (i.e. Western immunoblotting), the major protective antigens in the bacterin preparations of *V. anguillarum* and *V. ordalii* appear to be the heat stable lipopolysaccharides (LPS) (Chart and Trust, 1984; Smith, 1988; Bogwald et al., 1991). Also present in the outer membrane of these two vibrios are two minor proteins (49 - 51 kDa) that are strongly antigenic, and the major 40 kDa outer membrane protein that is weakly antigenic (presumably porin)(Chart and Trust, 1984). The antigens of *V. salmonicida* that are recognized by immune salmon sera were demonstrated to be numerous (Bogwald et al., 1991). Here it was demonstrated that sera from Atlantic salmon that were injection immunized against *V. salmonicida,* recognized a low molecular mass LPS of *V. salmonicida,* (presumably representing core antigen), while the sera from salmon immune to *V. anguillarum* recognized medium to high molecular mass LPS, (likely the O-polysaccharide of the LPS). These differences can be explained by the observation that the LPS of *V. salmonicida* is of the 'rough' type (little or no O-antigen), while the LPS of *V. anguillarum* is of the 'smooth' type (possessing O-antigen)(Bogwald et al., 1991). Also present in this immune sera were antibodies to numerous protein antigens, although these were minor compared to the response to LPS.

It appears that the majority of protective immunity to vibriosis, induced by injection or immersion vaccination, is apparently antibody mediated as several experiments have been able to demonstrate transfer of immunity via passive transfer of immune serum or antibodies from immunized rabbits or fish to naive fish (Harrell et al., 1975; Viele et al., 1980). Contrary to this evidence, oral vibriosis vaccination experiments that have resulted in protective immunity, have failed to identify circulating antibodies to any or all of these antigens (Smith, 1988).

Unfortunately, there could be a great deal of variation in the precise nature of the immune response generated by these vaccine preparations as the vaccines used were all

different. There are other possible explanations for this apparent controversy such as variation in the method of vaccine production (i.e. bacterial growth conditions), such that antigens that induce a humoral or cell mediated immune response may or may not have been included. Alternatively, the stimulation of the various branches of the immune system in fish could be highly dependent on route of vaccine administration.

**Furunculosis Vaccines: The Purpose of This Thesis.** In the past 50 years, furunculosis vaccines have probably received more attention than all other vaccines for salmonid diseases combined. The reasons for this are that the disease is both economically important, and a safe efficacious vaccine had yet to be developed. Sporadic success had been reported using any or all routes of administration, but a lack of consistently demonstrated protection has continually plagued furunculosis research (Austin and Austin, 1987; Ellis, 1988b). Vaccines based on whole/disrupted killed cells (bacterins), ECPs, and purified antigens have all been attempted with varying degrees of success (Duff, 1942; Klontz and Anderson, 1970; Paterson and Fryer, 1974; Michel, 1979; McCarthy et al., 1983; Tatner, 1987; Tatner, 1991; Hastings, 1988). Thus far, injection is the only route of administration that has afforded any reasonable levels of protection when using these types of vaccines (Hastings, 1988).

A number of studies have been carried out to elucidate which of the virulence factors and antigens of *A. salmonicida* are important in inducing long lasting protective immunity to furunculosis. These studies have revealed that *A. salmonicida* possesses a wide array of virulence associated factors including: the surface associated A-layer, (Kay et al., 1981); LPS, (Munn et al., 1982); high affinity iron sequestering systems (Chart and Trust, 1983; Hirst et al., 1991); and an overabundance of extracellular toxins and enzymes that are apparently associated with virulence (Fyfe et al., 1987). Of these virulence antigens, the A-layer has received the most attention by far (Kay et al., 1981;

Trust et al., 1982; McCarthy et al., 1983; Trust et al., 1983; Olivier et al., 1985; Kay et al., 1988).

It has been proposed that in order to resist infection by *A. salmonicida*, the fish immune system must recognize and respond to A-protein (Trust et al., 1982). In comparing the immunogenicity of various strains of *A. salmonicida*, Olivier *et al.* (1985) found that A-layer negative strains were inferior as immunogens in both fish and rabbits. In experiments that rely on the passive transfer of humoral immunity, McCarthy *et al.* (1983) demonstrated that passive immunity could only be transferred from rabbits to fish if the strain that immunized the rabbit was A-layer positive. Also, it was reported by these authors that of all bacterins tested, the only ones that conferred some level of immunity to fish were those bacterins made from a suspension of A-layer possessing *A. salmonicida* cells.

Assessments of antigens recognized by immune sera from bacterin vaccinated salmonids has revealed that antibodies rarely correlate with protection, but if present, are directed at A-protein and the C-antigen of the LPS (Chart et al., 1984; Hastings and Ellis, 1990). As far as antibodies to the ECP of *A. salmonicida*, it has been repeatedly demonstrated that the rabbit produced antibodies to between 15 and 25 different components (Hastings and Ellis, 1988; Hastings and Ellis, 1990). The antibody response of fish however, is apparently only to between 3 and 6 different components of the ECP (Ellis et al., 1988; Hastings and Ellis, 1990). Ellis *et al.* (1992) have recently developed subunit vaccines (based on purified antigen(s) that consist of two purified antigens that only weakly react with immune sera. Despite the fact that protection from other vaccines does not correlate well with antibody titre (Hastings, 1988), these authors found a strong correlation between protection and mean antibody titre in vaccinated fish. The major problem however, was that the mean antibody titre steadily declined throughout the trial (>40 weeks), and a subsequent boost only served to depress the serum titre dramatically,

coincidentally, the protection levels also fell. The authors suggest that possible explanations for this phenomenon may involve the development of tolerance to the antigens, or antigenic competition with other strong antigens in the vaccine preparation (Ellis et al., 1992). The actual antigens and precise formulation of the vaccine preparation were not disclosed by the authors, thus we can only speculate on the future of these types of vaccines.

The role of humoral immunity in the protection of fish from furunculosis has historically been assessed on the basis of either the passive transfer of immunity using either fish or rabbit sera raised against killed *A. salmonicida* cells, or by the examination of fish immune response following vaccination with a bacterin (Olivier *et al.*, 1986; Ellis *et al.*, 1988; Hastings and Ellis, 1990). Although humoral immunity has failed to correlated well with protection when measured by serum antibody titer (Olivier *et al.*, 1985; Tatner, 1991), a limited level of success has been achieved using passive transfer of anti-*A. salmonicida* antibodies from either fish (Cipriano, 1981) or rabbit sera (Marquis and Lallier, 1989), suggesting at least a partial role for humoral immunity in the prevention of furunculosis.

The above mentioned immune mechanisms of fish may have little or no effect on invading *A. salmonicida* due to the wide array of virulence factors this bacterium is equipped with. As previously mentioned, these factors exert a very complex debilitating effect on the defense of the host. The A-layer gives protection against the lytic activity of normal serum (Munn *et al.*, 1982). Fibronectin in the salmonid serum binding to the A-layer (Doig *et al.*, 1992) may shield underlying bacterial surface antigens from normal immunological recognition. Ability of the A-layer to bind immunoglobulins non-specifically (Phipps and Kay, 1988) may further shield the bacterium from other defense mechanisms including opsonic phagocytosis. The phagocytosed bacteria still have more than one chance to survive, as the assembled A-layer is highly resistant to proteolytic

attack (Chu *et al.*, 1991). Since proteases are a large part of the non-oxidative killing mechanisms of phagocytes (i.e. lysosomal degradative enzymes), the protease resistant A-layer shield may result in a decreased ability of a phagocytic cell to kill *A. salmonicida*. Phagocytes are not only unable to kill all the *A. salmonicida* cells in an established infection, but they themselves are killed after phagocytosing the bacterium. This is attributed to the cytotoxic substances produced by the bacterium that lead to a significant reduction in the amount of lymphocytes and macrophages. Macrophages infected with *A. salmonicida* cells suffer major cytoskeletal changes leading to pronounced cell rounding and complete smoothing of the cell surface. Eventually, these macrophages are unable to attach to their substratum, or lyse leaving a cytoskeletal ghost (Garduño et al., 1992).

Another of the many possible explanations for the lack of efficacy of the typical *A. salmonicida* bacterin preparations could be that antigens important for a protective immune response are not expressed by *A. salmonicida* grown in vitro using standard media preparations. Antigen expression in vivo has been examined in very few cases, likely due to unavailable or inappropriate host model systems, however, many pathogens have been examined using fluids derived from the host as growth media. These examinations have frequently revealed some novel antigen expression including the expression of capsule by *Staphylococcus aureus* (Johne et al., 1989; Karakawa et al., 1988) and *Mycoplasma dispar* (Almeida and Rosenbusch, 1991) and the expression of novel protein antigens by *Staphylococcus epidermidis* (Smith et al., 1991), *Klebsiella pneumoniae*, (Camprubi et al., 1992), *Salmonella typhimurium* (Buchmeier and Hefron, 1990), and *Campylobacter jejuni* (Panigrahi et al., 1992) just to name a few. The occurrence and significance of this phenomenon has been reviewed elsewhere (Brown et al., 1988; Smith, 1990), and these authors have stressed the importance of in vivo expressed antigens for the development of effective vaccines.

The inconsistent protection the available furunculosis vaccines provide may result from the lack of *A. salmonicida* antigens required for protective immunity in these preparations, or the necessary antigens may be improperly presented in the standard bacterin preparations. Thus, it was my goal to develop a live attenuated vaccine strain for the control of furunculosis. This thesis describes the construction of such a vaccine, and attempts to elucidate some possible explanations for its effectiveness.

# Chapter II

## Partial Characterization of D-Glucose Metabolism in Strains of *A. salmonicida.*

### Purpose:

The purpose of the experiments described in this section were to examine the metabolic pathways utilized by *A. salmonicida* for the metabolism of D-glucose.

### Summary:

Host range variants of the fish pathogen *Aeromonas salmonicida*, were examined with respect to D-glucose catabolism. The major pathway for glucose catabolism of 'typical' strains of *Aeromonas salmonicida* was the Entner-Doudoroff pathway, whereas 'atypical' strains and other members of the Vibrionaceae family, utilized the Embden-Meyerhoff-Parnas pathway. The tricarboxylic acid cycle of *A. salmonicida* was shown to be expressed only when cultured with excess glucose. However, during glucose limitation, the cycle became an unusual branched pathway characterized by high levels of isocitrate lyase activity and with no apparent ability to form α-ketoglutarate. The possible link between these findings to pathogenesis and the in vivo nutritional requirements of *A. salmonicida* is discussed.

## Materials and Methods:

**Bacterial strains and culture conditions.** All bacteria were grown in Luria broth (LB) supplemented with modified Davis salts (Somers et al., 1981), and were shaken at 250 rpm. When required, glucose was added to 1%. The solid medium used was Tryptic Soy Agar (TSA, Difco). *A. salmonicida* (typical strains A450 and A464; atypical strains A400 and A419); the fish pathogens *A. hydrophila* Ah65, and *V. anguillarum* Va775, were cultured at 20°C, and *E. coli* strain HB101 was cultured at 37°C. The media were supplemented with hemin (10 μg $ml^{-1}$) for the growth of atypical strains of *A. salmonicida*. Long term storage (>1 week) was carried out by freezing cultures at -70°C in 15% glycerol.

**Cell Fractionation.** Cell envelopes and cytosolic fractions were prepared in the following manner. Cells were harvested by centrifugation (5000 x g for 10 min at 4°C) from the appropriate growth media, washed twice in Tris buffered saline (TBS; 10 mM Tris pH 7.5, 0.15 M NaCl), and resuspended to 50 mg $ml^{-1}$ in TBS. Cell suspensions were then passed twice through a cold (4°C) French pressure cell at 16,000 psi. Unlysed cells and large cellular debris were removed by low speed centrifugation (1500 x g for 10 min at 4°C), and the membranes were then separated from the cytosolic fraction by centrifugation at 100,000 x g for 30 min at 4°C. Membranes were then resuspended in the appropriate buffer for the various enzyme assays at approximately 10 mg protein $ml^{-1}$. Membrane and cytosolic fractions were stored on ice prior to use. Protein concentrations were measured by the modified Lowry method of Markwell et al. (1978), using bovine serum albumin as a standard.

**Enzyme assays.** Phosphofructokinase (PFK; EC 2.7.1.11) activity was measured according to the method of Kotlarz and Buc (Kotlarz and Buc, 1982); 2-Keto-3-deoxy-6-phosphogluconate aldolase (KDGPA; EC 4.1.2.14) activity was measured according to the method of Allenza and Lessie (1982); Aconitase (EC 4.2.1.3) activity was measured according to the method provided by the Sigma Chemical Co. (St. Louis, MO); Isocitrate dehydrogenase (EC 1.1.1.41) activity was measured according to the method of Borthwick et al., (1984); Isocitrate lyase (EC 4.1.3.1) activity was measured according to the method of Ashworth and Kornberg (1963); α-Ketoglutarate dehydrogenase (EC 1.2.4.2) activity was measured according to the method of Smith and Neidhardt (1983); Succinate thiokinase (EC 6.2.1.4) activity was measured according to the method of Buck et al., (1985); Succinate dehydrogenase (EC 1.3.99.1) activity was measured according to the method of Sweetman and Griffiths (1971); Fumarase (EC 4.2.1.2) activity was measured according to the method of Penner and Cohen (1969); Malate dehydrogenase (EC 1.1.1.37) activity was measured according to the method of Courtright and Henning, (1970); and Malate synthase (EC 4.1.3.2) activity was measured according to the method of Kay (1972).

## Results:

**Enzymes of Intermediary Glucose Metabolism.** Subcellular fractions were assayed for the presence of the key enzymes of either the Embden-Meyerhof-Parnas (EMP) or the Entner-Doudoroff (ED) pathways, namely phosphofructokinase (PFK) or 2-keto-3-deoxy-6-phosphogluconate aldolase (KDGPA) respectively. The results from these assays are presented in Table 1. The typical strains of *A. salmonicida* (A450 and A464) were found to utilize the ED pathway for the metabolism of glucose. HB101, Va775, Ah65, and the atypical *A. salmonicida* strains A400 and A419, were all shown to preferentially utilize the EMP pathway for glucose metabolism. Also apparent from the enzyme activities is that in all cases where PFK activity was present (HB101, Ah65, A400, and A419), a basal level of KDGPA activity was also present, albeit minimal. For the typical *A. salmonicida* strains A450 and A464, only KDGPA activity could be detected, suggesting that this may be the only pathway utilized for $C_6$ carbohydrate metabolism by these strains.

**Enzymes of the Tricarboxylic Acid Cycle.** The results of an examination of the TCA cycle enzymes of *A.salmonicida* strain A450 and *E. coli* strain HB101 grown in the presence and absence of glucose are presented in Table 2. As expected, *E. coli* strain HB101 produced a full complement of TCA cycle enzymes during growth with no added glucose (Table 2). During growth with added glucose, the TCA cycle of *E. coli* was reduced to a branched pathway by the virtual elimination of α-ketoglutarate dehydrogenase activity (Table 2). Contrary to these findings, *A. salmonicida* only expressed the complete repertoire of TCA cycle enzymes when grown in the presence of

**Table 1. Key Enzymes of Glycolytic Pathways in *A. salmonicida* and Other Bacterial Strains.**

| Strain[a] | Specific Activity[b] PFK | KDPG Aldolase |
|---|---|---|
| *A. salmonicida* | | |
| Typical strains: | | |
| A450 | 0.0 | 73.1 |
| A464 | 0.0 | 54.6 |
| Atypical strains: | | |
| A400 | 50.7 | 0.3 |
| A419 | 33.8 | 0.7 |
| *V. anguillarum* Va775 | 296.5 | 1.9 |
| *A. hydrophila* Ah65 | 335.1 | 3.1 |
| *E. coli* HB101 | 178.0 | 6.4 |

[a]All strains were grown in the presence of 1% D-glucose.

[b]Specific activities are in units of nmol $min^{-1}mg^{-1}$.

**Table 2. Tricarboxylic Acid Cycle Related Enzymes of *A. salmonicida* A450 and *E. coli* HB101.**

| Enzyme | Specific Activity[a] *A. salmonicida* + Glc[b] | *A. salmonicida* - Glc | *E. coli* + Glc | *E. coli* - Glc |
|---|---|---|---|---|
| Pyruvate dehydrogenase | 45.9 | 30.8 | 62.5 | 58.3 |
| *cis*-Aconitase | 558.0 | 451.0 | 118.5 | 172.4 |
| *trans*-Aconitase | 462.0 | 570.0 | 11.1 | 5.3 |
| Isocitrate dehydrogenase | 307.0 | 0.0 | 113.9 | 437.2 |
| a-Ketoglutarate dehydrogenase | 26.5 | 0.0 | 0.7 | 54.8 |
| Succinate thiokinase | 6.1 | 5.8 | 18.4 | 12.7 |
| Succinate dehydrogenase | 16.6 | 0.0 | 119.2 | 357.0 |
| Malate dehydrogenase | 4.6 | 5.9 | 15.6 | 14.1 |
| Isocitrate lyase | 16.8 | 45.6 | 0.2 | 0.6 |
| Malate synthase | 0.0 | 0.0 | nd[c] | nd[c] |

[a] Specific activities are in units of nmol $min^{-1}mg^{-1}$

[b] +/- Glc refers to growth with or without 1% D-glucose.

[c] nd - not done.

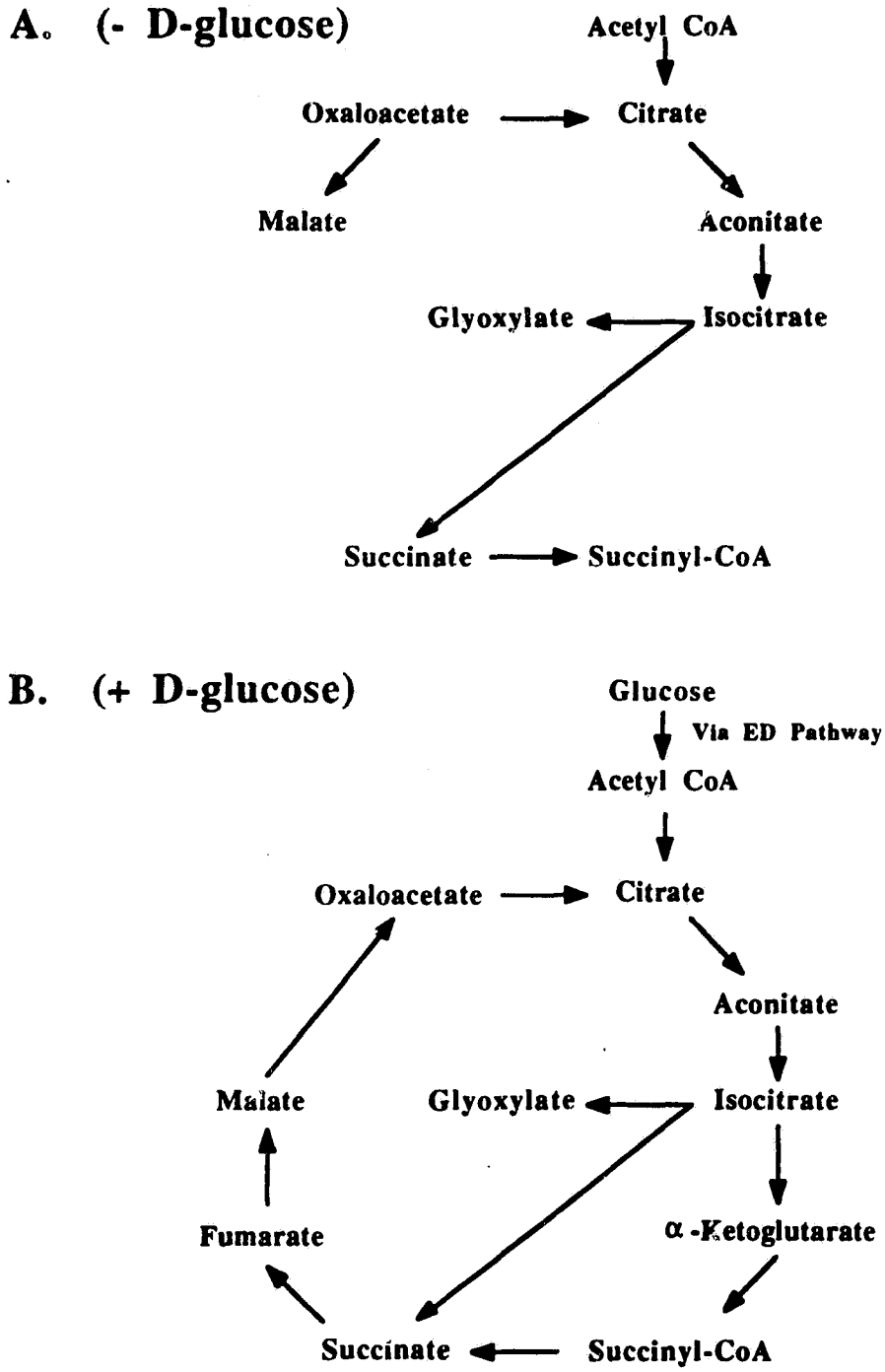


**Figure 1.** Flow of carbon in typical strains of *Aeromonas salmonicida* grown in the absence (A) or presence (B) of D-glucose. Arrows indicate the general of direction of catabolism as indicated in this study, and not the equilibrium the reaction.

glucose (Table 2; Fig. 1). Growth of *A. salmonicida* in a peptone based media without added glucose resulted in a branched TCA cycle that resembled the glyoxylate shunt (Table 2; Fig. 1). Isocitrate lyase (ICL) activity was present at all times, but was induced approximately 3 fold when *A. salmonicida* was grown in the absence of glucose. Another major difference between *E. coli* and *A. salmonicida* was demonstrated with isocitrate dehydrogenase (IDH) activity. In *E. coli*, IDH had a relatively high basal level when the cells were grown in the presence of glucose, and levels of IDH rose nearly 4 fold upon glucose limitation. However, in *A. salmonicida*, IDH activity was strictly regulated by the presence or absence of glucose, with no measurable activity when cells were grown in the absence of glucose and a high level of activity when the cells were grown with glucose.

Attempts to demonstrate the existence of the glyoxylate shunt (malate synthase), the glycerate pathway (tartronate semialdehyde reductase; EC 1.1.1.60), or the β-hydroxyaspartate pathway (*erythro*-β-hydroxyaspartate dehydratase; EC 4.1.2.38) were unsuccessful; thus the fate of the glyoxylate generated by ICL in *A. salmonicida* is unknown.

## Discussion:

These studies have demonstrated that typical strains of *A. salmonicida* appear to utilize the Entner-Doudoroff (ED) pathway for glucose metabolism. The utilization of the Embden-Meyerhof-Parnas (EMP) pathway appears to be common to all other members of the family Vibrionaceae including the atypical *A. salmonicida*. The finding that *A. salmonicida* utilizes the ED pathway (and thus gluconate), is significant, as the inability to oxidize gluconate has been regarded as a biochemical trait of *A. salmonicida* (Austin *et al.*, 1989). The negative results for gluconate oxidation were based on the detection of a pH change with an indicator such as phenol red, thus oxidization of the carbohydrate results in acid production which leads to a colour change in the peptone based media. This type of test is reliant on the lack of production of alkaline metabolites by the organism being tested (Smibert and Krieg, 1981). It was previously demonstrated that *A. salmonicida* constitutively expresses the arginine deiminase pathway (Shieh and Reddy, 1972), and the detection of acid production from carbohydrate metabolism is often masked if the peptone concentration of the assay media is high, due to the release of $NH_4$ (unpublished observations). The positive identification of KDGP aldolase activity, combined with the measurement of oxygen uptake by *A. salmonicida* in the presence of gluconate (Chapter III), essentially proves the existence of the ED pathway in *A. salmonicida* subsp. *salmonicida*. The preferential use of the ED pathway is apparently limited to typical strains of *A. salmonicida*, as only low background levels of KDGP-aldolase activity were detected of atypical strains.

The conversion of glucose to gluconate by some bacteria typically occurs in the periplasm (Gottschalk, 1986), thus allowing for the extracellular conversion of glucose to gluconate. Evidence, albeit circumstantial, that this enzymatic conversion of glucose to gluconate occurs in the periplasm of *A. salmonicida* rests with the observation that *A.*

*salmonicida* increases oxygen utilization when supplemented with extracellular 2-ketogluconate (Chapter III). It has been suggested that this type of strategy could confer a competitive advantage to the ED-utilizing bacteria as the uptake and use of gluconate is not as widespread in nature as is that of glucose, and thus this conversion reduces the effective concentration of usable carbon to other organisms competing for the same niche (Fraenkel and Vinopal, 1973; Lessie and Phibbs, 1984; Gottschalk, 1986).

Of particular interest with regard to *A. salmonicida* metabolism, is the ability to secure fish tissues as a niche. It is interesting to speculate that the degradation of fish muscle glycogen to glucose and subsequently to gluconate, confers a competitive advantage for *A. salmonicida* as no pathway exists for the utilization of gluconate in fish muscle tissue (T.P. Mommsen, personal communication). This scenario would allow for the exploitation of glycogen as a carbon source by *A. salmonicida*, without the competition with glycogen synthetic enzymes of the fish for the newly formed gluconate.

In *A. salmonicida*, unlike in *E. coli*, it was apparent that the TCA cycle was a branched pathway under glucose limitation, lacking in enzymes that produce the intermediates oxaloacetate and α-ketoglutarate (Fig 1). As these two TCA cycle intermediates are important in anaplerotic reactions, it is tempting to speculate that part of the nutritional fastidiousness of *A. salmonicida* is a requirement for compounds that are normally synthesized from these two TCA cycle intermediates. Evidence in support of this interpretation is the observation that *A. salmonicida* requires the amino acids Ala, Gly, Val, Thr, Cys, Met, His, Arg, Asp, Asn, Glu, and Gln for growth (Shieh and Reddy, 1972; Sakai, 1985). The amino acids Thr, Met, Asp, and Asn are all derived from oxaloacetate, and Glu, Gln, and Arg are synthesized from α-ketoglutarate. Ala and Val are synthesized from pyruvate, which could become limiting if gluconeogenesis were occurring, or if excessive amounts of succinate or succinyl-CoA were being removed for other anaplerotic reactions.

While this explanation for the fastidious nature of *A. salmonicida* is speculative, it stems from the inability of *A. salmonicida* to grow on any defined media tested so far. Clearly, as the development of attenuated vaccines for furunculosis in fish proceeds, the requirement for defined minimal media for the direct selection of auxotrophs will arise. The development of such media, has in all cases, been preceded by an intimate knowledge of the nutritional requirements, and thus the basic metabolism, of the organism for which the medium was developed. The pathways described in this chapter serve as a starting point to assess the nutritional requirements of *A. salmonicida*.

# Chapter III

## Isolation and Characterization of Attenuated Strains of *A. salmonicida.*

### Purpose:

The purpose of the experiments described in this section were to isolate attenuated strain(s) of *A. salmonicida* suitable for future vaccine development, and to characterize, if possible, the nature of the attenuating mutation(s).

### Summary:

A slow growing, aminoglycoside resistant mutant and a rapidly growing pseudorevertant were isolated from *Aeromonas salmonicida,* the causative agent of salmonid furunculosis. These mutants continued to elicit a variety of classical virulence factors associated with *A. salmonicida* pathogenesis. However, they differed morphologically from the wild-type and from one another with respect to A-layer organization, membrane antagonist sensitivity and particularly in aerobic metabolism. Both mutants were drastically altered in the architecture of the 2D crystalline surface array (A-layer), although both were similar to wild-type with respect to cell surface composition.

The slow-growing, antibiotic-resistant mutant differed significantly from the wild-type in an apparent loss of virtually all aerobic metabolism; the pseudorevertant had partially recovered the ability to aerobically metabolize certain carbon sources. The aminoglycoside resistant mutant of the fish pathogen *Aeromonas salmonicida*, A450-10S,

was demonstrated to be devoid of cytochromes. The partially compensating mutation in a pseudorevertant, A450-10SR, restored apparently all cytochrome types, but A450-10SR remained defective in $FADH_2$ coupling through complex II. Difference spectra of the cytochromes of the parent strain, A450, demonstrated the presence of cytochrome *c* , cytochrome *b*, cytochrome *o* oxidase and cytochrome *d* oxidase. 9-Aminoacridine fluorescence quenching indicated that these cytochromes normally allow for proton leakage, whereas membrane vesicles of the cytochrome deficient mutant A450-10S were capable of maintaining proton gradients induced by NADH for extended periods. The two mutants were both defective in their ability to generate a normal electrochemical gradient of protons, $\Delta p$, and were apparently more sensitive than the wild-type strain to acidification of the cytoplasm. Both mutants were avirulent and incapable of tissue persistence. The mutant strains A450-10S and A450-10SR were both disabled with respect to their ability to survive in different aqueous environments, suggesting that the mutations used for the construction of these strains are suitable for inclusion in vaccine strains that will be released into the environment.

Also, mutants of *A. salmonicida* strains lacking either the A-protein, O-antigen, or both of these major surface antigens were tested in Rainbow trout (*Oncorhynchus mykiss*, Walbaum) for virulence and thus their suitability as live vaccine candidates. All of these mutants were shown to be attenuated as fish receiving ~5 x $10^7$ of the respective strains showed no clinical signs of furunculosis.

## Materials and Methods:

**Bacterial strains and culture conditions.** The bacterial strains used in this study are listed in Table 3. Bacteria used in this study were grown in Luria broth supplemented with modified Davis salts (LBD, Somers et al., 1981), and were shaken at 250 rpm. When indicated the media was supplemented with glucose (1%) The solid media used was Tryptic Soy Agar (TSA, Difco). *Aeromonas salmonicida* strains were cultured at 20°C. *Escherichia coli* strain HB101 was cultured at 37°C. Long term storage of bacterial strains (>1 week) was carried out by freezing cultures at -70°C in 15% glycerol. The isolation of A-layer deficient strains was carried out by culture at 30°C as described previously (Ishiguro et al., 1981).

**Enzyme and siderophore assays.** Protease activity was assayed quantitatively using Hide Powder Azure (Calbiochem) according to the method recommended by the manufacturer, and qualitatively on skim milk agar (0.5% Difco proteose peptone No.3; 0.2% Difco yeast extract; 0.85% NaCl; 0.5% skim milk). Glycerophospholipid: cholesterol acyltransferase (GCAT) activity was measured as previously described (Thornton et al., 1988). Siderophore activity was assayed qualitatively by the media method of Schwyn and Neilands (1987) as modified by Barghouthi et al., (1989). Hemolytic activity was assessed on Human blood agar (HBA) (5% whole human blood in Difco Tryptic Soy Agar).

**Antibiotic sensitivity profiles.** Antibiotic sensitivities were determined on solid media using commercially available antibiotic discs. A 50 µl aliquot of a fresh LB grown culture containing approximately 1 x $10^9$ cells $ml^{-1}$ was spread evenly on an LB agar

**Table 3.** Bacterial strains.

| Strain | Phenotype[a] | Virulence | Source |
|---|---|---|---|
| A450 | $A^+LPS^+$ | + | Ishiguro *et al.*, 1981 |
| A450-1 | $A^+LPS^-$ | - | Ishiguro *et al.*, 1981 |
| A450-3 | $A^-LPS^+$ | - | Ishiguro *et al.*, 1985 |
| A450-10S | $A^+LPS^+$ | - | This study |
| A450-10SR | $A^+LPS^+$ | - | This study |
| A450-10SR-3 | $A^-LPS^+$ | - | This study |
| A450-1-3 | $A^-LPS^-$ | - | This study |
| A440 | $A^-LPS^+$ | - | ATCC 14174 |

[a] $A^{\pm}$ refers to whether the subunit of the A-layer is produced in detectable quantity by Coomassie Blue stained SDS-PAGE; $LPS^{\pm}$ refers to the production of O-antigen detectable by Silver stained SDS-PAGE.

plate and antibiotic discs applied prior to incubation at 20°C for two days. Values are reported as the radius of the zone of inhibition minus the radius of the disc.

**Sensitivity to membrane antagonists.** Sensitivity to membrane antagonists was assayed using sterile filter disks (6 mm) on which 50 µl of a saturated solution of the antagonist was dried. A 50 µl aliquot of a fresh LB grown culture containing approximately 1 x $10^9$ cells $ml^{-1}$ was spread evenly on an LB agar plate and the discs applied prior to incubation at 20°C for two days. Sensitivities were also expressed as the radius of the zone of inhibition minus the radius of the disk.

**Cell Fractionation.** Cell membranes were prepared in the following manner. Cells were harvested by centrifugation (5000 x g for 10 min at 4°C) from growth media, washed twice in Tris buffered saline (TBS; 10 mM Tris pH 7.5, 0.15 M NaCl), and resuspended to 50 mg wet cells $ml^{-1}$ in TBS. Cell suspensions were then passed twice through a cold (4°C) French pressure cell at 16,000 psi. Unlysed cells and large cellular debris were removed by low speed centifugation (1500 x g for 10 min at 4°C), and the membranes separated from the cytosolic fraction by centrifugation at 100,000 x g for 30 min at 4°C. Membranes were then resuspended in TBS at approximately 20 mg protein $ml^{-1}$. Outer membranes were prepared by solubilization of the inner membrane with 0.5% sodium lauryl sarcosinate (Filip et al., 1973). Periplasmic fractions were obtained by the sucrose-EDTA osmotic shock method of Willis et al. (1974). Protein concentrations were determined by the modified Lowry method of Markwell et al. (1978), using bovine serum albumin as a standard.

**Protein concentrations.** When necessary for visualization by SDS-PAGE, samples were concentrated prior to electrophoresis by ammonium sulfate or trichloroacetic acid (TCA) precipitation. Ammonium sulfate was added to 85% saturation at 0°C with gentle stirring, then left overnight at 4°C and centrifuged at 1.5 x $10^4$ x g for 30 min. TCA precipitation was accomplished by adding an equal volume of cold 20% TCA to samples on ice, followed by centrifugation and a -20°C acetone wash with subsequent drying in a desicator.

**Electrophoresis.** Samples were separated on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) according to the modified method of Laemmli (Ames, 1974). Proteins were visualized with Coomassie brilliant blue R250, and lipopolysaccharides (LPS) with the silver stain method of Tsai and Frasch (1982). Western immunoblots were performed essentially as follows: After SDS-PAGE, proteins were transferred to nitrocellulose paper as described by Towbin et al., (1979). Following blocking with 3% Skim milk, the blots were incubated with 1,000 fold dilutions of the appropriate polyclonal rabbit sera directed against formalinized whole cells. After extensive washing, these blots were then incubated with 1 $\mu g\ ml^{-1}$ alkaline phosphatase conjugated goat anti-rabbit antibodies (Caltag), and developed as described by Blake et al. (1984). All washes were performed in 1.5 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4 (NET buffer), containing 0.05% NP40 (Polyethylene glycol-p-isooctylphenyl ether; Calbiochem). All antibody incubations were performed in NET buffer containing 0.05% NP40 and 1% Skim milk.

**Lipid and fatty acid analysis.** Total lipids were extracted from 0.5 g wet cell paste according to the method of Folch (1957). Determination of lipid content was performed by silica gel G TLC analysis using Chloroform: Methanol: Water (75:25:3) as a solvent. Visualization of the phospholipids was carried out using iodine vapour. Fatty acids were

converted to methyl esters prior to separation and quantitation by gas-liquid chromatography (GLC). This was done by first resuspending half of the lipids from the Folch isolation in 1 ml benzene. To this, 2 ml of 0.5 M sodium methoxide in methanol was added, and the solution heated to 50°C for 10 min. After the heating, 0.1 ml glacial acetic acid and 5 ml water were added prior to extraction of the esterified fatty acids with 2 x 5 ml of hexane. The recovered hexane fractions were then dried over anhydrous sodium sulfate containing 10% potassium bicarbonate, and gravity filtered through Whatman 3 M paper. The hexane was removed under a stream of $N_2$, and the lipids resuspended in 200 µl chloroform. The lipids were stored under $N_2$ at -30°C until use.

GLC was performed using a Varian model 3700 gas chromatograph fitted with a Varian CDS 111 integrator. The column used was a 6' x $^1/_8$" Supelco EGSP-2 ($^{100}/_{120}$) - Gaschrome 2 IAA56, using $N_2$ as a carrier gas. Fatty acid esters were detected by flame ionization. Identification of fatty acid esters was done by comparisons of retention times with those of known standards.

**Electron microscopy.** Negative staining of whole cells and A-layer were carried out by taking direct impressions from bacterial colonies grown on TSA media (taken by touching the surface of a colony with a Formvar coated copper grid). The grids were then immediately floated on unbuffered saturated ammonium molybdate for staining.

Immunogold labeling was performed essentially by the method of DeMay and Moeremans (1986). Briefly, bacterial cells were fixed in 4% freshly depolymerized paraformaldehyde and 0.1% glutaraldehyde in 10 mM $Na_2HPO_4$; 0.85% NaCl; pH 7.4, (PBS), at room temperature for not less than 2 h. The fixed cells were pelleted and embedded in 0.3% agarose prior to dehydration and final embedding in LR White resin (London Resin Co. Ltd. Surrey, England). Thin sections, mounted on nickel grids, were

labelled *in situ* using polyclonal rabbit IgG directed against A-protein and 10 nM protein A-gold probe (Janssen Biotech, NV, Olen, Belgium).

Specimens were examined in a Philips EM-300 transmission electron microscope (Philips Electronic Instruments Inc., Mahwah N.J., U.S.A.) at an accelerating voltage of 60 kV.

**Dehydrogenase Assays.** Succinate dehydrogenase activity was measured in whole membranes by the reduction of 2,6-dichlorophenolindophenol (DCIP) according to the method of Sweetman and Griffiths (1971). Glycerol-3-phosphate dehydrogenase was assayed by measuring the reduction of 3-(4,5-dimethylthiazolyl-2-)2,5-diphenyltetrazolium bromide (MTT). The starting reaction mixture contained 50 mM Potassium phosphate buffer (pH 7.5), 1 mM KCN, 1 mM phenazine methosulfate, 50 µg MTT and 100 µl of whole membrane preparation (containing approximately 2 mg total protein). While monitoring at a wavelength of 550 nm, this mixture was allowed to react for 2-3 min prior to the addition of DL-α-glycerol phosphate to 100 mM in order to achieve a stable baseline. The reaction rate remained constant for 3-4 min.

**Oxygen Consumption.** The ability of the different strains of *A. salmonicida* to utilize different carbon sources aerobically was determined with a Clarke-style oxygen electrode. Briefly, whole cells, harvested from exponentially growing aerated cultures, were washed in TBS and starved for 6 hr in the same buffer in order to reduce levels of endogenous oxidizable carbon sources. These starved cells were resuspended to 5 mg wet cells $ml^{-1}$ in 3 ml modified Davis salts media and added to the incubation chamber of an oxygen electrode (YSI Inc., model 53). After monitoring the baseline for 5 min, the carbon source to be tested was added to 50 mM and the consumption of $O_2$ monitored for 3 min.

**Cytochrome Analysis.** Bacterial cytochromes were characterized spectrophotometrically using dithionite reduced-minus-peroxide oxidized spectra recorded at 77 K in a SLM-Aminco dual wavelength spectrophotometer. Briefly, cells were harvested from liquid media, washed with PBS, and resuspended to 20 optical density units at 650 nm. Duplicate samples of the cells were treated with sodium dithionite or hydrogen peroxide and placed in one of two cuvettes, frozen in liquid nitrogen, and differential spectra recorded with a wavelength scan between 450 and 700 nm.

**Fluorescence Quenching.** Energization of the bacterial membranes was assayed using the quenching of 9-aminoacridine (9-AA) fluorescence with an excitation wavelength of 412 nm and emission wavelength of 525 nm. Membranes, prepared as described, were homogenized in TBS, pH 7.5, at 100 mg wet weight $ml^{-1}$ to produce everted vesicles. The assays contained 50 µl of the membrane suspension in 5 µM 9-AA, 300 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES/KOH pH 7.5 in a final volume of 3 ml. NADH was added to a final concentration of 2.5 mM where required.

**Determination of ΔpH and ΔΨ.** Bacteria for the following determinations were harvested by centrifugation from LBD (50 ml each), washed, and resuspend to 10 $OD_{650}$ (1.5 mg dry wt $ml^{-1}$) in 100 mM Tris, 1 mM EDTA, pH 8.0. These cells were then incubated at 20°C for 5 min with shaking (200 rpm). The assays for cellular volumes, ΔpH, or ΔΨ all used these cell preparations within 3 hours of preparation.

Cellular volumes were calculated with $^3H_2O$ and [$^{14}$C]dextran in the following manner. Prepared samples of each strain (1 ml of a 1 $OD_{650}$ suspension), were mixed with $^3H_2O$ and [$^{14}$C]dextran to a final activity of $9.09 \times 10^{-5}$ µCi $\mu mol^{-1}$. Total radioactivity per assay was determined from duplicate 5 µl samples removed at time zero.

The remainder was incubated at 20°C for 10 min. After the incubation, triplicate 100 µl samples were filtered through 0.45 µm polycarbonate filters (Nucleopore) that had been presoaked in 1% polyvinylpyrolidine. The filters were placed in 5 ml of Scintillation fluid, left in the dark for 16 h, and radioactivity assayed in an LKB 1219 scintillation counter. Total water associated with the cell pellet was calculated as the $^3H_2O$ associated with the cell pellet divided by the total $^3H_2O$ per µl of reaction mix. Extracellular $H_2O$ was equal to the disintegration's per minute of [$^{14}$C]dextran associated with the filter divided by the total [$^{14}$C]dextran per µl of assay mix. Intracellular water was equal to the total water minus the extracellular water.

The method for ΔpH measurement involving the distribution of [$^{14}$C]benzoic acid across the cytoplasmic membrane was determined by incubating 1ml aliquots of 1 $OD_{650}$ of cells with 15 µM [$^{14}$C]benzoic acid (specific activity of $9.09 \times 10^{-5}$ µCi µmol$^{-1}$) for 10 min, followed by filtration through polycarbonate filters (0.45 µm) and washing with 1 ml of 0.1 M LiCl. Calculations of $[benzoate]_{in}$ and $[benzoate]_{out}$ were based on the cell volumes determined from the above measurements. The formula used to calculate ΔpH was:

$$\Delta pH = \log\{[benzoate]_{in}/[benzoate]_{out}\}$$

(Rottenberg, 1979)

The accuracy of this method was determined by comparing the values obtained by this formula with calculations based on a measured external pH, combined with calculated internal pH using the formula:

$$pH_{in} = \log[[benzoate]_{in}/[benzoate]_{out}\,(10^{pK+10^{pH_{out}}})-10^{pK}]$$

(Rottenberg, 1979).

In all cases the values of ΔpH determined by both methods were within 0.5%.

The measurement of $\Delta\Psi$ was carried out by examining the distribution of $^{86}Rb$ across the cytoplasmic membrane. Valinomycin was added to 20 μM in 5 ml of cells at 10 $OD_{650}$ in 100 mM Tris, 1 mM EDTA, pH 8.0., and the cells incubated for a further 10 min at 20°C with shaking (200 rpm). Following valinomycin treatment, the cells were harvested, washed, and resuspended to 5 $OD_{650}$ in 200 mM Na-Hepes buffer, pH 7.2, with 10 mM glucose (HG buffer). Cells were used within 0.5 hours after this treatment. $\Delta\Psi$ measurements were initiated by the addition of 20 μl of $^{86}Rb$ solution (1mM $^{86}RbCl$ at 20 μCi $ml^{-1}$) to 2 ml aliquots of prepared cells which were then incubated at 20°C for 4 min. Aliquots of these mixtures (0.5 ml, in triplicate) were then filtered through 0.45 μm (Millipore) filters using a manifold connected to a vacuum pump with an in line cold trap and a carbon filter. Each filter was then washed twice with 1ml of HG buffer, and assayed for radioactivity. Also, 100 μl of the remaining mixture was counted in the presence of an unused filter to determine total radioactivity per assay.

The formula used for the calculation of $\Delta\Psi$ was:

$$\Delta\Psi = -59.9(^{89}Rb_{in} / {}^{89}Rb_{out})$$

(Rottenberg, 1979).

**Analysis of Intermediates of Glucose Metabolism.** In order to examine the metabolic effects of cytochrome loss, cells were grown in the presence of [U-$^{14}$C]glucose, and the metabolites released to the culture supernatant were examined by cellulose thin layer chromatography. Cells were first grown to 5 x $10^8$ cfu $ml^{-1}$ (1 $OD_{650}$) in LBD at 20°C with shaking. Aliquots (2 ml) of these cells were harvested by centrifugation (1000 x g, at 20°C for 10 min), resuspended in 2 ml of fresh LBD with 2 μM [U-$^{14}$C]glucose (2μCi $ml^{-1}$), and incubated for 30 min at 20°C with constant shaking (200 rpm). Culture supernatants were separated from the cells by centrifugation (14,000 x g, at 20°C for 2

min), and acidified by the addition of 50 µl of 1 M HCl. The acidified supernatants were then lyophilized and resuspended in 100 µl distilled $H_2O$. These samples were then applied to a plastic backed cellulose TLC plate and resolved for approximately 45 min using anhydrous diethyl ether, formic acid, and distilled water (7:2:1) as a solvent. The TLC plate was then placed in an autoradiography cassette with Kodak XAR5 film for 4 days, and the autoradiogram developed.

**Virulence assays.** Virulence of the mutants was assayed by injection of the test organism into either juvenile rainbow trout (*Oncorhynchus tmykiss*, Walbaum), or juvenile Atlantic salmon (*Salmo salar*)(see specific tests), both of which were between 5 and 15 g. Briefly, overnight cultures were diluted to ~5 x $10^8$ cfu $ml^{-1}$ with sterile 0.85% saline and 0.1 ml (containing ~5 x $10^7$ cfu) was injected intraperitoneally. Any strains causing mortalities at this level of inoculation were determined to be still virulent.

Tissue persistence was assayed by either injection of target fish with 0.1 ml of saline containing 1 x $10^7$ cfu of the appropriate strain or by immersion in 1 x $10^7$cfu $ml^{-1}$ of the appropriate strain, the method of administration for the various groups is given in the data that follows. All immersion challenges of fish with the different strains were performed with constant aeration. At several timepoints, fish were sacrificed and *A. salmonicida* was cultured from various target tissues. The isolation of the test strain from the target tissues was carried out by incubation of 0.1 g tissue samples from euthenised fish in LB-Davis for 72 hr at 20°C, followed by examination of plated samples for typical pigment producing colonies on TSA with Congo red (30 µg $ml^{-1}$). Putative *A. salmonicida* colonies were positively identified by slide agglutination, or indirect immunofluorescence microscopy using polyclonal rabbit antiserum raised against *Aeromonas salmonicida*. To test for persistence in non-target species, the sculpin (a suspected carrier of *A. salmonicida*) was infected and tested as above.

Persistence of A450-10SR and A450-10SR-3 was also assessed in prednisolone treated fish. Prednisolone acetate was emulsified in molten (40°C) cocoa butter at 1.4 mg $ml^{-1}$. Rainbow trout (6-8 g) were anesthetized with MS222 (80 mg $l^{-1}$) then injected intraperitoneally (IP) with 0.1 ml of the molten emulsion giving a final dose of prednisolone acetate of 20 μg $g^{-1}$ of fish. Following a 15 minute recovery period, the fish were then divided into 4 groups of 35 fish for subsequent injections as follows:

**Group 1:** 0.1 ml sterile saline by IP injection.

**Group 2:** 0.1 ml of saline containing 1 x $10^7$ A450 (virulent wild type).

**Group 3:** 0.1 ml of saline containing 1 x $10^7$ 10SR (live vaccine strain #1)

**Group 4:** 0.1 ml of saline containing 1 x $10^7$ 10SR-3* (live vaccine strain #2)

(* 10SR-3 is a derivative of 10SR that lacks A-layer).

Ten days following initial prednisolone treatment, all surviving fish were given a second identical dose of prednisolone.

On day one, 5 fish from all groups were sacrificed and samples of spleen and kidney were cultured for the presence of bacteria to ensure that the initial exposure was sufficient to initiate an infection. All groups receiving bacteria tested positive. The tissues were removed aseptically and added to 5 ml of Tryptic soy broth. These cultures were then incubated at 20°C for 24 hours and plated on media for the identification of *Aeromonas salmonicida*. This media consisted of TSA with vancomycin (20 μg $ml^{-1}$) and Congo red (30 μg $ml^{-1}$). Identity was confirmed using slide agglutination with specific antisera for *A. salmonicida*. The control fish that received only saline and prednisolone were culture negative, while fish receiving A450, A450-10SR, and A450-10SR-3 were positive for these organisms. All experimental fish were maintained at

13°C(±1°C) in a continuous flow of dechlorinated city water before, and during experiments.

Serum resistance of bacteria was determined by the method of Munn et al. (1982) using fresh human sera. Control samples contained heat inactivated serum (55°C for 15 min).

**Survival of Attenuated *A. salmonicida* Strains in Aqueous Environments.** All survival experiments were carried out in 100 ml suspentions in 250 ml Erlenmeyer flasks, and were shaken (250 rpm) at the given temperatures. Sea water was collected locally, and the ground water was sampled from the input water to the aquatic facility at U. of Victoria. All water samples were filter sterilized ( 0.45 μm ). Starting cell densities were between $10^4$ and $10^5$ cells per ml. The experiment was performed three times with virtually identical results. All samples were plated in duplicate, and data on graphs represents average values. When starting cell densities were increased to $10^6$ cells per ml. (10 to 100 fold increase) the persistence was increased by less than 50%, likely due to cross feeding of live cells on debris of dead cells (data not shown).

## Results:

**Mutant isolation.** The wild-type *A. salmonicida* A450 was grown at 20 C to mid log phase (1.5 $OD_{650}$) in Luria broth (LB)-Davis + 2% glucose. At this time, streptomycin was added to 100 $\mu g\ ml^{-1}$, and allowed to grow for a further 16 hr at 20 C. Aliquots containing approximately 1 x $10^8$ cfu were plated on LB-Davis agar containing streptomycin at 250 $\mu g\ ml^{-1}$ and incubated at 20°C for 72 h. Slow growing streptomycin resistant colonies ($Str^r$) which arose at a frequency of 1 x $10^{-7}$, were then restreaked on the same medium for purity and cultures from single colony isolates frozen at -70°C in 15% glycerol-LB. One such mutant, A450-10S, typical of these slow growers, was found to be resistant to streptomycin to levels in excess of 2 mg $ml^{-1}$, and grew more slowly and to a lesser degree than the wild-type strain (Fig. 2). When A450-10S was grown in the absence of streptomycin, fast growing streptomycin sensitive ($Str^s$) apparent revertants, or pseudorevertants, arose at a frequency of 1 x $10^{-7}$ above the slow growing A450-10S background. One of these, A450-10SR, was selected for further study and purified by repeated single colony isolation. In growth experiments A450-10SR was similar to the wild-type A450 with respect to growth rate and yield (Fig. 2). Repeated attempts to revert A450-10SR to a slow growing, $Str^r$ phenotype by selection for streptomycin resistance on plates containing 100 or 250 $\mu g\ ml^{-1}$ were unsuccessful suggesting a separate non-reverting mutation, perhaps due to a deletion or genomic rearrangement , led to strain A450-10SR.

**Phenotypic properties.** When the mutant A450-10S and apparent revertant A450-10SR were screened for the classical virulence phenotypes associated with virulent *A. salmonicida* (Trust, 1986), it was found that both strains resembled the virulent parental

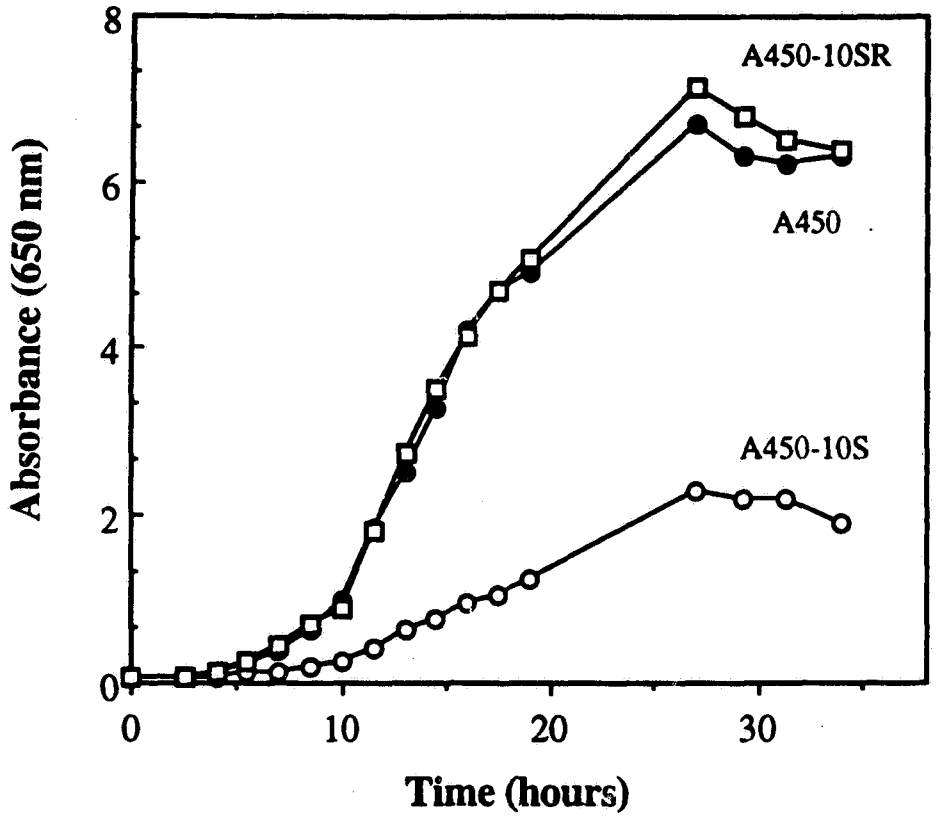


**Figure 2.** Growth curve of *A. salmonicida* A450 in comparison to the mutant strains A450-10S and A450-10SR. All cultures were 100 ml in 500 ml flasks, and were grown at 20°C with shaking at 250 rpm in LB. One absorbance unit is equivalent to 0.35 mg dry wt cells $ml^{-1}$.

strain A450 with respect to these markers. Thus these strains were agglutinated by specific antiserum to the A-protein of the A-layer (Kay et al., 1981), and to lipopolysaccharide (LPS) (Chart et al., 1984), and were positive for the extracellular factors: hemolysin and proteases (Ellis et al., 1981), as well as siderophores (Chart and Trust, 1983). Therefore, the ability of these mutants to synthesize the principle surface antigens and the secretion of the usual extracellular proteins was judged to be qualitatively unimpaired.

The antibiotic sensitivity profiles (Table 4) indicated that A450-10S harbors a mutation that confers general resistance to aminoglycoside antibiotics, and that A450-10SR had, at least with respect to this phenotype, reverted to wild-type sensitivity. It is interesting to note that the mutant and the apparent revertant have wild-type sensitivities to both penicillin and tetracycline.

**Cell composition.** To obtain a quantitative assessment of the cell surface composition, both mutants (A450-10S and A450-10SR) and wild-type (A450) cells were fractionated into inner membrane, outer membrane, periplasm, and whole cell lysates. These fractions were analyzed by SDS-PAGE combined with protein, silver, and immunochemical staining (Fig. 3). Whole cell lysates (Fig. 3A), while naturally complex due to the large number of proteins represented, were similar in most aspects, although small changes would not easily be seen. The outer membrane fractions (Fig. 3B) were not strikingly different since all three strains contained the two principal proteins of this fraction, A-protein (Kay et al., 1981), the main component of the 2D crystalline array (Dooley et al., 1989), and the lower band representing the major outer membrane porin of this strain (Darveau et al., 1983). The ratio of these proteins appeared to be reversed in A450-10S, but returned to normal in A450-10SR. The minor protein bands appearing in the outer

**Table 4. Antibiotic sensitivities of A450, A450-10S, and A450-10SR**

| | Sensitivity (mm)[a] | | |
|---|---|---|---|
| Antibiotic[b] | **A450** | **A450-10S** | **A450-10SR** |
| Sm | 4 | 0 | 4 |
| Nm | 5 | 0 | 4.5 |
| Tm | 6 | 1 | 6 |
| Vm | 0 | 0 | 0 |
| Km | 6 | 1.5 | 6 |
| Gm | 6.5 | 0.5 | 6 |
| Am | 1 | 0 | 1 |
| Pen | 15 | 16 | 16 |
| Tc | 9 | 9.5 | 9 |

[a] Values represent the zones of growth inhibition, (radius of zone minus disk radius).

[b] Sm: Streptomycin; Nm: Neomycin; Tm: Tobramycin; Vm: Vancomycin; Km: Kanamycin; Gm: Gentamycin; Am: Aureomycin; Pen Penicillin G; Tc: Tetracycline.

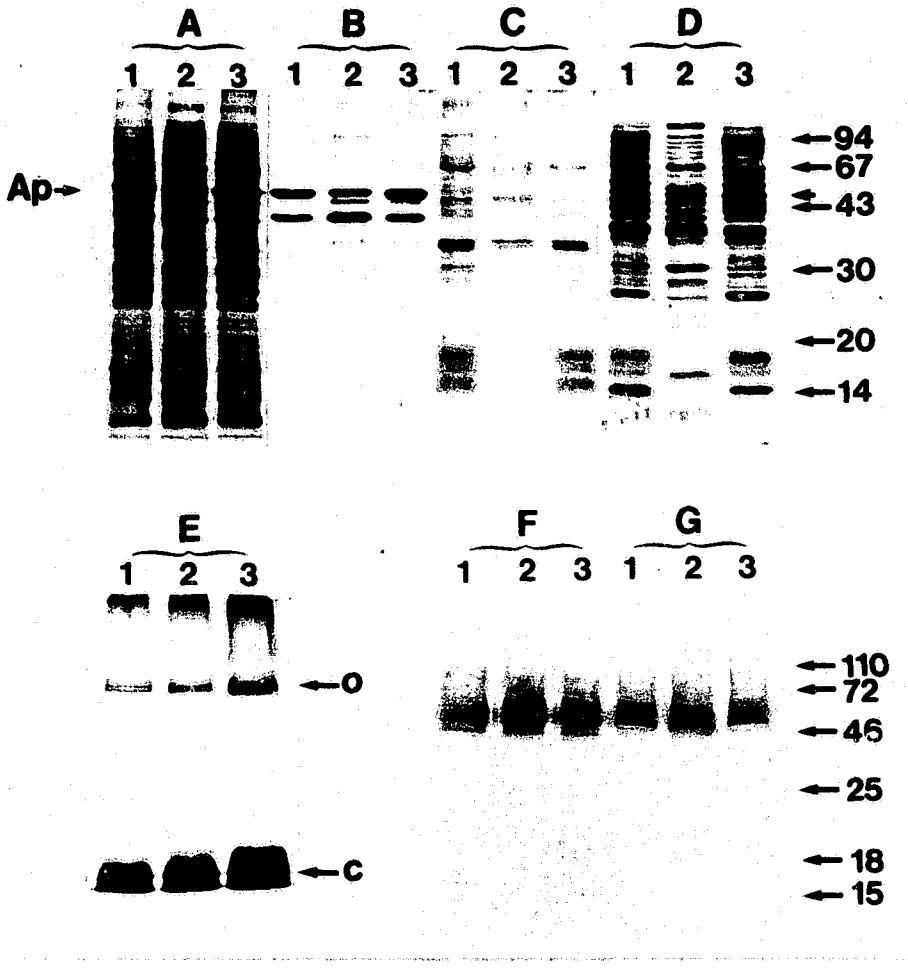


**Figure 3.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cellular fractions from: *A. salmonicida* A450 (lane 1), A450-10S (lane 2), and A450-10SR (lane 3). A-D represent Coomassie blue stained samples of whole cells, outer membrane, inner membrane, and periplasm respectively; Ap indicates A-protein. E represents silver stained lipopolysaccharides from the three strains, O, indicates lipopolysaccharide, and C indicates core lipopolysaccharide. F and G represent immunoblots of whole cell lysates and outer membranes respectively. The primary antibody was rabbit immune serum raised against formalin fixed whole A450 cells. The secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase.

membrane, particularly with A450-10S, were unidentified but were virtually absent once more in strain A450-10SR. The inner membrane fraction (Fig. 3C), indicated that several proteins were either absent - especially those of lower $M_r$ - from stain A450-10S or were grossly underrepresented, a pattern which was partially corrected with respect to strain A450-10SR. However, the periplasmic fraction (Fig. 3D) showed the greatest and clearest difference in protein composition, especially for strain A450-10S. The apparent revertant, A450-10SR, displayed a periplasmic protein profile overall only slightly different from the wild-type but still with distinct minor differences indicating once more that a complete reversion to wild-type had not occurred. All three strains still produced their normal complement of lipopolysaccharides (Fig. 3E), comprising the core region lipooligosaccharide (lower band) and the O antigen containing complete LPS, although strain A450-10SR appeared to produce slightly more than the other strains. In addition, Western blots of whole cell lysates (Fig. 3F) as well as the isolated outer membrane fraction (Fig. 3G), using antisera raised against whole cells of the parent strain, indicated that all three strains were qualitatively and quantitatively immunogenically similar. Importantly, with respect to the major cell surface components and immunogens these three strains were strikingly similar.

In addition to A450-10S and A450-10SR, other mutant strains which were included in virulence assays for comparison (see Table 1 for descriptions) were electrophoretically analysed in order to verify the presence or absence of the major antigens LPS and A-protein. Figures 3 and 4 show that the only mutant strain possessing both A-protein (Fig. 4A, lane 2) and a complete LPS (Fig. 4B, lane 2) is A450-10SR. Strains A450-10SR-3 (Fig. 4A, lane 3), A450-3 (Fig. 4A, lane 4), A450-1-3 (Fig. 4A, lane 6), and A440 (Fig. 4A, lane 7) all lack A-protein as a result of culture at elevated temperatures. In Figure 4B, a silver stain of proteinase K digested cell lysates, strains A450-1 and A450-1-3 are both shown to be lacking in the high molecular weight, O-

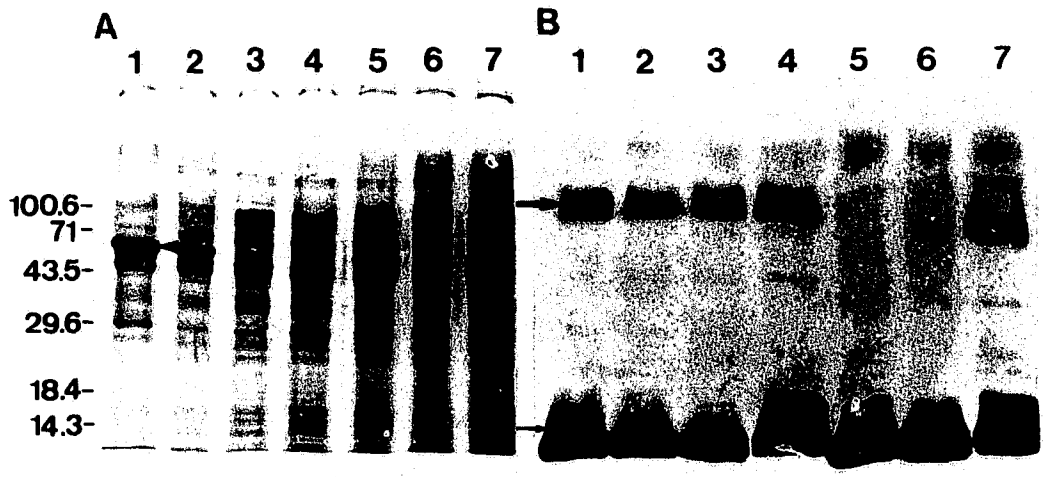


**Figure 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of attenuated *A. salmonicida* strains. **A** represents Coomassie blue stained whole cell lysates and **B** represents silver stained proteinase K digested cells. The position of A-protein, the subunit of the regular surface array, or A-layer, is indicated (arrow head) as is high MW LPS (large arrow) and core antigen(small arrow). The lane contents are MT26 (lane 1), 10SR (lane 2), 10SR-3 (lane 3), A450-3 (lane 4), A450-1 (lane 5), A450-1-3 (lane 6), and A440 (lane 7). Positions of MW standards are indicated (kDa).

antigen containing LPS. Other than these major differences, all strains are apparently qualitatively similar with respect to both protein and carbohydrate content.

**Lipid and fatty acid content of the cells.** Analysis of the major lipids by TLC revealed no differences between the wild type strain A450, and the mutants A450-10S and A450-10SR (data not shown). However, analysis of the fatty acid contents of the three strains by gas chromatography (Table 5) revealed that A450-10S had undergone a significant decrease in the relative amount of long chain fatty acids. It appears that the unsaturated fatty acid linoleic acid(18:1) was only 65% of the level found in A450 and A450-10SR, while palmitoleic acid (16:1) in A450-10S was approximately 112% of the level found in these other two strains. The inclusion of *E. coli* HB101 as a control illustrated the vast difference between *A. salmonicida* and *E. coli* with respect to individual fatty acids, but also demonstrated that the overall ratio of long to short fatty acids is comparable.

**Electron microscopy.** Electron microscopic observation of these strains demonstrated some interesting features of these mutants (Fig. 5). Panel A depicts the cell morphologies of the mutants in comparison to the wild-type. Both A450-10S and A450-10SR cells are slightly smaller and are, in general, coccoid in form. Panel B represents a negative stain of the A-layer of these organisms. The wild-type A-layer shows the typical 2D tetragonal array of this layer, while the layers from the two mutants contained regions that clearly differ in pattern from the wild-type. The A-layer from A450-10S displayed an altered morphology with regular white or unstained areas running at 45° to the usual tetragonal array; the A-layer from A450-10SR is seen in some areas to be very similar to the wild-type array (upper split frame), but in preparations from A450-10SR colonies older than three days the array was commonly without regular pattern perhaps due to a high degree of stacking of this layer (lower split frame). Panel C represents thin sections of cells

**Table 5. Fatty Acid Content[a] of strains A450, A450-10S, A450-10SR and *E. coli* HB101.**

| Fatty Acid[b] | A450 | A450-10S | A450-10SR | HB101 |
|---|---|---|---|---|
| < C-15 | 3.38 | 4.52 | 3.32 | 1.85 |
| C-16:0 | 22.72 | 22.71 | 22.42 | 44.90 |
| C-16:1 | 53.22 | 59.61 | 53.64 | 5.41 |
| C-18:1 | 20.45 | 13.16 | 20.61 | 4.51 |
| Less than C-16:2[c] | 79.33 | 86.84 | 79.38 | 81.93 |
| Greater than C-16:2[c] | 20.67 | 13.16 | 20.62 | 18.07 |

[a] Relative amounts of different fatty acids are expressed as a percent of the total.

[b] Only the individual percentages of the major fatty acids are shown.

[c] These calculations are based on all fatty acids detected by gas chromatography.

treated with anti-A protein immunoglobulin and Protein A-colloidal gold conjugate. In the parent strain the colloidal gold label is fairly uniformly distributed at the cell periphery, but becomes unevenly distributed in A450-10S and even more so in A450-10SR. These results demonstrate that in all cases the layer is present and peripheral to the outer membrane, but that it adopts different arrangements, and disposition in each of the three strains.

**Cell envelope integrity.** To assess whether these mutants were impaired with respect to membrane integrity, their sensitivity to a panel of potential membrane antagonists was assessed, the results are depicted in Figure 6. Strain A450-10S was unusually sensitive to nonionic (NP40), anionic (SDS), and cationic detergents (CTMB, ATMB, and THA). Although altered, A450-10SR was more similar to the wild-type in its sensitivity pattern, being insensitive to nonionic detergents, less sensitive to the anionic detergent, but slightly more sensitive to the cationic detergents. These studies suggested that changes had occurred in the integrity of the mutants cell surface and/or membranes; the mutant A450-10S became more sensitive and the apparent revertant A450-10SR partially regained the properties of the wild-type strain A450.

**Capacity for Aerobic Metabolism.** The observed reduction in growth rate and yield of A450-10S, led to the suspicion that a major dysfunction in cellular metabolism and/or energy metabolism was present. Using a Clarke electrode to monitor oxygen consumption as an indicator of oxidative metabolism it was shown that both mutants differed from A450 in their ability to oxidize various carbon sources (Table 6). Strain A450-10S was severely impaired, unable to oxidize any of the carbon sources tested, whereas A450-10SR regained, or in some cases partially regained, the ability to oxidize some carbon sources tested.

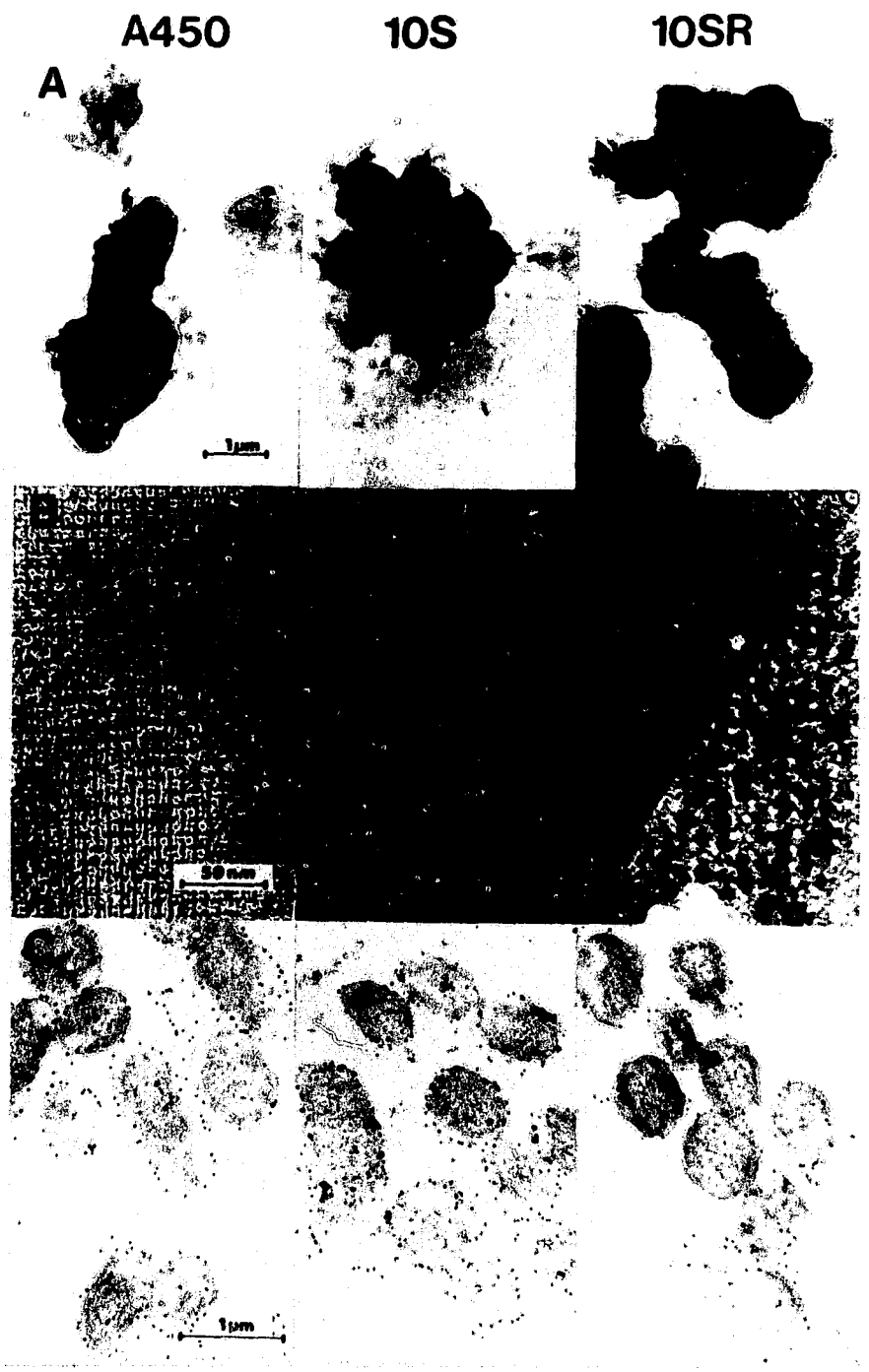


**Figure 5.** Electron micrographs of negatively stained whole cells (A), A-layer (B), and immunogold labelled thin sections (C) of the strains *A. salmonicida* A450, A450-10S and A450-10SR. In (C ), the primary antibody was affinity-purified anti-A-protein IgG. The secondary label was protein A-colloidal gold conjugate. The scales for A, B, and C are indicated by the bar in the middle column.

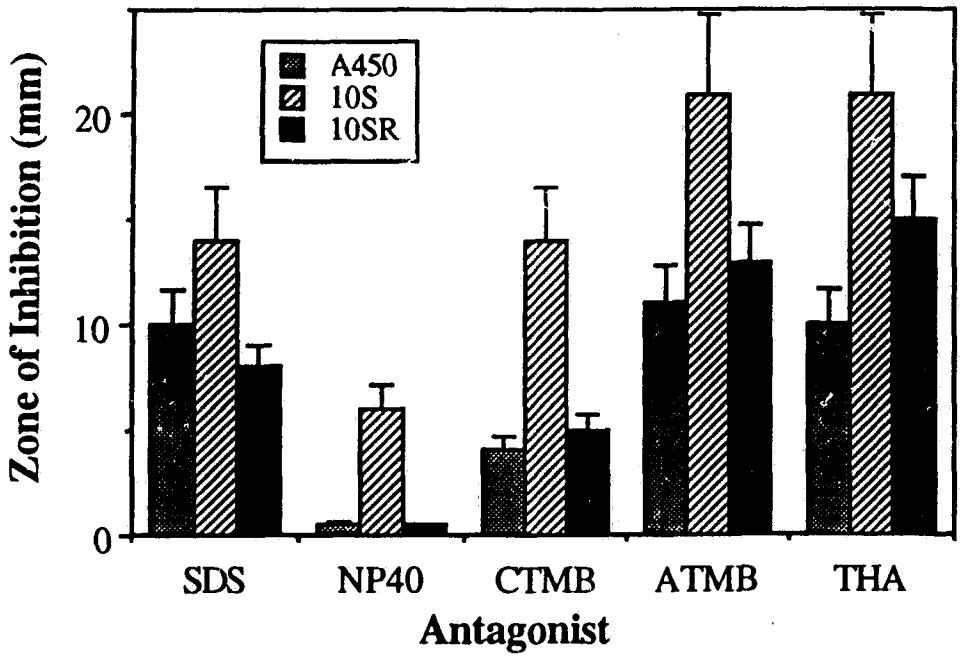


**Figure 6.** Sensitivity of *A. salmonicida* strains A450, A450-10S, and A450-10SR to membrane antagonists. The membrane antagonists are: SDS, sodium dodecyl sulfate; NP40, nonidet P40; CTMB, cetyltrimethyl ammonium bromide; ATMB, alkyltrimethyl ammonium bromide; THA, trihexylamine. Error bars indicate standard deviation calculated from two trials.

**Table 6. Substrate Oxidation of *A. salmonicida* strains**

| | $O_2$ Consumption ($ml\ O_2\ h^{-1}\ mg_{(wet\ cells)}^{-1}$) | | |
|---|---|---|---|
| **Substrate** | **A450** | **A450-10S** | **A450-10SR** |
| Glucose | 4.14 | 0 | 0 |
| Sucrose | 2.64 | 0 | 0 |
| Gluconate | 5.40 | 0 | 0 |
| 2-Ketogluconate | 1.70 | 0 | 0 |
| Lactose | 0.55 | 0 | 0 |
| Xylose | 1.15 | 0 | 0 |
| Sorbitol | 1.73 | 0 | 0 |
| Glycerol | 3.60 | 0 | 0 |
| Succinate | 0.48 | 0 | 0 |
| Malate | 1.0 | 0 | 0.22 |
| Peptone | 1.24 | 0 | 1.38 |
| Galactose | 1.91 | 0 | 1.55 |
| Mannose | 2.16 | 0 | 2.15 |
| Maltose | 2.80 | 0 | 1.20 |
| Lactate | 1.46 | 0 | 1.27 |

**Cytochrome Analysis.** The defects observed in the aerobic metabolism of A450-10S and A450-10SR suggested serious defects in terminal respiration. In order to examine whether this was due to alterations in the electron transport chain, cryoscopic difference spectra were recorded for the mutants and compared to the wild type parental strain. Reduced minus oxidized difference spectra demonstrate that the cytochromes present in wild type *A. salmonicida* show major absorbance maxima that correspond to *c* (~550 nm), *b* (~557 nm and ~595 nm), and *d* (625-635 nm) type cytochromes (Fig. 7). Cytochrome *o* was detected in A450 using room temperature difference spectra of cells treated with CO (data not shown). A450-10S was devoid of all cytochrome types, showing only a small peak at approximately 550 nm (Fig. 7). The area under this 550 nm absorption maxima of strain A450-10S represents approximately 5% of the area under this absorption maxima of the wild type strain A450. A450-10S also contained no cytochrome *o* (data not shown). The pseudorevertant, A450-10SR, appeared to have regained a full complement of cytochromes at apparently normal levels (Fig. 7), including cytochrome *o* (data not shown).

**Succinate and Glycerol-3-phosphate Dehydrogenase Activity.** In order to assess the nature of the defect in A450-10SR that resulted in a deficiency of most aerobic capabilities despite an apparently complete cytochrome repertoire, activities of succinate dehydrogenase (SDH) and glycerol-3-phosphate dehydrogenase (GPDH), both of which pass electrons into the electron transport chain at complex II via FAD, were determined from crude membrane preparations. The activities of these two enzymes are presented in Table 7. It is evident from the data that the three strains A450, A450-10S, and A450-10SR all produce SDH and GPDH at similar levels.

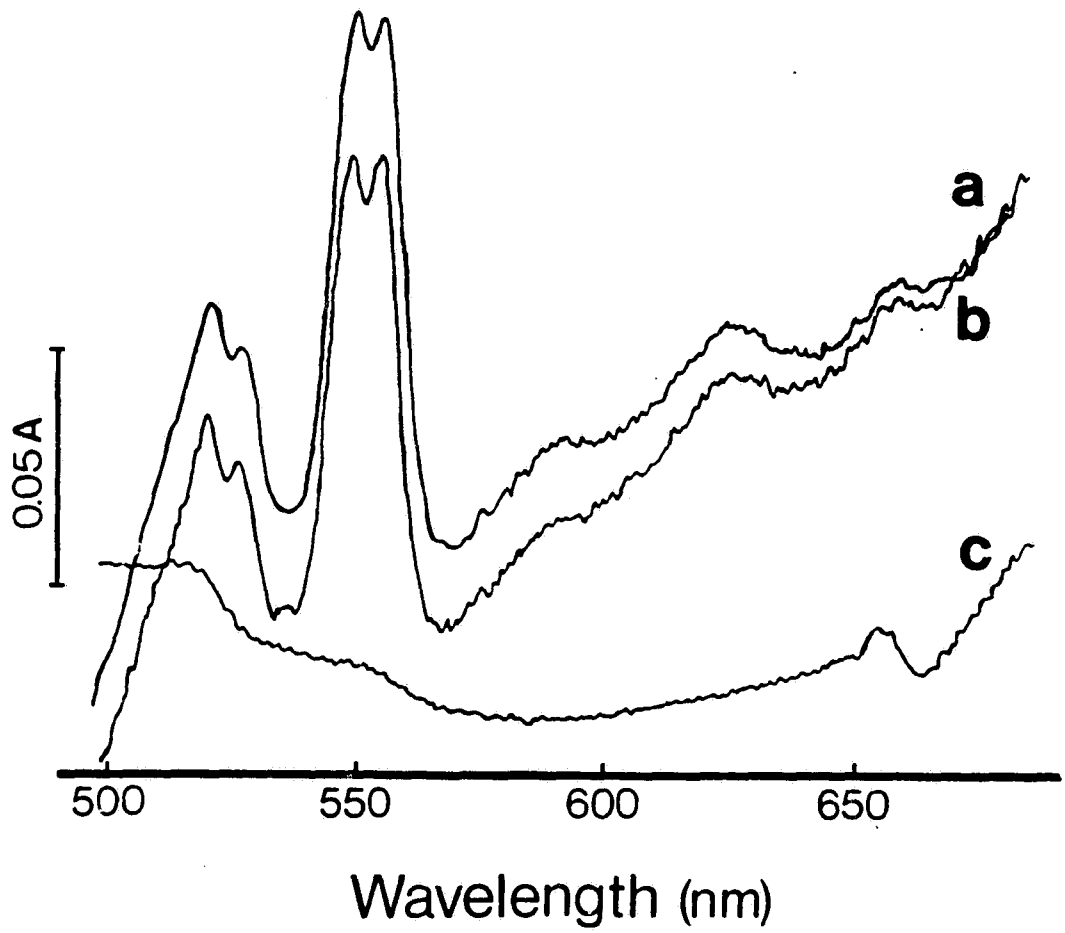


**Figure 7.** Dithionite reduced minus peroxide oxidized difference spectra (77 K) showing the cytochromes of *A. salmonicida* wild type strain A450 (**a**), and the mutants A450-10SR (**b**), and A450-10S (**c**). Each spectra represents an average of three individual scans.

**Table 7. Activities[a] of NADH oxidase, glycerol-3-phosphate dehydrogenase, and succinate dehydrogenase in *A. salmonicida* strains.**

| | Strain | | |
|---|---|---|---|
| Enzyme | A450 | A450-10S | A450-10SR |
| NADH oxidase | 7.8 ± 2.7 | 9.3 ± 4.0 | 11.4 ± 3.8 |
| SDH | 21.4 ± 1.5 | 19.8 ± 3.7 | 22.7 ± 1.1 |
| GPDH | 44.3 ± 7.1 | 52.1 ± 11 | 32.6 ± 9.3 |

a Enzyme specific activities are expressed in units of nmol $min^{-1}mg^{-1}$.
Values are expressed ± standard deviation (n=2).

**Fluorescence Quenching.** Using 9-aminoacridine fluorescence quenching as a fluorescent probe of membrane potential (Rottenberg, 1979), the ability to energize the membrane was compared between the two mutant strains, A450-10S and A450-10SR, and the wild type strain A450 (Fig. 8). Everted membrane vesicles of A450 showed a general NADH generated, fluorescence quenching profile, followed by a phase of refluorescence normally attributed to a collapse or equilibration of the membrane energization (Fig. 8A). Membrane vesicles of A450-10S were unable to energize the membrane to the same degree as A450, and interestingly showed no evidence of collapse of membrane energization that is seen in both wild type *A. salmonicida*, A450, and the pseudorevertant, A450-10SR. While the collapse in A450 and A450-10SR were approximately 13% and 25% respectively of the total fluorescence quenching, A450-10S displayed less than 2% collapse even over an extended monitoring period (Fig. 8C). In addition, the sensitivity of A450-10S to the protonophore FCCP, and the ionophores valinomycin and gramicidin were extremely reduced. In fact, the NADH induced gradient of A450-10S was apparently resistant to both valinomycin and gramicidin, and only slightly sensitive to the protonophore FCCP (Fig. 9). The gradient induced by NADH in A450 was sensitive to both FCCP and gramicidin, but was apparently resistant to valinomycin. The NADH induced gradient of the pseudorevertant A450-10SR, like that of A450, was sensitive to both FCCP and gramicidin but was also slightly sensitive to valinomycin (Fig. 9). These results raised the question as to whether the degree of fluorescence quenching seen with A450-10S was due to membrane energization, more specifically an electrochemical gradient of protons.

**Determination of ΔpH and ΔΨ.** In order to more closely assess the energetic effects of cytochrome deficiency in *A. salmonicida*, ΔpH and ΔΨ were measured at different stages of growth. Mid log phase cultures were used to represent actively growing cells (ΔpH

and $\Delta\Psi$), early stationary phase cultures were used to represent cells entering starvation mode ($\Delta$pH only), and late stationary phase cultures, where the media was depleted, was used to represent cells entering death phase ($\Delta$pH and $\Delta\Psi$; Table 8 and Fig. 10). The most obvious difference between A450 and the two mutant strains A450-10S and A450-10SR, was the magnitude of $\Delta$p for the actively growing cells (Table 8). Here, A450-10S which lacks cytochromes, is capable of producing a $\Delta$p of only 60% of the wild type (A450) level. The pseudorevertant (A450-10SR) was also deficient in the magnitude of $\Delta$p even though it apparently possesses a complete repertoire of cytochromes. It is apparent from the data that both mutants are affected in both components of $\Delta$p, namely $\Delta$pH and $\Delta\Psi$. With the late stationary phase cells, all strains appeared to lose $\Delta$pH and $\Delta\Psi$ simultaneously, resulting in an overall decrease in $\Delta$p. In unbuffered culture media, A450-10S and A450-10SR metabolizing glucose extensively acidify the media (Fig. 10). Here A450-10S was incapable of maintaining a $\Delta$pH greater than -50 mV or 0.85 pH units. A450-10SR was capable of creating higher $\Delta$pH values (~65 mV or 1.1 pH units), but incapable of maintaining this $\Delta$pH during later stages of growth. The $\Delta$pH data, in combination with the growth curve data indicates that the mutants A450-10S and A450-10SR both showed a steady decline in the interior pH and $\Delta$pH even before culture began losing viability, while the wild-type strain A450 maintained $\Delta$pH and therefore interior pH, until the culture entered stationary phase. The data also suggest that the regain of cytochromes in A450-10SR was not entirely functional in coupling metabolic oxidative reactions to electron transport and the formation and maintenance of $\Delta$p.

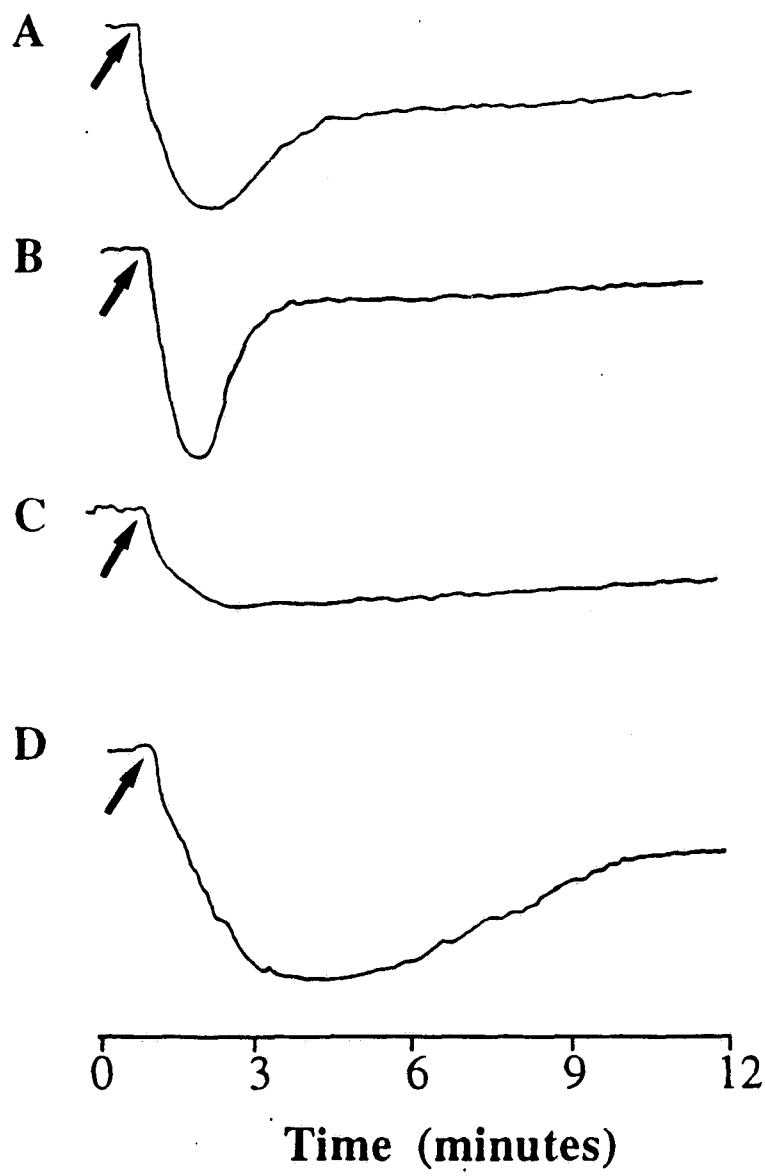


**Figure 8.** 9-Aminoacridine fluorescence quenching traces of membrane vesicles of *A. salmonicida* wild type strain A450 (**A**), compared with the mutant strains A450-10SR (**B**) and A450-10S (**C**), and *E. coli* HB101 (**D**). NADH addition is indicated by the arrows. Fluorescence quenching is in the downward direction, and the fluorescence units are arbitrary and therefore no scale is included. The traces shown are a typical representation of numerous experiments.

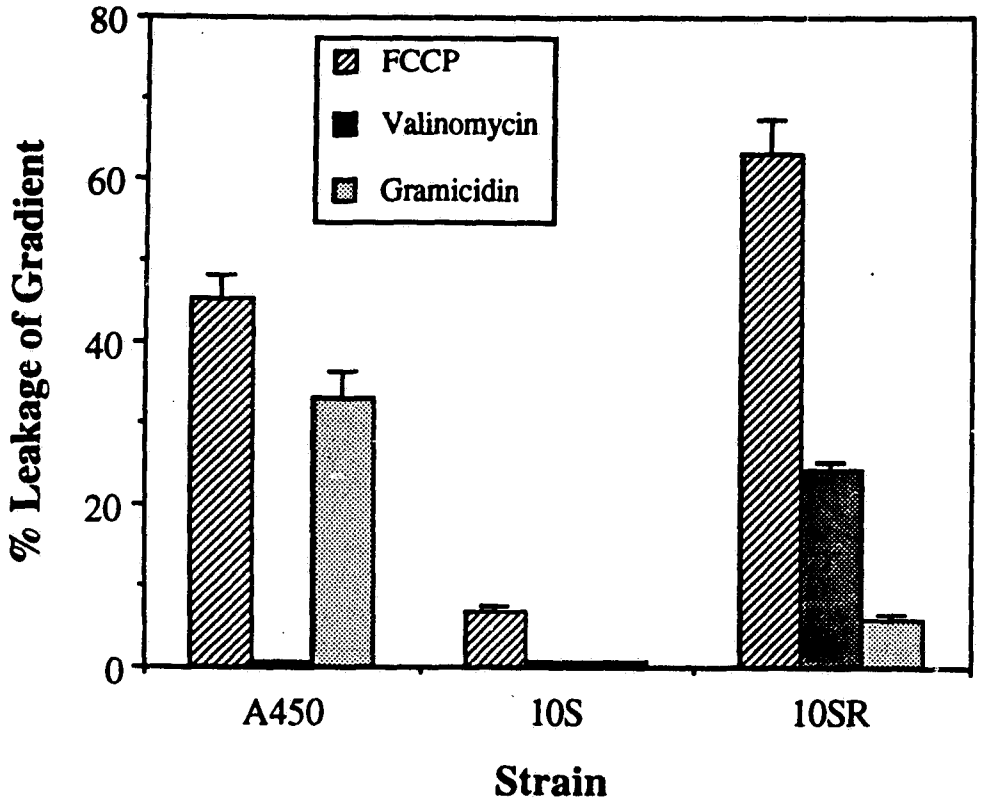


**Figure 9.** Effects of the ionophores FCCP, Valinomycin, and Gramicidin on the NADH induced fluorescence quenching by everted membrane vesicles of *A. salmonicida* strains A450, A450-10S and A450-10SR. The data represents the percentage of peak fluorescence collapsed immediately after the addition of the ionophore. Values shown are the mean of three trials, and error bars represent standard deviation.

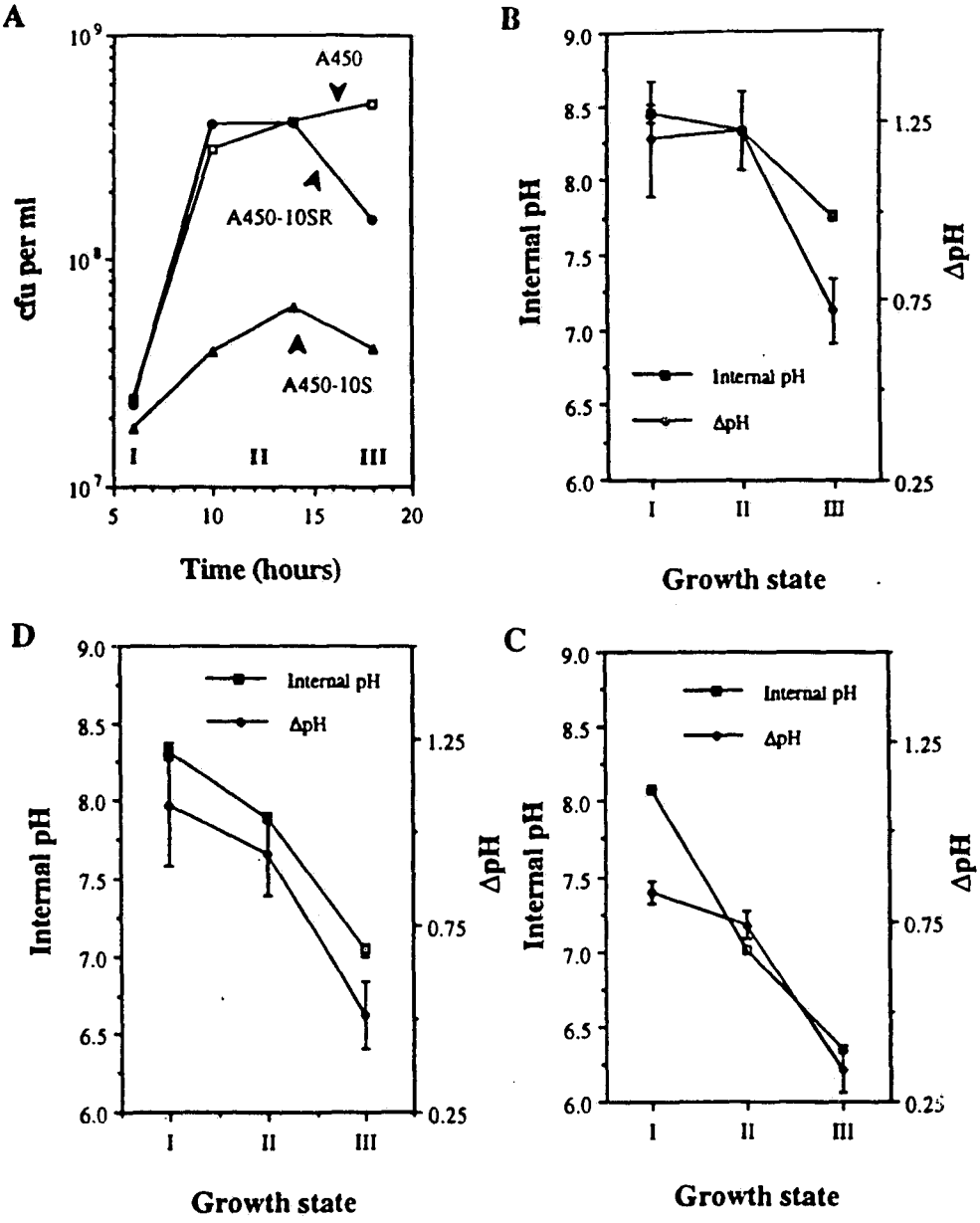


**Figure 10.** The effects of external pH on internal pH and ΔpH with respect to the growth state of *A. salmonicida* strains A450, A450-10S, and A450-10SR. **A** represents a typical growth curve comparing the wild type strain A450 to the mutants A450-10S and A450-10SR. The samples labelled I, II, and III on **B** (A450), **C** (A450-10S), and **D** (A450-10SR) correspond to cells removed from culture at the indicated points on the growth curve (A). Where: **I** represents culture in early exponential phase, **II** represents culture in early stationary phase, and **III** represents culture in late stationary-early death phase.

**Table 8. Changes in ΔpH and ΔΨ with respect to growth stage for *A. salmonicida* strains.**

| | Strain | | | | | |
|---|---|---|---|---|---|---|
| | A450 | | A450-10S | | A450-10SR | |
| | ML[a] | LS[b] | ML | LS | ML | LS |
| $pH_{out}$ | 7.25 | 7.04 | 7.25 | 6.01 | 7.25 | 6.53 |
| $pH_{in}$ | 8.45 | 7.76 | 8.08 | 6.35 | 8.32 | 7.04 |
| $\Delta pH^{c}$ | -72.3±9 | -43.2±9 | -49.7±2 | -21.0±4 | -63.8±9 | -30.7±2 |
| $\Delta\Psi^{c}$ | -183.9±14 | -80.8±7 | -110.6±1 | -70.9±5 | -105.7±9 | -84.5±20 |
| $\Delta p^{c}$ | -255.3±23 | -124.0±16 | -160.4±3 | -91.9±9 | -169.6±18 | -115.2±22 |

[a] ML-Mid log phase cells.

[b] LS-Late stationary phase cells.

[c] Units are in millivolts

**Analysis of Excreted Metabolites.** In order to understand why the two mutant strains A450-10S and A450-10SR acidified the media much more rapidly than wild type *A. salmonicida*, the metabolites released into the media were examined after pulsing a culture of wild type A450, and the mutant strains A450-10S and A450-10SR with D-[U-$^{14}$C]glucose. $R_f$ values calculated from a thin layer chromatograph (TLC) of the released radiolabelled metabolites are presented in Table 9. The radioactive metabolites released by the wild type strain A450 consisted mainly of unidentified compounds of low relative mobility ($R_f$), i.e. highly polar, and a weak spot corresponding to pyruvate. Also apparent, is that not all of the glucose was metabolized by A450. The profile of metabolites for the mutants A450-10S and A450-10SR differed radically from that of A450. Both mutants appeared to release large amounts of pyruvate and lactate, with little or none of the low $R_f$ compounds excreted by A450. Strain A450-10S, like wild type, failed to utilize all of the glucose present. But, this strain did produce an unidentified metabolite of high $R_f$ not seen in either of the other two strains. Strain A450-10SR was unique in that the only radiolabelled metabolites seen on the TLC were pyruvate and lactate. Unlike the other two strains, A450-10SR had apparently metabolized all the added glucose.

**Virulence of mutant strains.** In order to test the virulence of all mutants, 30 juvenile salmonids (*O. tshawytscha*, *O. mykiss*, or *S. salar*) (5-15g) were injected intraperitoneally (IP) with an excessive dose ($5 \times 10^7$ cfu, ~$10^5 \times LD_{50}$ of the virulent parental strain A450) of live cells of each of the mutant *A. salmonicida* strains. The results of these challenges demonstrated that A450-1, A450-3, A440, A450-1-3, A450-10S, A450-10SR and A450-10SR-3 were completely avirulent, as no fish receiving these injections died.

**Table 9. Thin layer chromatography analysis of metabolites released by *A. salmonicida* strains A450, A450-10S, and A450-10SR.**

| Metabolite[a] | $R_f$ x 100 | | | |
|---|---|---|---|---|
| | Standard[b] | A450[c] | A450-10S[c] | A450-10SR[c] |
| Unk.[d] | na | - | 97 | - |
| Lactate | 85 | - | 85 | 85 |
| Pyruvate | 67 | 66 | 67 | 67 |
| Glucose | 49 | 49 | 49 | - |
| Unk. | na | 37 | - | - |
| Citrate | 29 | 29 | - | - |
| Unk. | na | 18 | - | - |
| Unk. | na | 11 | - | - |

[a] Metabolites were identified on the basis of $R_f$ values for known compounds.

[b] na indicates the lack of a match of metabolite from bacteria with standards tested.

[c] - indicates corresponding metabolite is absent from respective TLC profile.

[d] Unk. indicates that the identity of the spot on the TLC remains unknown.

**Tissue persistence.** The tissue persistence of A450-10SR, the least incapacitated and most promising vaccine candidate of the two energetically defective mutants, was examined at various times after a 30 minute immersion of 7-10g Rainbow trout and Atlantic salmon in a 0.85% saline bath containing 1 x $10^8$ cfu $ml^{-1}$ of the mutant strain. Specific target tissues were cultured and examined by plating for the presence of the invading bacteria strain. Sculpins, a non-target, but suspected *A. salmonicida* reservoir, were also tested for carrier status with strain A450-10SR. The persistence of 10SR in trout was compared to the spread and persistence of the parent strain A450 in fish tissues (Table 10). Strain A450-10S was not examined as it was deemed to have no potential as a vaccine candidate due to its low growth rate. Strain A450-10SR spread throughout the examined tissues in an apparently identical pattern to that of A450. A450-10SR could only be isolated from kidney up to 48 h post infection, but could not be isolated from any tissue tested by 72 h.

The persistence data in prednisolone acetate treated fish demonstrated that only in the group that received A450 were *A. salmonicida* recovered in mortalities after day four (Table 11). This suggested that A450-10SR and A450-10SR-3 do not persist in immunocompromised fish tissues, and are thus, completely attenuated.

**Serum resistance.** Virulent *A. salmonicida* strains are noted to be highly resistant to serum killing by the cytolytic action of both immune and non-immune complement. This resistance is due to both LPS as well as an intact A-layer (Munn et al., 1982). Serum killing experiments (Fig. 11) revealed that A450-10S and A450-10SR were far more sensitive to serum killing than A450. Two other attenuated strains, A450-3, (a mutant of A450 that is LPS$^+$ A-protein$^-$), and A450-1, (a mutant of A450 that is LPS$^-$ A-protein$^-$), were also tested. It is interesting that both A450-10S and A450-10SR were more

**Table 10. Tissue Persistence Comparison of Strains A450 (wild type) and the Mutant A450-10SR.**

| | Time (days) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **4** | **6** | **9** | **10** | **12** | **15** |
| | # positive (# tested) | | | | | | | |
| **A450 (wild type)** | | | | | | | | |
| **Rainbow trout (13°C):** | | | | | | | | |
| Kidney | 3 (4) | 4 (4) | 4 (4) | nd[a] | nd | nd | nd | nd |
| Liver | 2 (4) | 2 (4) | 3 (4) | nd | nd | nd | nd | nd |
| Spleen | 1 (4) | 3 (4) | 4 (4) | nd | nd | nd | nd | nd |
| **10SR** | | | | | | | | |
| **Rainbow trout (13°C):** | | | | | | | | |
| Kidney | 2 (4) | 3 (4) | 0 (4) | 0 (4) | nd | nd | nd | nd |
| Liver | 1 (4) | 2 (4) | 0 (4) | 0 (4) | nd | nd | nd | nd |
| Spleen | 3 (4) | 1 (4) | 0 (4) | 0 (4) | nd | nd | nd | nd |
| **Atlantic salmon:** | | | | | | | | |
| **Trial 1: 7°C** | | | | | | | | |
| Feces | 0 (5) | 0 (5) | 0 (5) | 0 (5) | 0 (5) | nd | 0 (5) | 0 (5) |
| Spleen+kidney | 0 (5) | 2 (5) | 0 (5) | 0 (5) | 0 (5) | 0 (5) | 0 (5) | 0 (5) |
| **Trial 2: 15.5°C** | | | | | | | | |
| Feces | nd | 0 (5) | nd | 0 (5) | nd | 0 (5) | nd | nd |
| Spleen+kidney | nd | 1 (5) | nd | 0 (5) | nd | 0 (5) | nd | nd |
| **Sculpin: 14°C (Infected by Immersion)** | | | | | | | | |
| No persistence at any time. | | | | | | | | |

[a] For rainbow trout experiments, nd indicates no testing of the samples due to excessive mortalities in the wild type A450 challenge, or no further persistence with the attenuated strain A450-10SR.

**Table 11. Persistence of Attenuated Strains in Trout Treated with Prednisolone acetate.**

| Day | None | A450 | A450-10SR | A450-10SR-3 |
|---|---|---|---|---|
| | (number of mortalities)[a] | | | |
| 1 | 0 | 0 | 0 | 0 |
| 2 | 0 | **3** | 0 | 0 |
| 3 | 1 | **7** | 0 | 0 |
| 4 | 0 | **13** | **1** | 0 |
| 5 | 0 | **5** | 0 | 0 |
| 6 | 0 | **2** | 0 | 0 |
| 7 | 1 | - | 0 | 0 |
| 8 | 0 | - | 0 | 0 |
| 9 | 0 | - | 0 | 0 |
| 10 | 0 | - | 0 | 0 |
| 11 | 1 | - | 1 | 0 |
| 12 | 1 | - | 2 | 1 |
| 13 | 0 | - | 0 | 0 |
| 14 | 2 | - | 1 | 3 |
| 15 | 0 | - | 0 | 0 |
| 16 | 0 | - | 0 | 0 |
| 17 | 0 | - | 0 | 0 |
| 18 | 0 | - | 0 | 0 |
| 19 | 0 | - | 0 | 0 |
| 20 | 0 | - | 0 | 0 |
| Total Mortality | 20% | 100% | 17% | 13% |
| Total Mortalities testing positive for *A. salmonicida* | 0% | 100% | 3% | 0% |

[a] Bold type mortalities indicate that *A. salmonicida* was cultured from the fish.

sensitive to serum killing than even A450-3, even though the aforementioned mutants are still apparently normal with respect to LPS production and both have been demonstrated to possess an A-layer, albeit an altered layer in both cases. This suggested that the A-layer was not entirely intact, and that serum complement had access to the cell membrane.

**Survival of Attenuated *A. salmonicida* Strains in Aqueous Environments.** The mutant strains A450-10S and A450-10SR are both defective in their ability to survive in dilute environments compared to the wild type parental strain A450 (Figs. 12 and 13). These figures demonstrate that the potential vaccine strain A450-10SR dies rapidly in both sea water and ground water, and in no circumstances was A450-10SR more capable to survive than the wild type strain A450. Not surprisingly, survival of all strains was generally better when peptone was added to 0.05% to mimic polluted conditions, with death occurring at a later time than in non-polluted conditions. This death likely occurs only when the carbon source (peptone) was depleted.

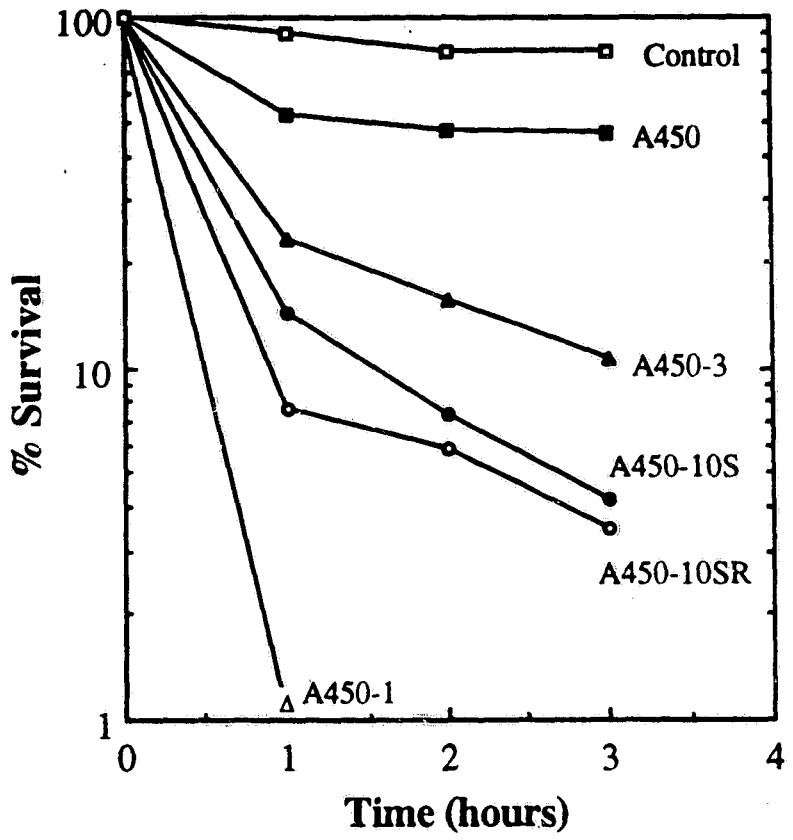


**Figure 11.** Non-immune serum killing of *A. salmonicida* strains. Values are expressed as percent survival compared to the original viable cell counts. Strains shown are: wild-type A450 (■); an A-protein$^-$ lipopolysaccharide$^+$ derivative A450-3 (▲); an A-protein$^-$lipopolysaccharide$^-$ derivative A450-1 (△); and the strains A450-10S (●); and A450-10SR (○).

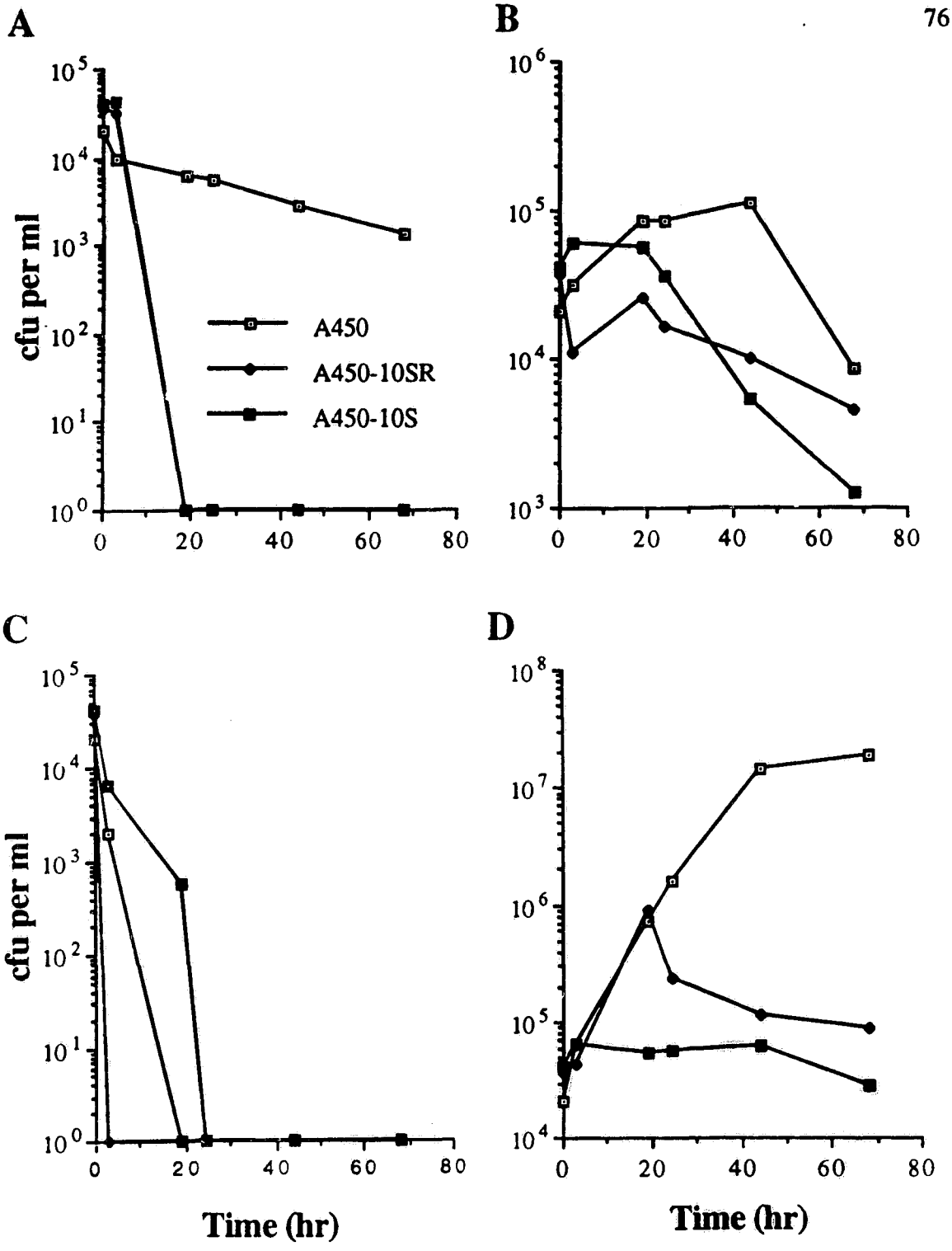


**Figure 12.** Survival of the *A. salmonicida* strains A450, A450-10S, and A450-10SR in ground water. The survival of A450 (⊡), A450-10S (■), and A450-10SR (◆) was measured in ground water at 4°C (A), polluted ground water at 4°C (B), ground water at 20°C (C), and polluted ground water at 20°C (D).

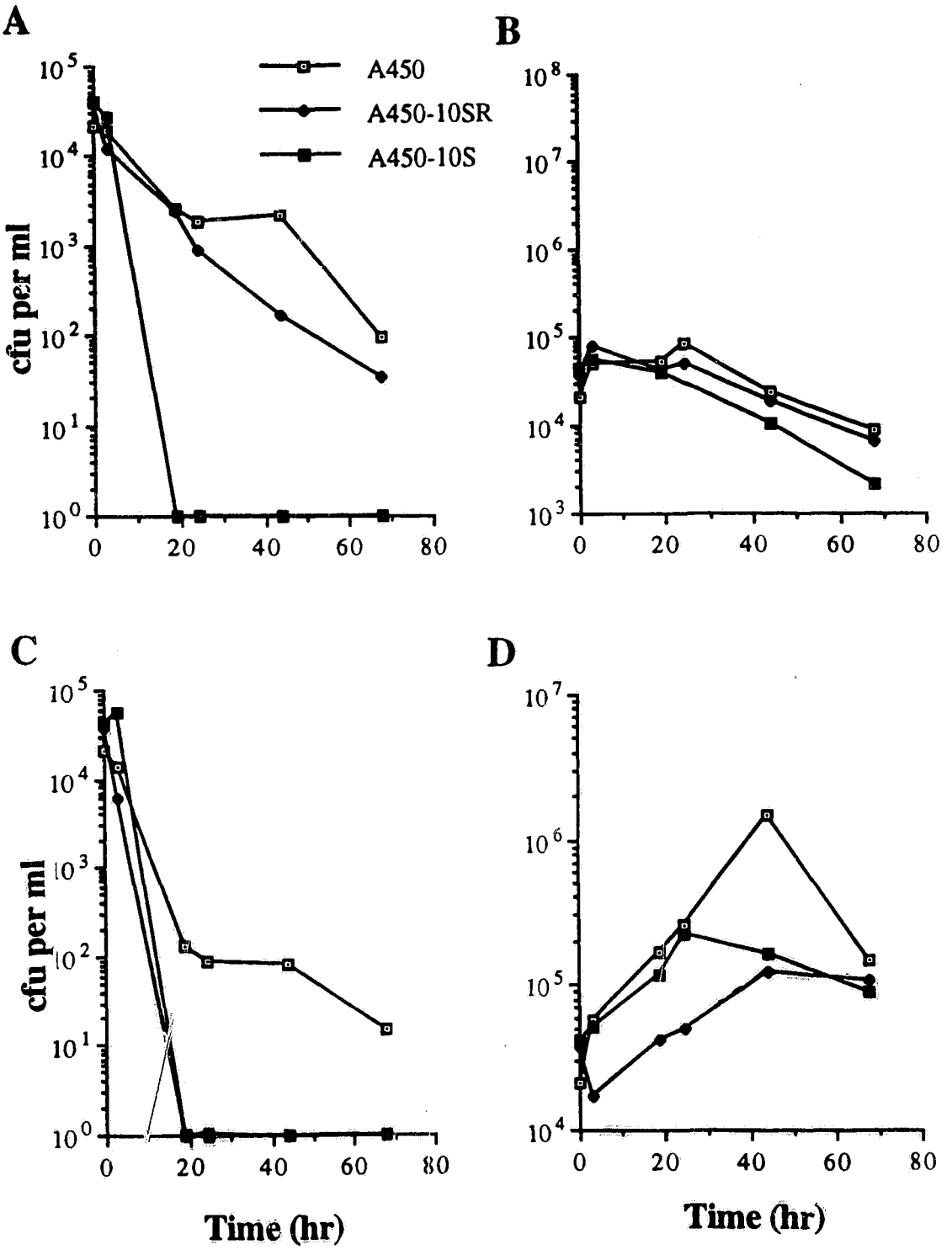


**Figure 13.** Survival of the *A. salmonicida* strains A450, A450-10S, and A450-10SR in sea water. The survival of A450 (▣), A450-10S (■), and A450-10SR (◆) was measured in sea water at 4°C (A), polluted sea water at 4°C (B), sea water at 20°C (C), and polluted sea water at 20°C (D).

## Discussion:

The isolation of a unique spontaneous streptomycin resistant, ($Str^r$), slow-growing mutant of *A. salmonicida* as well as an apparent streptomycin sensitive, ($Str^s$), rapidly growing pseudorevertant appears to be applicable to the attenuation of *A. salmonicida* strains, and thus may be applied to the development of live furunculosis vaccines. This isolation strategy embodies at least two mutational events in the creation of A450-10S and three mutational events in the creation of A450-10SR, all being spontaneous and thereby having a fair probability of being due to stable deletions or genetic rearrangements. Equally attractive is the pleiotropic nature of these mutations, a broad phenotypic response increasing the likelihood of the expression of several detrimental physiological effects. The degree of aminoglycoside resistance of A450-10S is highly unusual in that it empowers A450-10S to specifically resist near-saturating levels of this antibiotic, unlike typical $Str^r$ bacterial mutants more commonly found to be altered in ribosome function (Grieco, 1982). The precise mechanism of resistance in A450-10S is unknown, but the fact that it is specific for aminoglycosides argues against a non-specific impermeability effect. The influence of the defects in terminal respiration will be discussed below. Similar uncharacterized mutants of *Yersinia pestis* (Brubaker and Surgalla, 1962), were also found to be attenuated, an observation that gave rise to the approach used here. Both A450-10S and A450-10SR produced a seemingly normal array of the extracellular virulence factors associated with *A. salmonicida* such as hemolysin (Lee and Ellis, 1989; Lee and Ellis, 1990), proteases (Shieh and Maclean, 1975; Lee and Ellis, 1989), and siderophores (Chart and Trust, 1983), yet they are both avirulent. This suggests that together these extracellular virulence factors described for *A salmonicida* (Trust, 1986) are not by themselves enough to confer virulence. That they are still

expressed in these mutants suggests they will be available *in vivo* to participate in the stimulation of an antitoxin immunity.

The usual cell surface antigens, A-protein and LPS, of *A. salmonicida* were still well expressed and accessible in both mutants. The normal arrangement for the A-layer of *A. salmonicida* is an intricate, 2D, tetragonal interconnecting array of protein subunits tethered to the cell-surface by LPS (Belland and Trust, 1987; Dooley et al., 1989). However, the cell surfaces of both the mutant A450-10S and the pseudorevertant A450-10SR are apparently disorganized. While difficult to specifically structurally ascribe these differences to specific biochemical changes, the pattern observed with A450-10S is typical of those created by $Ca^{2+}$ depletion of wild-type cells which interferes with normal subunit assembly. Surprisingly, the apparent genetic compensation exploited by A450-10SR, while correcting certain growth and membrane dysfunctions, resulted in an even more disordered appearance of the A-layer, especially as observed in older cells. In addition, the A-protein containing A-layer appeared not to be uniformly disposed on the cell surface as demonstrated by immunogold labeling. This evidence, as well as serum and detergent sensitivity of these two strains suggests the layer has diminished structural integrity. SDS-PAGE and immunoblot data indicated that both A450-10S and A450-10SR were quantitatively normal with respect to A-protein and LPS. This could indicate that the A-layer of these mutants is multilayered or aggregated rather than a monomolecular layer, giving rise to unusual Moiré patterns. As it is not clear whether immunity to furunculosis is mediated via cellular and/or humoral responses, this may carry an unanticipated advantage in that such mutants may expose critical cell surface protective antigens normally tightly shielded by the highly organized A-layer (Kay and Trust, 1991), thus allowing for the possible participation of B-cells in T cell independent immune responses. In addition, since A-layer deficient strains secrete extracellular toxins

more readily than A-layer sufficient cells (unpublished observations), it is possible that the avirulent strains we have described here have the potential of delivering a higher level of extracellular factors for immunity.

Physiologically, it appeared that A450-10S harbors mutations that completely abrogates oxidative metabolism while A450-10SR had undergone a further mutational event that partially restored the ability to oxidize certain carbon sources, principally carbohydrates, thereby restoring the energy available for growth and somehow regaining sensitivity to aminoglycosides. This may indicate that aminoglycosides enter *A. salmonicida* illicitly by an active transport pathway. The slow growth rate, reduced growth yield and complete lack of aerobic metabolism suggests that the mutation in A450-10S effects energy metabolism of which little is known for this organism. The fact that a partially compensating mutation or partial reversion such as is seen in A450-10SR, can be achieved, suggests that *A. salmonicida* may have an alternative means to aerobically metabolize some carbon sources when the primary mechanism is impaired. This hypothetical metabolic/energetic mutation present in A450-A450-10S, and partially compensated for in A450-10SR, does not affect the overall composition of the cell envelope, nor of the cell membrane since only minor changes in the fatty acid composition of the phospholipids in these cells have been seen. However, it does affect its architecture, suggesting that the maintenance of surface integrity is somehow energy-dependent.

The appearance of some accentuated bands on SDS-PAGE in particular of the periplasm of A450-10S (Fig. 3) suggests that the effect may be incurred at an early assembly stage. A-protein, for instance, appears to move through this cell compartment on its assembly pathway (Belland and Trust, 1987). However, there are obviously whole sets of proteins in both the periplasmic and inner membrane that are under-represented in A450-10S, especially in the low $M_r$ range, suggestive of incomplete processing to the

level of this compartment. Proteins over represented here may be accumulating because of an inability to pass into or beyond the outer membrane.

It has already been amply demonstrated that virulent strains, such as A450, rapidly disseminate to a variety of host tissues, aided by professional phagocytes (Trust, 1986). It is apparent from the tissue persistence of A450-10SR that its spread through the tissues is similar to that of the wild-type strain, though it is cleared within 60 h by the host defenses in all salmonids tested. Clearance may be due to both cellular and humoral killing of mutants not fully protected by their A-layer (Kay and Trust, 1991). The consequences of tissue entry and persistence to immunity provided by live vaccines have been discussed elsewhere (Sigwart *et al.*, 1989; Smith, 1990). It is important that the resident attenuated pathogen express the appropriate protective antigens in vivo, as well as stimulate the correct type of host immunity in the affected target tissues.

As a general deficit in aerobic metabolism was shared by both mutants, defects in the electron transport chain of the mutants A450-10S and A450-10SR were suspected. Thus, an examination of the cytochrome content and cellular energetics of the mutant strains in comparison with wild type *A. salmonicida* was performed.

The difference spectra of the membranes of strain A450 demonstrated that wild type *A. salmonicida* possesses a common repertoire of bacterial cytochromes, cytochrome *c* , cytochrome *b*, cytochrome *o* oxidase and cytochrome *d* oxidase. The presence of heme *c* suggests the respiratory chain of *A. salmonicida* is not like that in *E. coli*, which flows from dehydrogenases (NADH or succinate) to ubiquinol then to terminal oxidases of either the *bo* or *bd* type. The respiratory chain of *E. coli* is characterized by a lack of the *c* type cytochromes (Anraku, 1988). It is probable that the flow of electrons in *A. salmonicida* is from primary dehydrogenases to ubiquinol then to the terminal *o* and *d* type cytochrome oxidases via cytochrome *c*.

It was not completely surprising to find that the mutant strain A450-10S was deficient in terminal respiration, as mutants of *E. coli* and *Pseudomonas aeruginosa* that are resistant to high levels of aminoglycoside antibiotics, and also defective in terminal respiration, have been isolated (Bryan et al., 1980; Bryan and Kwan, 1981; Nichols and Young, 1985). Also, aminoglycoside resistance has been effectively used for the selection of heme auxotrophy, and thus defective terminal respiration, in *Bacillus subtilis* (Anderson and Ivanovics, 1967). The two classes of aminoglycoside resistant *P. aeruginosa* previously identified were demonstrated to be defective in nitrite reductase and cytochrome *d* (Bryan and Kwan, 1981), or nitrate reductase and cytochrome $c_{552}$ (Bryan et al., 1980). The relationship between a functioning respiratory chain and aminoglycoside uptake has been established previously, and it has been concluded that the uptake of streptomycin and other polycationic aminoglycosides is dependent on the magnitude of $\Delta\Psi$ (reviewed by Taber et al., 1987). Thus defects in energy metabolism would be readily selected by aminoglycoside resistance.

It was surprising however, that A450-10S was apparently lacking in all cytochrome types. To our knowledge, no other mutants that lack all cytochrome types that are not mutants in heme biosynthesis, have been described for any aerobic or facultative anaerobic bacterial genus or species. The isolation of a mutant, such as A450-10S, that is completely cytochrome deficient by selecting for high level streptomycin resistance is also, to my knowledge, a unique finding.

The minor peak at 550 nm observed in the differential spectra of A450-10S may represent free heme synthesized by A450-10S that remains unincorporated due to a lack of cytochrome proteins, alternatively the absorbance peak may represent heme from the yeast extract in the growth media bound with the A-layer of *A. salmonicida*. It has been previously demonstrated that *A. salmonicida* possesses a regular surface array, known as the A-layer, that is capable of binding heme with a dissociation constant of 0.63 μM (Kay

et al., 1981; Kay et al., 1985). Of course, this small peak at 550 nm may represent real components of electron transport produced by A450-10S, but present at such a low concentration that detection of aerobic metabolism by respirometry (i.e. a Clarke style electrode) is impossible.

In order to assess whether the mutant A450-10S was deficient in cytochromes due to a form of heme auxotrophy, cytochrome scans were performed on strains after growth in media containing hemin (data not shown). Neither an increase in the frequency of mutations giving rise to A450-10SR, nor a restoration of normal, or near normal, growth rates for A450-10S were seen when ∂-aminolevulinic acid, protoporphyrin IX, or hemin were added to the media (unpublished observations). However, slight increases in absorption maxima at ~552 nm and ~557 nm were observed in the differential spectra for A450-10S grown in the presence of hemin. As it was thought that the high level streptomycin resistance was a result of the defect in aerobic metabolism, A450-10S was grown with the hemin prior to the cytochrome scan in the absence of streptomycin. In these cultures it was seen that A450-10SR-like mutants arose at a frequency of $10^{-7}$, the same frequency that occurs in the absence of heme or its precursors (data not shown). This mutational frequency, in combination with the fact that A450-10SR has a doubling time less than half of that for A450-10S (Fig. 2), suggests that the slight increases in cytochromes *b* and *c* seen in the spectra are probably due to the outgrowth of A450-10SR in the culture of A450-10S regardless of whether heme was added. It is important to note that the absorption spectra for heme has very strong absorption maxima in the regions we examined, thus the magnitude of any or all observed peaks in the strains may only reflect confounded data from the binding of excessive amounts of heme to the A-layer of the *A. salmonicida* strains tested.

The pseudorevertant, A450-10SR, which was also demonstrated to be defective in some aspects of aerobic metabolism, had apparently regained a full complement of

cytochromes through some form of compensatory mutation (Fig. 7). The precise reason why A450-10SR remains defective in aerobic metabolism was initially unclear. The carbohydrates which A450-10SR remains unable to oxidize include glycerol, succinate, glucose, gluconate, lactose, xylose, sorbitol, and 2-ketogluconate, while those tested that A450-10SR has regained the ability to utilize included lactate, galactose, mannose, maltose, and peptones (Table 6). It has been demonstrated that *A. salmonicida* utilizes the Entner-Doudoroff pathway for glucose metabolism via gluconate (Chapter III), and is devoid of phosphofructokinase activity. It has also been demonstrated that in some *Pseudomonas* spp., glucose can be oxidized to gluconate and to 2-ketogluconate via periplasmic FAD linked systems (Lessie and Phibbs, 1984), and glycerol can be oxidized by the flavoprotein *sn*-glycerophosphate dehydrogenase (Gottschalk, 1985), while examples for NADH/NADPH dehydrogenases and for mannose and galactose entering metabolism as hexose phosphates via various kinases and isomerases (i.e. Phosphoenolpyruvate:Sugar Phosphotransferase systems; Postma, 1987), exist in other bacteria. It is interesting to speculate that the mutations in A450-10S involve a complete loss of cytochromes while the associated enzymes of complex II (succinate- and glycerolphosphate dehydrogenases) that feed into electron transport remain active. This hypothesis is in agreement with the observation that both A450-10S and A450-10SR can couple the oxidation of succinate and glycerol-3-phosphate to artificial electron acceptors, yet are apparently unable to couple these oxidations to the electron transport chain and subsequently to the reduction of $O_2$. A450-10SR however, has apparently regained all the primary cytochrome components by an unknown compensatory mutation, yet remains unable to couple various oxidative reactions to the electron transport chain to subsequently reduce oxygen. It is evident from the types of carbon sources that A450-10SR is unable to oxidize that it is defective in electron transport complex II, the site at which succinate dehydrogenase and *sn*-glycerophosphate dehydrogenase as well as other

FAD requiring primary dehydrogenases act. This would then result in the observed phenotype of A450-10SR, an organism that is incapable of capturing the redox potential of substrates that enter electron transport at sites other than that for NADH. It is unlikely that transport systems utilized by *A. salmonicida* for the uptake of these compounds may be lacking due to the pleiotropic effects of one or more of the mutations in A450-10S and A450-10SR as whole membrane preparations of these strains, like whole cells, are unable to utilize either succinate or glycerol-3-phosphate with simultaneous $O_2$ consumption (data not shown). The examination of metabolites secreted by the mutant strains after growth on [U-$^{14}$C]glucose also suggests that the cytochromes seen in A450-10SR are not functional, as the TLC profile indicates that only the end products of fermentation, pyruvate and lactate, are produced from glucose. Thus the glucose metabolism of A450-10SR closely resembles that of A450-10S which seems only to ferment glucose.

As it was determined that A450-10S and A450-10SR still contain NADH oxidase activity (Table 7), the ability to energize the membrane was compared between the two mutant strains and the wild type strain using 9-aminoacridine fluorescence quenching, and by measuring the two components of $\Delta p$ ($\Delta pH$ and $\Delta\Psi$). Membrane vesicles of A450-10S showed none of the spontaneous collapse of the proton gradient that was seen in both wild type A450 and A450-10SR . The reason for this maintenance of the proton gradient in A450-10S vesicles remains unclear. It has been demonstrated that *E. coli* mutants that have a defective ATPase are proton leaky (Altendorf and Harold, 1974), however the lack of proton leakiness coupled with the lack of cytochromes in A450-10S suggests that the cytochrome chain may play a part in the natural proton leak observed in *A. salmonicida* vesicles. This line of reasoning is strengthened by the concomitant regain of proton leakiness coincidentally with the reappearance of a seemingly complete, although nonfunctional, of cytochrome components in A450-10SR. The induced gradient of A450-10S was apparently resistant to both valinomycin and gramicidin, and only

slightly sensitive to the protonophore FCCP. The gradient in A450 was sensitive to both FCCP and gramicidin, but was apparently resistant to valinomycin. The gradient of the pseudorevertant A450-10SR, like that of A450, was sensitive to both FCCP and gramicidin but was also slightly sensitive to valinomycin. The sensitivities to FCCP is indicative that the gradient is maintained as a proton gradient and is not unexpected, and as gramicidin has poor selectivity between protons and monovalent cations it is likely that the collapse observed upon addition of gramicidin it due to the uncoupling of a proton gradient. However, the sensitivity of the gradient of A450-10SR to valinomycin suggests that at least part of the proton gradient induced was converted to a potassium gradient.

Measurements of $\Delta$pH and $\Delta\Psi$ at different states of growth demonstrated some basic energetic differences between the mutants A450-10S and A450-10SR and the wild type strain of *A. salmonicida*, A450. The difference between A450 and the two mutant strains in the magnitude of $\Delta$p for the actively growing cells demonstrates that A450-10S and A450-10SR are capable of producing a $\Delta$p of only 60-70% of the wild type level. Apparently, both mutants are essentially deficient in both components of $\Delta$p, namely $\Delta$pH and $\Delta\Psi$. By late stationary phase, cultures from all strains appeared to lose $\Delta$pH and $\Delta\Psi$ simultaneously, resulting in an overall decrease in $\Delta$p of the population, this phenomenon coincided with a decrease in viability of the cultures. While the previous report that aminoglycoside uptake is dependent on the magnitude of $\Delta\Psi$ (Taber et al., 1987), correlates well with the aminoglycoside resistant mutant A450-10S, it is in apparent contradiction with the aminoglycoside sensitive mutant A450-10SR. The data presented in Table 8 show that values of $\Delta\Psi$ for both A450-10S and A450-10SR were virtually identical for actively growing cultures, and that not until late in stationary phase that the value of $\Delta\Psi$ was less for A450-10S. There is however a slight difference for the two mutants in the value of $\Delta$p, and this difference becomes larger in later stages of growth. The largest energetic difference between the two mutants lies in the magnitude of $\Delta$pH.

Thus it may be that the uptake of aminoglycoside antibiotics in *A. salmonicida* is dependent on some other effect exerted by a lack of cytochromes.

In order to test the cells acid tolerance, cultures were grown in unbuffered media where A450-10S and A450-10SR extensively acidify the media (Fig. 10). A450-10S was incapable of maintaining a ΔpH greater than -50 mV or 0.85 pH units. A450-10SR was capable of creating ΔpH values of ~65 mV or 1.1 pH units, but incapable of maintaining this ΔpH during later stages of growth. These data, in combination with the growth curve in Fig. 10, suggests that the mutants A450-10S and A450-10SR have both lost at least part of the acid tolerance observed in the wild type strain A450. It is interesting to speculate that this increased sensitivity to low pH has a great influence on virulence. *A. salmonicida* has been demonstrated to be a facultative intracellular pathogen (Garduño et al., 1993b), that is capable of growth in the macrophages of fish. As the phagosome is acidified following phagosome-lysosome fusion, the survival of *A. salmonicida* through this event would absolutely require tolerance to acidic conditions, and the ability to maintain a ΔpH during such a time.

The selection for energetically impaired bacteria results in effective attenuation. An aminolevulinic (ALA) acid, or heme auxotroph constructed in *Vibrio cholerae*, like A450-10S, displayed a reduced growth rate compared to wild type under aerobic conditions (Rijpkema et al., 1993). It was suggested that these heme auxotrophs showed poor growth aerobically due to a lack of catalase activity thus a hypersensitivity to reactive oxygen species (Elliot and Roth, 1989). We have shown that the cytochrome deficient mutant A450-10S is catalase positive (data not shown), thus the impaired growth rate of A450-10S is assumed to be due to the lack of effective energy conversion associated with an anaerobic, fermentative metabolism. The ALA auxotroph of *V. cholerae* was demonstrated to be attenuated and effective as a live vaccine, but it was

suggested that the lack of persistence and thus reduced virulence was due to an inability of the ALA auxotroph to effectively colonize the epithelia of the gut where the oxygen tension is reportedly high (Bornside et al., 1976). It has been demonstrated that A450-10S was completely unable to persist in fish and was thus likely not effective as a live vaccine due to the lack of persistence, thus aerobic capabilities may also play an important role in the colonization of fish by *A. salmonicida*.

The precise reasons for the attenuation of the mutants remain unclear although it is not likely to be a result of an individual effect such as acid intolerance affecting intracellular survival, a lack of aerobic capabilities playing an important role in the colonization of the fish, the slightly altered fatty acid composition of the lipids, or the effects of a disorganized surface architecture. Most probably, the attenuation is the result of the pleiotropic nature of the mutations harbored in the mutants A450-10S and A450-10SR (see Table 12 for summary of effects). These types of attenuating mutations that affect the fundamental physiology of the pathogen, are much safer than attenuating mutations that result in simple auxotrophy, as the complementation of an auxotroph may readily result from dietary supplements of the recipients of that live vaccine.

Finally, it was noticed that a further phenotype associated with the mutations in A450-10S and A450-10SR is the inability of the mutants to persist in dilute aqueous environments. This phenotype may prove to be useful in the licensing of such mutants as commercial vaccines. Although the in vitro assay used to determine persistence can only serve to partially mimic conditions in the environment, it does indicate that environmental impact of these mutants would be negligible, especially if the vaccine strains are released only in areas where *A. salmonicida* is an enzootic problem.

**Table 12. Summary of Phenotypes of *A. salmonicida* strains A450, A450-10S, and A450-10SR.**

| Phenotype | Strain A450 | A450-10S | A450-10SR |
|---|---|---|---|
| Growth | + | ± | + |
| Aminoglycoside resistance | - | + | - |
| A-layer | + | + | + |
| LPS | + | + | + |
| ECP | + | + | + |
| Capsule | + | nd | ± |
| Surface architecture | + | - | ± |
| Serum resistance | + | - | - |
| Aerobic metabolism | + | - | ± |
| Cytochromes | + | - | + |
| Resistance to reactive oxygen species[a] | + | - | - |
| Tissue persistence | + | - | ± |
| Invasiveness[a] | + | nd | + |
| Cytotoxicity[a] | + | nd | ± |

[a] R.A. Garduño, personal communication, 1993.

# Chapter IV

## Novel Antigens Expressed by *Aeromonas salmonicida* Grown In Vivo

### Purpose:

The successful attenuation described in Chapter III raised questions regarding the antigenic appearance of *A. salmonicida* when harbored inside a fish. The purpose of chapter IV is to describe the changes in antigenic profile that occur when *A. salmonicida* is cultured in vivo in chambers implanted into the peritoneal cavity of Rainbow trout (*Oncorhynchus mykiss*).

### Summary:

Virulent and avirulent *Aeromonas salmonicida* strains grown inside intraperitoneal implants in Rainbow trout (*Oncorhynchus mykiss*) were examined for unique antigen expression. Western immunoblots using immune rabbit serum raised against in vivo grown cells revealed several unique antigens. With the exception of lipopolysaccharide (LPS), these novel antigens were destroyed following proteinase K treatment. The majority of these antigens were not induced in vitro in response to either iron limitation or anaerobiosis. In addition, electron microscopy demonstrated the presence of a putative capsule on in vivo grown cells. Purification and fractionation of this carbohydrate material from cells grown in carbon rich synthetic media resulted in the isolation and separation of an antigenically distinct LPS not seen with cells grown in standard media. Antiserum raised against in vivo grown cells recognized both this LPS

and the typical LPS of *A. salmonicida* apparent in in vitro grown cells. Antiserum raised against in vitro grown cells recognized only the in vitro expressed LPS. Antisera directed against in vivo grown cells was approximately 10 times more sensitive than sera directed against in vitro grown cells in detecting *A. salmonicida* in infected fish kidney tissue.

## Materials and Methods:

**Bacterial strains.** *A. salmonicida* strains A450 and MT26 are wild type isolates virulent for salmonids. The avirulent mutants A450-3 and 10SR were derived from A450. Strain A450-3 is an isogenic, A-layer deficient derivative of A450. Strain A450-10SR was developed as an attenuated furunculosis vaccine and is described in chapter III.

**In vitro media and culture conditions.** All *in vitro* grown bacteria were grown in Tryptic Soy Broth (TSB, Difco) with 0.2% glucose. Solid media was Tryptic Soy Agar (TSA, Difco). Media used for the production of the putative capsule was prepared as described by Garrote *et al.*, (1992). *A. salmonicida* was cultured at 20 C. Iron limitation was imposed with ethylene-diamine-di-(0-hydroxyphenylacetic acid) (EDDA) as described by Chart and Trust (1983). Anaerobic growth was performed by shaking (100 rpm) cultures for 48 hours in anaerobic jars with a $CO_2/H_2$ anaerobic gas-pak (BBL).

**In vivo culture of *A. salmonicida*.** Cells of the A-layer$^+$ wild type strain A450, the attenuated mutant strain A450-10SR, and an isogenic A-layer$^-$ strain (A450-3) were grown in the peritoneal cavity of 400-500 g rainbow trout (*Oncorhynchus mykiss*) in a surgically implanted 1 ml chamber fitted with a 0.45 µm Millipore filter as described elsewhere (Garduño et al., 1993a). Briefly, 5 x $10^8$ washed cells suspended in 1 ml PBS (10 mM $NaH_2PO_4$; 0.85% NaCl; pH 7.25) were aseptically introduced into the chamber. The chamber was then surgically implanted into the peritoneal cavity of the fish, and allowed to remain for 7 days. The fish were then sacrificed, and the chamber recovered. Cells from the chambers were then washed gently 5 times with sterile PBS. These cells were then used immediately for antisera preparation or electrophoresis samples (see

below). The yield of cells from the peritoneal implants was similar to those described previously (Garduño et al., 1993a).

**Antigen and Antisera preparation.** Washed whole cells of A450 and A450-3 from both in vivo and in vitro growth were formalin fixed prior to injection as follows. The cells were resuspended to 1 O.D. at 650 nm, and 35% formalin was added to a final concentration of 5%. These suspensions were incubated at 4°C for 16 hours. Antiserum was prepared by injecting New Zealand White rabbits subcutaneously with 1 x $10^8$ cells in Complete Freunds Adjuvant. Fourteen days later the rabbits received a booster injection consisting of 1 x $10^8$ cells in Incomplete Freunds Adjuvant. On day 28, the rabbits were exsanguinated and the collected serum was stored at -20°C. Serum samples from these rabbits taken prior to the injections were used as control sera. Antiserum against in vivo grown A450-3 (anti-in vivo A450-3) and antiserum against both in vitro and in vivo grown A450 (anti-in vitro A450 and anti-in vivo A450) were used in subsequent experiments.

**Electrophoresis.** Samples were prepared for electrophoresis by resuspending 5 mg washed wet cell pellets in 250 µl of solubilization buffer followed by incubation at 100°C for 5 minutes (Ames, 1974). For LPS visualization, Proteinase K was added to these samples at a final concentration of 10 µg/ml, and the samples were incubated at 55°C for 3 hours. Samples were then separated on 12.5% sodium dodecylsulfate-polyacrylamide gels (SDS-PAGE) according to the modified method of Laemmli (Ames, 1974). Proteins were visualized with Coomassie brilliant blue R, and lipopolysaccharides (LPS) with the silver stain method of Tsai and Frasch (1982). Western immunoblots were performed essentially as described by Towbin *et al.*, (1979). Briefly, after SDS-PAGE proteins were

transferred to nitrocellulose paper, followed by blocking with 3% skim milk in 1.5 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4 (NET buffer); the blots were then incubated with a 2000 fold dilution of polyclonal rabbit sera directed against the formalinized A450 whole cells. After extensive washing, these blots were then incubated with 1 $\mu g\ ml^{-1}$ alkaline phosphatase-conjugated goat anti-rabbit antibodies (CALTAG Laboratories, South San Francisco, Calif.), and developed as described by Blake *et al.* (1984). All incubations and washes were performed in NET buffer, containing 0.05% NP40 (Sigma Chemical Co.).

**Characterization of carbohydrate antigens.** Growth of cells on carbon rich complex media for the production of capsule was as previously described (Garrote et al., 1993). The purification of putative capsule was as follows: cells were harvested from the appropriate media by centrifugation at 5000 x g, at 4°C, and incubated for 16 hours in 0.9 M $CaCl_2$. Both the culture supernatant and the $CaCl_2$ extraction supernatant were precipitated with ethanol at a final concentration of 80% for 16 hours at -20°C. The resulting precipitate was harvested by centrifugation at 20,000 x g and 4°C. The pellet was resuspended in 10 mM Tris; 1 mM $MgCl_2$ (pH 8.0), treated with 10 U DNase (Boerhinger Mannheim) and 10 U RNase (Boerhinger Mannheim) for 2 hours, and incubated at 50°C for 3 hours with Proteinase K (10 $\mu g\ ml^{-1}$). The sample was then mixed 1:1 with 0.4 M NaCl, 0.5% Sodium deoxycholate (DOC), 2 mM EDTA, 10 mM Tris (pH 8.0), heated to 50°C for 10 minutes, and applied to a 50 cm x 25 mm Sephadex G-200 column equilibrated with 0.2 M NaCl, 0.25% DOC, 1 mM EDTA, 10 mM Tris, 0.02% $NaN_2$, (pH 8.0). The column was eluted with the same buffer at 6 ml $hour^{-1}$ collecting 1 ml fractions. Aliquots (0.25 ml) of the fractions were then subjected to total carbohydrate analysis to locate peak fractions. The carbohydrate assay consisted of

mixing a 0.25 ml sample with 0.15 ml 5% phenol and 1 ml 18 M $H_2SO_4$. This was reacted at 20°C for 20 m. and the absorbance read at 485 nm.

**ELISA Method.** Aliquots (50 µl) of the column fractions were incubated in 96 well microtitre plates for 3 hours at 37°C. The wells were washed with PBS with 0.05% Tween 20 (PBS-Tween), and blocked with 3% skim milk in PBS-Tween for 3 hours at 37°C. Antisera, either anti-in vivo grown or anti-in vitro grown *A. salmonicida*, was added at a 1:500 dilution to each well in PBS-Tween with 1% skim milk and incubated for 1 hour. The wells were subsequently washed extensively with PBS-Tween, after which, alkaline phosphatase-conjugated goat anti-rabbit antibody (Caltag) was added at a 1:4000 dilution. The colour reaction was developed using p-nitrophenyl phosphate (1 mg $ml^{-1}$) in 0.95 M ethanolamine; 5 mM $MgCl_2$ (pH 9.0), the reaction was stopped using 1 volume of 1 M sodium citrate pH 4.5.

ELISA of infected Chinook salmon kidney samples were carried out by diluting infected and non infected salmon kidneys 1:3000 with PBS-Tween and incubating these in wells of a standard ELISA plate for 1 hour. The first antibodies (anti-in vivo and anti-in vitro *A. salmonicida*) were purified using ImmunoPure IgG purification kit (Pierce) according to the manufacturers instructions (antibodies were lyophilized and resuspended to 0.1 mg $ml^{-1}$ in sterile water). After blocking (as described above), these purified IgG samples were used in this ELISA format in serial 1:3 dilutions starting at 1 µg per well. The washing and developing steps were carried out as described above.

**Electron microscopy.** Washed cells were fixed as previously described (12) in 1.25% formaldehyde, 2.5% glutaraldehyde, 0.03% tannic acid, and 0.5% Alcian blue in Hanks balanced salt solution (HBSS). Post fixation was in carried out in 1% osmium tetroxide in HBSS for 1 hour at 4°C. Cells were then embedded in 0.3% agarose, washed with

HBSS, and dehydrated with ethanol. The resulting pellets were embedded in EPON-812. Thin sections (<0.1 μm) were then stained on grids with 2% uranyl acetate and 0.2% lead citrate. Sections were examined in a Philips EM-300 transmission electron microscope (Philips Electronic Instruments Inc., Mahwah N.J., U.S.A.) at an accelerating voltage of 60 kV.

## Results:

**SDS-PAGE and Western Blot Analysis of Cell Associated Antigens.** When in vivo or in vitro grown whole cell lysates of *A. salmonicida* were analyzed by SDS-PAGE (Fig. 14A) no major differences were seen with the exception of A-protein mutants ($A^-$)(lanes 3 and 6). This was hardly unexpected as differences in a complex mixture of proteins would have to be major to be noticed without prior fractionation. Western blots of these samples reacted with rabbit antisera to in vitro grown A450 reveal no differences between in vivo and in vitro grown cells (data not shown). However, immunoblots of these samples reacted with rabbit antisera to in vivo grown A450 cells (Fig. 14B) and A450-3 cells (Fig. 14C) revealed a number of novel antigens expressed by *A. salmonicida* when cultured in vivo, particularly antigens of high $M_r$. Proteinase K treatment of the cell samples followed by Western immunoblotting with rabbit anti-in vivo A450 resulted in an apparent loss of all antigens with the exception of lipopolysaccharide (LPS)(Fig. 14D), indicating that the majority of the antigens were protein. Both antisera failed to detect these antigens in naive fish sera or peritoneal exudate (data not shown) indicating that these antigens are of bacterial origin.

It is interesting that the antigenic profile of the attenuated vaccine strain A450-10SR was not drastically altered by in vivo growth (Fig. 14B and 14C, lanes 2 and 5). However, both antigenic profiles of A450-10SR more closely resemble that of in vivo grown wild type cells than in vitro grown wild type cells.

From the immunoblot using antisera raised against in vivo grown, A-layer deficient strain A450-3 (Fig. 14C), there appeared to be antibodies present against either the A-protein, the protein subunit of the A-layer, or to a protein of similar $M_r$. This is surprising considering that A-protein is not clearly visible in either the Coomassie stained

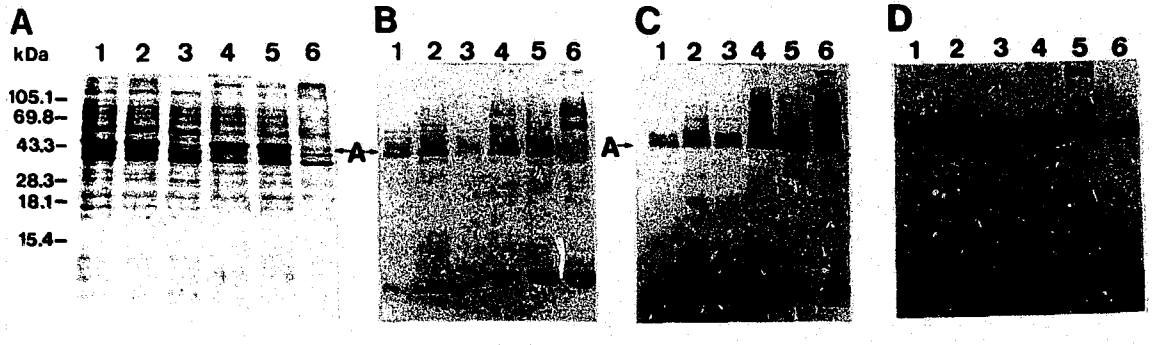


**Figure 14.** In vitro and in vivo expressed antigens of *A. salmonicida*. SDS-PAGE and immunoblots of the *A. salmonicida* strains grown in vitro and in vivo. In vitro grown A450 (lane 1), A450-10SR (lane 2), and A450-3 (lane 3); in vivo grown A450 (lane 4), A450-10SR (lane 5), and A450-3 (lane 6). Panel **A** represents a Coomassie Blue stained gel. Panel **B** is a Western immunoblots of whole cell lysates developed with rabbit anti-in vivo A450, and panel **C** with rabbit anti-in vivo A450-3. Panel **D** is a Western immunoblot of proteinase K digested cells developed with rabbit anti-in vivo A450. The position of the surface layer protein, A-protein, is indicated. Molecular weight standards are indicated in kDa.

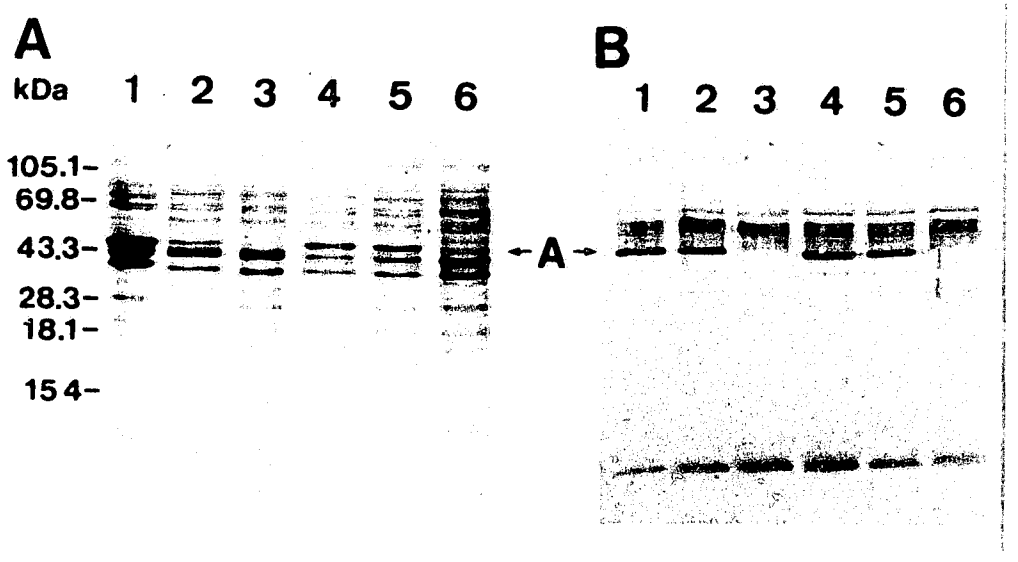


**Figure 15.** Effect of anaerobiosis and iron restriction on antigen expression in *A. salmonicida*. SDS-PAGE and immunoblots of *A. salmonicida* strains: anaerobically grown A450 (lane 1), anaerobically grown A450-10SR (lane 2), anaerobically grown A450-3 (lane 3), iron restricted A450 (lane 4) iron restricted A450-10SR (lane 5), and iron restricted A450-3 (lane 6). Panel **A** is a Coomassie Blue stained gel, and panel **B** is a Western immunoblot developed with rabbit anti-in vivo A450. The position of the surface layer protein, A-protein, is indicated. Molecular weight standards are indicated in kDa.

gels (Figs. 14A and 15A, lanes 3 and 6), or immunoblots of A450-3 using rabbit anti-in vivo A450 (Fig. 14B lanes 3 and 6), nor is the A-layer visible in electron micrographs of negatively stained in vivo or in vitro grown whole cells of A450-3 (data not shown).

Lee and Falkow (1990) recently demonstrated that anaerobiosis was an important factor in *Salmonella* virulence and antigen expression, in order to examine if this state induced any of the in vivo expressed antigens seen in the *A. salmonicida*, cells were cultured in an anaerobic atmosphere. Although some differences were apparent in the overall protein profiles there were no detectable differences with respect to antigenic profiles from cells cultured under standard in vitro conditions (Fig. 15A).

Since it is common for iron assimilation systems to be associated with in vivo growth (Smith, 1990), the antigenic profiles of cells grown under iron limited conditions were examined. From Western blots of these cells using anti-in vivo A450 serum (Fig. 15B), it is apparent that the majority of the in vivo expressed antigens were not expressed in response to iron limitation. However, there were 3 or 4 minor proteins ($M_r$ between 17,000 and 28,000) that were clearly recognized by the anti-in vivo serum and were only present in visible amounts when the cells were cultured in vitro if iron is limiting.

**Electron microscopy.** Thin sections of A450-3 were stained with the acidic polysaccharide stain, Alcian blue, revealing a diffuse layer surrounding the cell that was only present on in vivo grown cells (Fig. 16). This A-layer deficient strain was used for the electron microscopy to eliminate visual obstruction of the putative capsule by the A-layer present on wild-type strains. Other evidence has suggested that this material was hydrophilic and not of host origin (Garduño et al., 1993b), which taken together, indicated that this layer is a capsular polysaccharide.

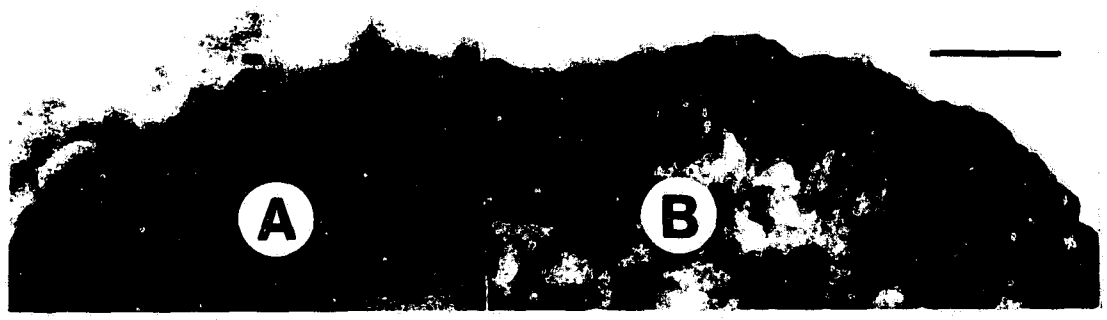


**Figure 16.** Electron microscopy of in vitro and in vivo grown *A. salmonicida.* Composite electron micrograph of thin sections of in vivo grown A450-3 (**A**) and in vitro grown A450-3 (**B**) stained with the acidic polysaccharide stain, Alcian blue, revealing a diffuse layer only present on the in vivo grown cells. The bar scale represents 0.1 μm.

**Partial Purification and Characterization of the Putative Capsule.** As the samples recovered from the peritoneal implants were, in general, to small to permit subcellular fractionation, the crude purification of a putative capsule was carried out using cells grown on a carbon rich media as recently described for the production of an undefined capsule by *A. salmonicida* (Garrote et al., 1992). The sample isolated prior to gel permeation chromatography closely resembled *A. salmonicida* LPS on SDS-PAGE (Fig. 17A, lane A), having both fairly homogenous length O-antigen and fast migrating, free core polysaccharide; however there was no evidence of a high MW capsular polysaccharide. Further fractionation of this sample by Sephadex G-200 column chromatography in the presence of detergent resulted in an apparent separation of the putative O-antigen containing LPS into two broad peaks of nearly identical mobility ($M_r$ ≈ 50,000 to 65,000) on SDS-PAGE (Fig. 17A) with peak I containing fractions 17 to 37, and peak II containing fractions 40 to 52. This second peak was not seen when *A. salmonicida* grown in TSB was used as a source of carbohydrate (data not shown). The fractions shown in Fig. 17 represent only the first half of the elution profile, with core antigen eluting in fractions 88-95. Also visible on silver stained SDS-PAGE are several minor bands from within peaks I and II, that were size fractionated as expected from gel permeation chromatography suggesting that although peaks I and II are broad, there are sufficient differences within the molecules of these peaks to allow some measure of separation. These minor bands that were fractionated had $M_r$ of approximately 30 kDa, (fractions 20-30), 20 kDa (fractions 37-41), and 15 kDa (fractions 45-52)(Fig. 17A). None of the latter peaks (after fraction 52) displayed the antigenic differences observed as

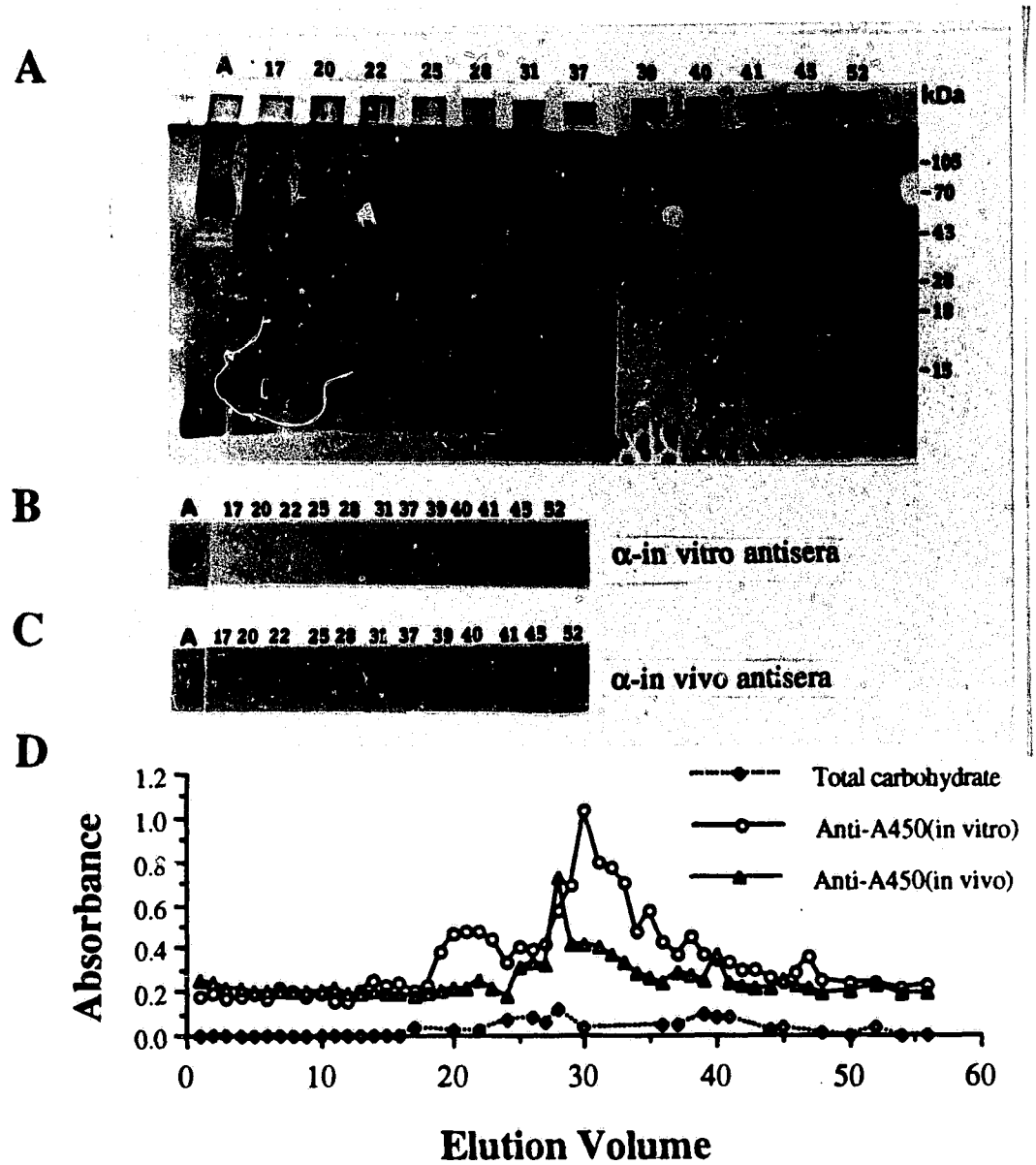


**Figure 17.** Partial purification of the putative capsular polysaccharide from *A. salmonicida*. Panel **A** is a silver stained SDS-PAGE of the fractions from the Sephadex G-200 column. Fraction numbers are above the lanes, also shown is the original sample that was applied to the column (A). Panels **B** and **C** are Western immunoblots of the LPS region of the gels of the same fractions shown in panel A. The sera used for developing was rabbit anti-in vitro A450 (**B**) and rabbit anti-in vivo A450 (**C**). Panel **D** is a representative ELISA of the column fractions showing the relative differences in antigen elution. Also shown for comparison is the elution profile based on total carbohydrate content. Peaks I and II contain fractions 17 to 37, and 40 to 52 respectively (see text). Void volume of the column is 17 ml.

in peaks I and II. Included in peak I fractions are one or more bands that partially obscure the lower $M_r$ O-antigen bands ($M_r$ < 55,000), this region is not as clearly visible in peak II (Fig. 17A).

The immunoblots of the column fractions revealed significant differences in antigen recognition between the anti-in vitro A450 and anti-in vivo A450 antisera. The anti-in vitro A450 serum immunoblot, although heavily developed, only revealed antigens associated with the major fractions of peak I from the column (Fig. 17B), while the anti-in vivo A450 serum immunoblot revealed antigens associated with most of the column fractions of both peak I and II (Fig. 17C), albeit a weaker response. These differences were seen in each of three identical trials, indicating that these differences are not due to a poor transfer of antigens to the nitrocellulose in the immunoblot procedure. Other differences in antigen recognition by the two serum samples seen by immunoblotting are that the anti-in vitro A450 serum only detected a narrow $M_r$ range of high MW LPS, while the anti-in vivo A450 serum seemed to detect the a broader $M_r$ range of LPS. As mentioned above, there are minor bands associated with the two LPS fractions visible by silver staining (Fig. 17A), and it is possible that the two immune sera are detecting different antigens within the respective fractions. Both types of antisera failed to recognize core antigen from *A. salmonicida.* This phenomenon appears to be a common feature of *A. salmonicida* LPS (Thornton, Garduño and Kay, unpublished observations).

Using antisera raised against in vitro vs. in vivo grown cells, the ELISA pattern of the carbohydrate containing column fractions was shown to be similar for the two different antisera, however there were subtle differences. As seen in Figure 17D, the peaks occurred at different points, again suggesting that the anti-in vivo A450 and the anti-in vitro A450 sera recognize different antigens. Evidence for these differences can be seen in the western blots depicted in Figures 17B and 17C. Antisera raised against in

vitro A450, while apparently higher in titer than anti-in vivo A450, reacted only with the first O-antigen type eluted from the column. The anti-in vivo A450 serum was capable of reacting with most, if not all, fractions that contained O-antigen like material, suggesting the possibility that this second O-antigen like fraction contains a novel, in vivo expressed molecule that may be either LPS or some form of carbohydrate capsule.

**In situ Detection of *A. salmonicida*.** In order to assess the relative sensitivity of the two sera for in situ detection of *A. salmonicida* cells, an ELISA was carried out using affinity purified antibodies raised against in vitro and in vivo grown cells and a 3000 fold dilution of *A. salmonicida* infected salmon kidney samples as an antigen. It was previously determined that this dilution of antigen represented the minimum number of *A. salmonicida* cells for consistent detection (data not shown). As can be seen in Figure 18, although the concentration of the two antibodies was constant, the ELISA using antibodies against in vivo grown cells was approximately 10 times more sensitive than with antibodies against in vitro grown cells in the range from a 1 μg to 10 μg of added antibody. Additionally, the antisera raised with in vivo grown cells recognized in vivo grown cells better than in vitro grown cells, antisera raised with in vitro grown cells recognizes these cells better, indicating antigenic specificity dependent on in vitro vs. in vivo growth.

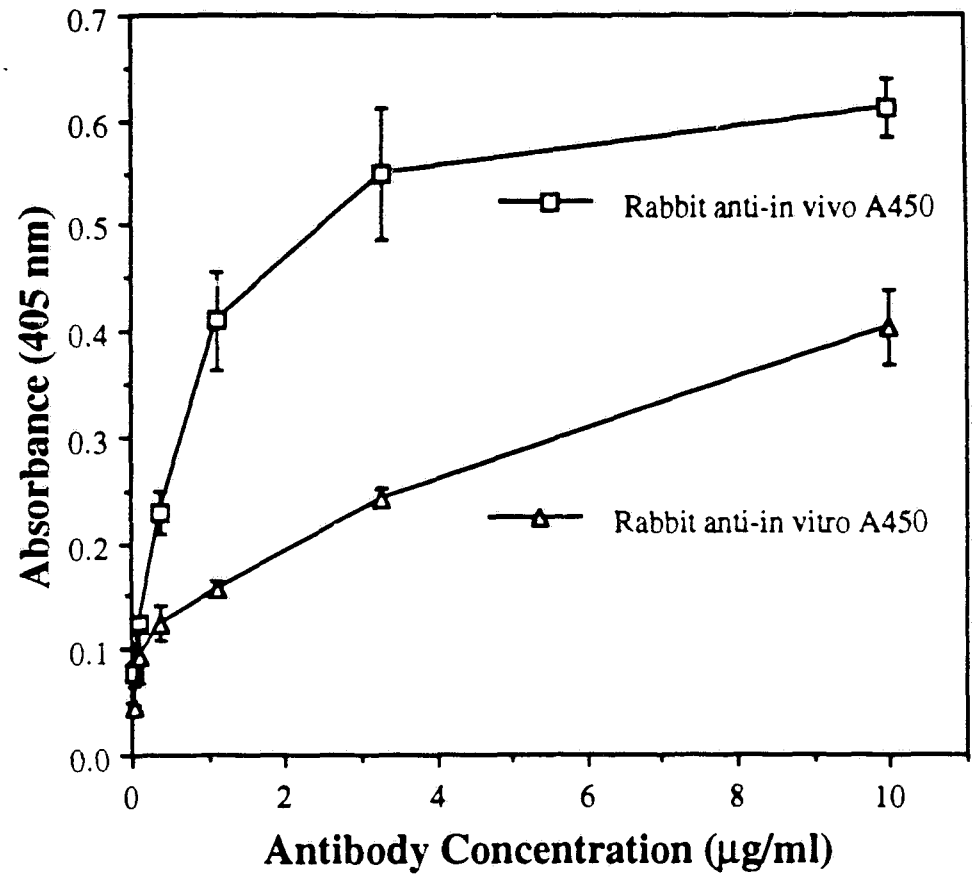


**Figure 18.** Comparison of anti-in vitro and anti-in vivo antibodies in the detection of *A. salmonicida* in infected salmon kidneys. ELISA were used to compare the sensitivity of affinity purified rabbit anti-in vitro A450 IgG (△) with rabbit anti-in vivo A450 IgG (□). Both antibody preparations were purified, standardized, and used at identical concentrations. Error bars represent standard deviation calculated from duplicate experiments.

## Discussion:

The data presented here demonstrate that *A. salmonicida* produces unique antigens in response to extracellular growth in vivo. That bacterial cells grown in vivo and those grown in vitro can differ has been recognized for at least forty years (Smith et al., 1953; Keppie et al., 1955). The most thoroughly documented cases of in vivo antigen expression are probably for those antigens involved in iron sequestration (Sciortino and Finkelstein, 1983; Griffiths et al., 1985; Shand et al., 1985; Smith, 1990; Morck et al., 1991). The in vivo expression of antigens by pathogenic bacteria is recognized as an important tool not only for understanding pathogenesis, but also for the development of effective vaccines and diagnostic assays (Brown et al., 1988; Williams, 1988; Smith, 1990).

We have found that raising antisera in rabbits with in vivo grown cells is a sensitive method for the detection of novel antigens. Other methods, many of which have been useful for the development of effective vaccines, may not result in the identification of many in vivo expressed antigens for the following reasons. Attempts to examine in vivo expressed antigens from bacterial pathogens using sera from rabbits immunized with cells grown under various in vitro conditions usually reveals only differences in the level of expression of in vitro expressed antigens and few, truly novel antigens are seen. The use of antiserum to cells grown under varied in vitro conditions (e.g. iron restriction) restricts the study to those antigens expressed in response to specific in vivo conditions recreated in the laboratory. With convalescent antisera, only those antigens with potential for stimulating humoral immunity will be seen, and thus effective candidates for either diagnostic assays or antigens involved in the stimulation of cell mediated immunity may be overlooked.

The vast majority of the in vivo expressed antigens detected were demonstrated to be proteinase sensitive, and were not induced in response to either iron restriction or anaerobiosis. It is interesting that few of the major in vivo antigens are expressed in response to iron limitation in vitro. There was no evidence of the high molecular weight, iron regulated proteins described by Chart and Trust (1983) in both immunoblots and Coomassie blue stained SDS-PAGE, but rather, there were minor proteins with apparent molecular weights between 17,000 and 28,000 $M_r$ that clearly reacted with the anti-in vivo serum and were only present in visible amounts when the cells were cultured in vivo or if iron was artificially restricted. It is known that in the case of the fish pathogen *Vibrio anguillarum* the high affinity iron sequestration system is required for virulence (Crosa, 1980), thus it can be assumed that iron is limiting in fish tissues. Therefore, it is possible that the proteins involved in iron assimilation for *A. salmonicida* described by Chart and Trust (1983) are either poor immunogens in rabbits, or that an iron sequestration system different than that described by these authors is utilized by *A. salmonicida* strain A450 cells when grown in vivo. Anaerobiosis has recently been demonstrated to induce up to 29 different proteins involved in *Salmonella* virulence (Lee and Falkow, 1990). However, *A. salmonicida* cultured in an $H_2/CO_2$ environment did not express these in vivo induced antigens. Difficulty in determining the precise environmental signals that stimulate the expression of in vivo expressed antigens is not surprising considering the complexity of the in vivo environment. In many cases, in vivo antigens expressed by bacterial pathogens are merely members of the stress response family of proteins (Buchmeier and Hefron, 1990).

An interesting observation was that the live vaccine strain A450-10SR was shown to have virtually the same antigenic profile on western blots regardless of whether it was cultured in vivo or in vitro (Fig. 14). As this strain was demonstrated to persist free in fish tissues for only 48 to 72 hours (Chapter III), it is attractive to propose that a high level of

protection may be conferred by this live vaccine due to the presence of these previously unknown in vivo expressed antigens of *A. salmonicida*, rather than just to the known antigens.

The data that demonstrates that A450-3 may produce A-protein (Figs. 14 and 15) was unexpected, and may be explained as follows. As A-protein is not visible in A450-3 samples when using sera derived from in vivo grown A450 in Western blots (Figs. 14B and 15B), it may be possible that the 50 kDa band apparent in all samples of the Western blot developed using anti-in vivo A450-3 sera, (Fig. 14C) is not A-protein, but rather an antigen expressed by *A. salmonicida* when cultured *in vivo*. Alternatively, A450-3 may produce small amounts of A-protein sufficient for an immune response in rabbits, but insufficient for visualization by SDS-PAGE or electron microscopy.

The presumptive identification of a polysaccharide capsule on in vitro grown *A. salmonicida* has been recently reported (Garrote et al., 1992). We have confirmed the existence of capsular material on *A. salmonicida*, but this material, isolated using the same procedure of Garrote et al. (1992) appears, by SDS-PAGE, to be a unique form of LPS that *A. salmonicida* expresses both in vivo (based on recognition by antibodies raised with in vivo grown cells) and in the media of Garrote *et al.* (1992), and is possibly not a true capsule as suggested by these authors. The potential for the existence of more than one LPS type in *A. salmonicida* has been described elsewhere (Lee and Ellis, 1991), but as these authors did not completely characterize the two forms, no direct comparisons could be made.

As SDS-PAGE separates molecules on the basis of molecular weight only if the samples have a constant charge to mass ratio, it is unknown whether these two LPS populations are of the same size. If the sizes are indeed the same then possibly the second major peak (peak II) from the G-200 column is comprised of a population of LPS that interacts differently with the column matrix, thus accounting for the longer retention

time. Conversely, if relative mobility on SDS-PAGE is an artifact, the two major peaks must be sufficiently different in size so as to alter their mobility through G-200. Factors that could affect the elution profile of these molecules could include modification of the individual sugars or a different repeating O-oligosaccharide structure. The fact that both peaks of LPS-like material react differently with different antisera by both immunoblotting and ELISA is compelling evidence that the molecules of these two peaks differ antigenically, although the precise nature of the antigen(s) being detected is not known at this time. In vivo variation of O-antigens has also been described for *Klebsiella pneumoniae* (Whitfield et al., 1991) and *Pseudomonas aeruginosa* (Cochrane et al., 1988), while the O-antigen of *Neisseria gonorrhoeae* is sialylated in vivo by host derived mechanisms (Mandrell, 1992; Wetzler et al., 1992). Clearly, as we can stimulate *A. salmonicida* to produce this material in vitro, the change in antigenicity of the LPS-like material is not due to host factors as is the case for *Neisseria gonorrhoeae* (Mandrell, 1992; Wetzler et al., 1992).

The presumption that the capsule observed on in vivo grown cells consists of this LPS-like material is based on the observations that no new major protein of sufficient abundance to account for the coat observed by transmission electron microscopy was seen by SDS-PAGE of in vivo grown cells, and that the capsule purification method of Garrote et al. (1992) failed to reveal any other likely carbohydrate candidate. As the antisera against the in vivo cells contains antibodies to A-protein (Fig. 14), we must conclude that this putative capsule or LPS does not shield the entire surface of the cell. The possibility that the material surrounding the in vivo grown cells is A-layer has been ruled out by immunogold staining cells with monospecific polyclonal anti-A-protein antibodies (Garduño et al., 1993b). Here it was found that even A-layer expressing cells do not react well with anti-A-protein antibodies after in vivo growth, even though A-layer was visible by transmission electron microscopy, suggesting that the material shields the

A-layer enough to block immunogold labeling. Finally, it must be emphasized that the carbon rich media used to induce capsule could only serve to mimic some in vivo conditions and in no way could it exactly recreate them, thus there still may be a novel capsule expressed in vivo that is separate from this antigenically distinct form of LPS.

The increased sensitivity of an anti-in vivo antibody based diagnostic test (ELISA) for *A. salmonicida* present in infected tissues serves to emphasize that unique antigens are expressed in vivo. This assay also suggests that some of these in vivo expressed antigens are surface exposed. In conjunction with the observation that the A-layer is apparently shielded on in vivo grown cells (Garduño et al., 1993b), this leads to one possible explanation of the inability to correlate specific antibody titers to the known surface antigens (A-layer and LPS) with protection from current vaccines derived from in vitro grown cells (Tatner, 1987; Hastings, 1988; Hastings and Ellis, 1990). Simply, the antigens that stimulate protective immunity may not be present on in vitro grown cells. Thus, it is likely that the inability of current furunculosis vaccines to provide high levels of protection is due simply to a lack of appropriate antigens in the vaccine preparation.

It should be emphasized that since *A. salmonicida* is a facultative intracellular pathogen, the unique antigens identified here may not be the only in vivo expressed antigens. Clearly, the data and methods presented in this chapter for the examination of in vivo expressed antigens on *A. salmonicida* in response to extracellular growth have opened new avenues to exploit in the design and construction of effective furunculosis vaccines and diagnostic assays.

# Chapter V

## Efficacy of Attenuated Live Vaccines for the Control of Salmonid Furunculosis

### Purpose:

Whereas the previous chapters document fundamental in vitro and in vivo physiological properties of attenuated strains of *A. salmonicida*, this chapter describes the culminating experiments that demonstrate the protection afforded against challenge with virulent *A. salmonicida* by the different mutant strains, described in chapter III, when used as live vaccines.

### Summary:

The rapidly-growing, antibiotic-sensitive pseudorevertant A450-10SR, when administered by immersion, effectively protected salmonid fish from challenges of a heterologous virulent strain suggesting its candidature as a live attenuated furunculosis vaccine.

This mutant A450-10SR and other mutants of *Aeromonas salmonicida* strains lacking either the A-protein, O-antigen, or both of these major surface antigens were tested in Rainbow trout (*Oncorhynchus mykiss*, Walbaum) for their suitability as live vaccines (LV). Immersion vaccination of fish in $5 \times 10^7$ cfu $ml^{-1}$ of these strains with an identical immersion dose fourteen days later, resulted in significant protection by all strains from challenge with a heterologous virulent strain of *A. salmonicida* five weeks later. The levels of protection conferred were all greater than or equal to that provided by

an injected bacterin using the same vaccination schedule. With one exception, all LV strains that still possessed a functional O-antigen provided protective indices (PI) 4 - 7 fold greater than the PI for the fish injected with bacterin. When antibody responses of vaccinated fish were compared, it was found that only vaccination by bacterin gave rise to a measurable agglutinating titer. Western immunoblots using the immune fish sera failed to reveal any major differences in antigen recognition in fish that received any of the vaccines tested. These data suggest that the immune response generated by the use of live vaccine strains is different from that generated by a bacterin, and that these useful mutations may be incorporated into furunculosis LV strains for attenuation.

## Materials and Methods:

**Bacterial strains and culture conditions.** The *A. salmonicida* strains used in this study are listed in Table 3, Chapter III. Bacteria were grown in Tryptic Soy Broth (TSB, Difco) with 0.2% glucose, and were shaken at 250 rpm. Solid medium was Tryptic Soy Agar (TSA, Difco). *A. salmonicida* was cultured at 20°C, unless otherwise stated. Long term storage (>1 week) was carried out by freezing cultures at -70°C in 15% glycerol. The isolation of A-layer deficient strains was carried out by culture at 30°C as described previously (Ishiguro et al., 1981).

**Preparation of polyclonal rabbit anti-trout Ig.** Keyhole limpet hemocyanin (KLH) in complete Freunds adjuvant, was injected intraperitoneally into two 500 g rainbow trout (*O. mykiss*) at 5 mg per fish. Two weeks following this, the fish were boosted with a further dose of 5 mg KLH in incomplete Freunds adjuvant. Twenty-eight days following this booster dose the fish were euthanized with MS-222 (100 mg $l^{-1}$), and their blood collected from the caudal vein. The pooled blood was left at 4°C overnight to clot, and serum was separated by centrifugation at 1000 x g for 15 min at room temperature. The separated serum was aseptically aliquoted into 2 ml samples and stored at 4°C.

The 2 ml aliquots of serum were then passed twice through a 5 ml KLH-conjugated Aminolink™ column prepared according to the manufacturers directions (Pierce Chemical Company, U.S.A.). The column was then washed with 10 column volumes of PBS. Elution of bound fish serum proteins was performed with 3 M NaCl, 0.01 M sodium phosphate buffer (pH 7.5). The eluate was collected in 0.5 ml fractions and the absorbance of individual samples was measured at 280 nm. A single peak consisting of 3 fractions was eluted. The contents of this peak was determined by SDS-

PAGE. The affinity purified sample was demonstrated to contain major bands of approximately 75-80 kDa and 25-30 kDa (likely heavy and light chains respectively), as well as other minor bands (data not shown). This sample was dialyzed into 10 mM Tris, pH 7.5, and concentrated with the Centriprep-30™ system (Amicon) according to the manufacturers directions. A New Zealand White rabbit was then immunized with 100 µg of the protein in complete Freunds adjuvant, with 50 µg booster doses in incomplete Freunds adjuvant 2 and 4 weeks later. One month after the final booster dose, the rabbit was sacrificed and the blood collected. The immune rabbit serum was collected, and frozen in 5 ml aliquots. Testing of the rabbit anti-trout Ig (RAT) was carried out by both ELISA and Western immunoblotting. In ELISA, the RAT was tested by first coating ELISA plate wells with KLH, followed by the trout anti-KLH immune sera as a first antibody. Second antibody was the RAT, and the third antibody was alkaline phosphatase conjugated goat anti-rabbit Ig (CALTAG). The ELISA methodology was essentially the same as that used in Chapter IV with the above noted changes. The titre of the RAT was determined to be the dilution at which the signal to noise ratio was still greater than three, and was determined to be approximately $1000^{-1}$. In Western Immunoblotting, the RAT was used at a 1:400 dilution as a first antibody on a gel in which the samples were rainbow trout and chinook salmon sera. Second antibodies in the Western blot was alkaline phosphatase conjugated goat anti-rabbit Ig (CALTAG). The western blot demonstrated that the RAT was detecting major bands of ~80 kDa in both samples, and ~28 kDa in the chinook sera sample, as well as a few other minor bands. As a control an identical blot was developed using the commercially available goat anti-trout Ig (Kirkegaard and Perry Laboratories Inc., U.S.A.). In this blot all bands visible in a Coomassie blue stained SDS-PAGE gel were visible, suggesting that the RAT sera developed here was more selective in antigen recognition than the commercially available product.

**Electrophoresis.** Samples were prepared for electrophoresis by resuspending 5 mg washed wet cell pellets in 250 µl of solublization buffer followed by incubation at 100°C for 5 minutes (Ames, 1974). Western immunoblots were performed essentially as described by Towbin et al., (1979). Briefly, after SDS-PAGE, proteins were transferred to nitrocellulose paper, followed by blocking with 3% skim milk in 1.5 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4 (NET buffer); the blots were then incubated with a 100 fold dilution of immune fish sera collected from a pool of five fish prior to challenge. The next antibody was rabbit anti-trout Ig (prepared in this laboratory), followed by 1 µg $ml^{-1}$ of alkaline phosphatase-conjugated, goat anti-rabbit antibodies (CALTAG Laboratories, South San Francisco, Calif.). All antibody incubations were carried out in NET buffer with 1% skim milk for 1 hr at room temperature. After extensive washing, these blots were then developed as described by Blake et al., (1984). All washes and incubations were performed in NET buffer, containing 0.05% NP40 (Sigma Chemical Co.).

**Virulence and protection studies.** All experimental fish were maintained at 13°C(±1°C) in a continuous flow of aerated, dechlorinated city water before, and during experiments. Fish densities were maintained between 10 and 15 $kg/m^3$. Virulence of the mutants was determined by intraperitoneal (IP) injection of the test organism into juvenile (5-10 g) rainbow trout (*Oncorhynchus mykiss*, Walbaum), and the results indicated that all strains used as vaccines were attenuated (see chapter III). Briefly, overnight cultures were diluted to ~5 x $10^8$ cfu $ml^{-1}$ with sterile 0.85% saline and 0.1 ml (containing ~5 x $10^7$ cfu) was injected IP. Any strains causing mortalities at this level of inoculation were determined to be unsuitable for further use.

For injection vaccinations, fish were injected IP with bacterin, (approximately 100 µg total antigen), in 0.1 ml 0.85% saline. Immersion vaccinations were carried out by immersing fish (at a density of 15 kg $m^{-3}$) in 5 x $10^7$ cells $ml^{-1}$ of the appropriate strain

for 10 minutes. For experiments in which a booster vaccination was used, vaccinates were given an identical treatment 14 days later. The vaccinated and control fish were challenged by immersion exposure to the highly virulent *A. salmonicida* strain MT26, which was isolated from a local epizootic in chinook salmon (*O. tschawytcha*), two to five weeks after vaccination (see tables for individual trial method).

The fish were challenged by two different methods, ones in which LD50 values were calculated, and the others in which the relative percent survival (RPS) were calculated.

The LD50 method included multiple groups that were challenged with the virulent *A. salmonicida* strain MT26. For each of three challenge doses (see tables for individual trials), groups of fish were immersed in the appropriate amount of MT26 for 15 minutes. Mortalities were attributed to furunculosis if *A. salmonicida* could be recovered from kidney, spleen, and liver samples by standard plate culture methods. These challenge experiments were terminated when mortalities attributable to *A. salmonicida* ceased, but not in less than 18 days. $LD_{50}$ values were calculated according to the method of Reed and Muench (1938), and the protective index (PI) was calculated as a ratio of the $LD_{50}$ for vaccinated fish to the $LD_{50}$ for control fish.

The single dose challenge, or RPS method involved challenging groups of fish with a single dose of MT26 determined to kill >70% but <100% of non-vaccinated control fish. This dose was determined by empirical methods. RPS was calculated by the formula:

$$RPS = [\,1 - (\%\text{Mortality of Vaccinates}/\%\text{Mortality of Controls})] \times 100.$$

All challenges were carried out in 0.85% saline with constant aeration.

**Serum agglutination.** At the same time as the challenge experiments, five fish from each vaccination were sacrificed and their sera pooled. The antigen consisted of a

washed suspension (1 $OD_{650}$) of A450-3 cells (A-layer$^-$) that had been fixed in the presence of 5% formalin at 4°C for 16 h. Serum samples (100 µl) were added to the first well of a 96 well microtitre plate and serially diluted with phosphate buffered saline (0.01 M $Na_2HPO_4$, 0.15 M NaCl, pH 7.4) containing 0.05% NP40 (Calbiochem) and 1% skim milk. To each of these wells 100 µl of the antigen suspension was added, and the plate was incubated for 3 h at 20°C. Rabbit serum raised against wild type *A. salmonicida* was used as a positive control, and non-immunized fish sera was used as a negative control. Each serum sample was tested in duplicate assays.

## Results:

**Identification of major antigens.** The various live vaccine strains used were electrophoretically analysed in order to verify the presence or absence of the major antigens LPS and A-protein in Chapter III (see figure 4).

**Virulence and protection studies.** Virulence testing of all potential live vaccine strains (Chapter III; Table 3) were carried out by injection at a dose of $5 \times 10^7$ cfu per 5-7 g rainbow trout. As this dose represents approximately a $5 \times 10^5$ fold increase over the wild type strain $LD_{50}$ value by injection (unpublished observations), any strains unable to cause disease at this level were determined to be effectively attenuated (Table 3).

The data from challenge experiments using the different live vaccines are presented in Tables 13 through 15. The *A. salmonicida* strain A450-10SR was compared to A-layer deficient mutants (A450-10SR-3, A440, and A450-3), a mutant lacking O-antigen (A450-1), and its A-layer deficient counterpart (A450-1-3). Also for comparison, fish vaccinated with a commercially available bacterin of *A. salmonicida* were included. All of the live vaccines were demonstrated to induce protective immunity when administered to fish by immersion (Table 13). Clearly, the most effective immunity was stimulated in response to the strains A450-10SR, A450-10SR-3, and A450-3. These vaccinates yielded PI values of approximately 400-700. The $LD_{50}$ and PI values for the vaccine strains A450-10SR, A450-10SR-3 and A450-3 are estimates as no mortalities were recorded for fish vaccinated with A450-10SR, while only one fish at the lowest challenge dose ($1 \times 10^6$ cfu $ml^{-1}$), and one fish at the highest dose ($1 \times 10^8$ cfu $ml^{-1}$) died in the groups vaccinated with A450-10SR-3 and A450-3, respectively (Table 13). The mutants lacking O-antigen (A450-1 and A450-1-3) and one lacking only the A-layer

**Table 13. Immunity Provided by Various Attenuated *A. salmonicida* strains compared to a bacterin.**

| Vaccine[a] | # Mortalities(# Tested) Challenge Dose (cfu $ml^{-1}$) $1 \times 10^6$ | $1 \times 10^7$ | $1 \times 10^8$ | $LD_{50}$[b] | PI[c] |
|---|---|---|---|---|---|
| None (control) | 5(10) | 8(10) | 10(10) | $1.4 \times 10^6$ | 1 |
| 10SR | 0(10) | 0(10) | 0(10) | $>1 \times 10^9$ | >714 |
| 10SR-3 | 1(10) | 0(10) | 0(10) | $9.2 \times 10^8$ | 657 |
| A450-3 | 0(10) | 0(10) | 1(10) | $6 \times 10^8$ | 428 |
| A440 | 0(10) | 1(10) | 2(10) | $2.9 \times 10^8$ | 203 |
| A450-1 | 0(10) | 0(10) | 3(10) | $2.5 \times 10^8$ | 178 |
| A450-1-3 | 0(10) | 1(10) | 3(10) | $1.9 \times 10^8$ | 135 |
| Bacterin | 1(10) | 1(10) | 3(10) | $1.6 \times 10^8$ | 114 |

[a] Administered with an identical boost at day 14

[b] $LD_{50}$ values were calculated according to the method of Reed and Muench (1938). If the LD50 value exceeded the highest challenge dose, that value was extrapolated from the survival/killing curves generated.

[c] PI = $LD_{50}$ Vaccinates / $LD_{50}$ Controls.

(A440) are seemingly reduced in their efficacy, giving PI values of 100-200. Fish vaccinated by injection with a bacterin of strain MT26 also show a modest level of protection. Here, the $LD_{50}$ and PI values ($1.6 \times 10^8$ and 114 respectively) are comparable only to the fish vaccinated with the mutant lacking both A-layer and LPS even though both of these antigens are present in the bacterin.

Table 14 compares the live vaccine strain to a bacterin when administered by different routes. In control experiments, unvaccinated fish were killed in proportion to the level of challenge. The bacterin, administered intraperitoneally (IP), protected fish well at all levels of challenge, but when the bacterin was administered by immersion, a dramatic reduction in the level of protection for the two highest challenge levels was observed (data not shown). Interestingly, 10SR was not as effective as the bacterin when administered IP, but was protective by immersion and fish vaccinated in this way resisted higher levels of challenge by the highly virulent heterologous strain MT26. $LD_{50}$ values were increased from 10 fold (for administration by injection) to 35 fold (for administration by immersion) for fish vaccinated with 10SR (Table 14).

Table 15 demonstrates the efficacy of the vaccine strains A450-10SR and A450-10SR-3 in Atlantic salmon (*Salmo salar*). This experiment demonstrates that protection, as measured by RPS, is nearly twice that provided by the immersion bacterin. During a subsequent trial with Atlantic salmon, an epizootic outbreak of saproligniosis occurred. This is a fungal problem commonly associated with juvenile Atlantic salmon. Vaccinated and non-vaccinated fish were observed during this outbreak, at it was noted that a non-specific RPS against saproligniosis of approximately 60% was observed for only those fish receiving A450-10SR or A450-10SR-3, but not for those fish receiving a bacterin.

**Serum antibody response.** Sera from vaccinated fish were compared for the ability to agglutinate A450-3. This strain was chosen as it is A-layer deficient and as a result does

**Table 14. Efficacy of live vaccine (10SR) in Rainbow trout compared to a bacterin.**

| Vaccine[a] | Method of Administration | $LD_{50}$ | PI |
|---|---|---|---|
| None | NA[b] | $3.1 \times 10^6$ | 1 |
| Bacterin | I.P. injection | $1.0 \times 10^8$ | 32.3 |
| | Immersion | $1.6 \times 10^7$ | 5.2 |
| Live (10SR) | I.P. injection | $3.2 \times 10^7$ | 10.3 |
| | Immersion | $1.1 \times 10^8$ | 35.5 |

[a] These experiments were carried out using a single dose of vaccine and results tabulated using the $LD_{50}$ method (Reed and Meunch, 1938).

[b] NA - Not applicable.

**Table 15. Efficacy of live vaccines (10SR and 10SR-3) in Atlantic salmon compared to a bacterin.**

| Vaccine[a] | # mortalities (n) | % Mortality | RPS[b] |
|---|---|---|---|
| None (control) | 24 (30) | 80 | na |
| Bacterin | 10 (30) | 33 | 59 |
| 10SR | 4 (30) | 13 | 84 |
| 10SR-3 | 5 (30) | 17 | 79 |

[a] All vaccinations were by the immersion method.

[b] This experiment was carried out using the RPS method (see Materials and Methods).

not autoagglutinate; thus antibodies against A-layer were not detected. None of the serum samples from fish vaccinated with a live vaccine strain were capable of agglutinating A450-3, while serum from fish vaccinated by injecting a formalinized bacterin of *A. salmonicida* had an agglutinating titer of $128^{-1}$ (data not shown).

Western immunoblotting using the immune fish serum at a $10^{-2}$ dilution gave only a weak, unconvincing response (Fig. 19). The immunoblot developed with serum from bacterin immunized fish (lanes 1 and 2) was not substantially different from all of the immunoblots developed with the different serum samples from fish vaccinated with the live vaccines (lanes 3-14). In all cases the dominant antigens were approximately 14, 39, 71, 75, and 100 kilodaltons (kDa) as determined by Western immunoblots using whole cell lysates of the challenge strain MT26 as antigen source (odd numbered lanes). MT26 cells grown in peritoneal implants by previously described methods (Garduño et al., 1993a), were used as a source of in vivo expressed antigens for immunoblots (even numbered lanes). Curiously, the antigens recognized by all serum samples included high molecular weight LPS, and the 14 and 29 kDa bands. Only sera from bacterin vaccinated fish detected the 100 kDa band seen in the lanes containing in vitro antigen. Although the total amount of protein loaded was constant between the in vivo and in vitro grown cells, the immune fish sera reacted less well with the antigens from in vivo grown cells. Sera from non-immunized fish did not react with any antigens, indicating that the responses were not merely artifacts. The Western immunoblots were identical when a commercially available alkaline phosphatase-conjugated goat anti-trout antibody was used to develop the immunoblots (data not shown).

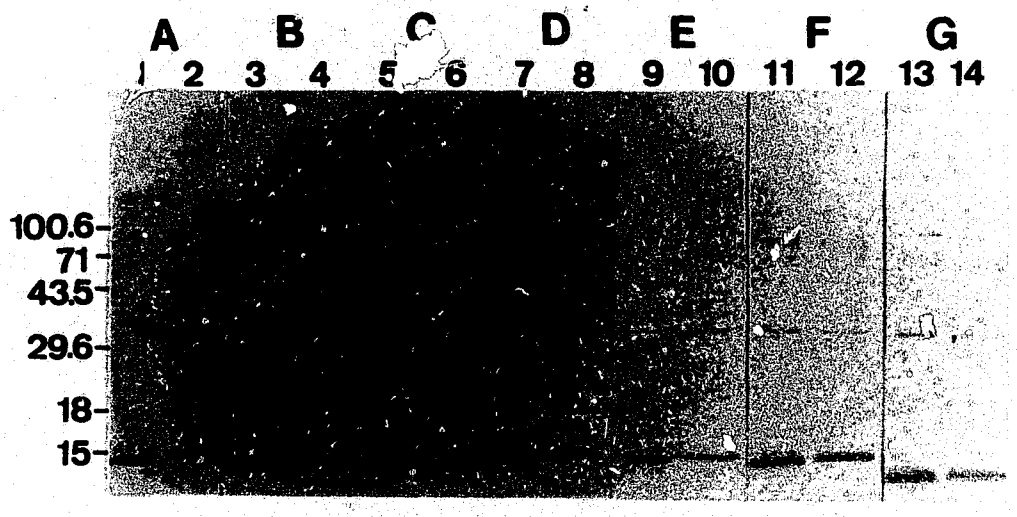


**Figure 19.** Western immunoblots of in vitro (odd numbered lanes) and in vivo (even numbered lanes) grown *A. salmonicida* strain MT26 cells. The immunoblots were developed using immune sera from fish vaccinated with a bacterin (**A**), or the live vaccine strains 10SR (**B**), 10SR-3 (**C**), A450-3 (**D**), A450-1 (**E**), A450-1-3 (**F**), and A440 (**G**). Positions of MW standards are indicated (kDa).

## Discussion.

Aside from assessing the levels of protection conferred to salmonids by the live vaccines, the aim of this study was to determine which, if any, of the major surface antigens of *A. salmonicida* are necessary for the induction of immunity when live vaccines are used for the prevention of salmonid furunculosis. Mutations altering the integrity of A-layer are known to reduce virulence (Ishiguro et al., 1981; Olivier et al., 1985; this study), therefore mutants deficient in A-layer and O-antigen were tested in conjunction with the live vaccine candidate A450-10SR.

The role of humoral immunity in the protection of fish from furunculosis has historically been assessed on the basis of either the passive transfer of immunity using either fish or rabbit sera raised against killed *A. salmonicida* cells, or by the examination of fish immune response following vaccination with a bacterin (Olivier et al., 1986; Ellis et al., 1988; Hastings and Ellis, 1990). Although humoral immunity has failed to correlated well with protection when measured by serum antibody titer (Olivier et al., 1985; Tatner, 1991), a limited level of success has been achieved using passive transfer of anti-*A. salmonicida* antibodies from either fish (Cipriano, 1981) or rabbit sera (Marquis and Lallier, 1989), suggesting at least a partial role for humoral immunity in the prevention of furunculosis.

The results from vaccination trials using the various live vaccines demonstrated that effective protective immune responses are generated by these live strains with or without the A-layer, and that mutants lacking even the LPS O-antigen still provided protection at least equivalent to that of an injectable bacterin (Table 13). This surprising result, especially in conjunction with the lack of agglutinating antibodies, leads to the possible conclusion that the branch(es) of the immune system that is stimulated by these live vaccines is at least partly different than that stimulated by a simple bacterin. Another

possible explanation is that the antigens responsible for protection may differ between attenuated vaccines and simple bacterins. It is now known that *A. salmonicida* expresses a series of novel antigens when grown in vivo (described in Chapter IV), thus if the live strains persist in tissues long enough, it is probable that these novel antigens will be expressed and stimulate an immune response. It is important to indicate that these two conclusions are by no means mutually exclusive, for example the novel antigens expressed may stimulate different branches of the fish immune system from those stimulated by antigens in bacterin preparations. The inconclusive results from Western immunoblots suggest that if humoral immunity is involved in the high levels of resistance after vaccination with live strains, it likely plays only a minor role as only a weak, nonagglutinating serum response was achieved. The anecdotal evidence for protection against saproligniosis, indicated a general increase in non-specific immunity for those fish receiving the live vaccines A450-10SR and A450-10SR-3. The true nature of this non-specific immunity is unknown.

The reduced efficacy of strains lacking O-antigen (A450-1 and A450-1-3), compared to mutants only lacking A-layer, is likely due to the extreme sensitivity of these strains to complement lysis, as both a functional A-layer and intact LPS are known to increase serum resistance of *A. salmonicida* (Munn et al., 1982; Chart et al., 1984). This increased sensitivity to complement likely results in highly reduced tissue persistence, and thus reduced exposure of the cells of the immune system to antigen.

Historically it has been indicated that both A-protein and LPS, which are expressed in vivo and in vitro are required antigens in bacterin preparations (Munn, et al., 1982; McCarthy et al., 1983; Cipriano and Pyle, 1985; Lund et al., 1991; Olivier et al., 1985). As the levels of protection afforded by bacterin injection are minimal, it may be that these antigens are only of secondary importance to a protective response, and only

the live vaccines provide the novel in vivo expressed antigens for the stimulation of high level protective immunity.

The safety of the environmental release of live vaccine strains is of utmost importance, thus the incorporation of mutations that not only affect virulence but also affect the environmental persistence of the organism must be considered. It has been proposed that the presence of A-layer increases the survival of *A. salmonicida* in environments such as river beds (Sakai, 1987). This is reportedly due to the charge imparted by A-layer allowing for the interaction of A-layer possessing cells ($A^+$) with amino acids associated with the humic acid coating of silt and sand. Also, the hydrophobic nature of $A^+$ cells has been recently implicated in increasing the apparent concentration of *A. salmonicida* at the air-water interface, thus increasing the potential bacterial load for fish at or near the surface (Enger & Thorson, 1992). These two attributes of $A^+$ *A. salmonicida* become relevant in the event, albeit a remote possibility when more than two mutations are involved, that the vaccine strain in question reverts to a wild-type virulence.

Another important aspect of virulence that is commonly overlooked in the construction of live vaccines is the role of toxins in both immunity and undesirable side effects after exposure to a live vaccine. Recent reports on the toxins of *A. salmonicida* have revealed that the major toxic factor produced by *A. salmonicida* is a combination of protease, glycerophospholipid: cholesterol acyltransferase (GCAT), and LPS (Lee & Ellis, 1989, 1990, &1991). More specifically, it was shown that the addition of LPS to GCAT stabilized and enhanced toxicity of this enzyme. Thus the exclusion, or alteration of LPS in live vaccine strains should aid in the reduction of the toxic effects of the extracellular components of *A. salmonicida*, while still having the proteins produced by the live strain to aid in the stimulation of a protective immune response.

For these reasons, and for the fact that $A^-$ and $LPS^-$ cells are still effective vaccines, the inclusion of one or both of these mutations into a live vaccine strain for the control of furunculosis, while not essential, may play an important role in the construction of effective live vaccines without potential for adverse biological and environmental impact.

# REFERENCES

Actis, L.A., W. Fish, J.H. Crosa, K. Kellerman, S.R. Ellenberger, F.M. Hauser, and J. Sanders-Loher. 1986. Characterization of anguibactin, a novel siderophore from *Vibrio anguillarum* 775 (pJM1). J. Bacteriol. 167: 57-65.

Acton, R.T., P.F. Weinheimer, S.J. Hall, W. Niedermeier, E. Shelton, and J.C. Bennet. 1971. Tetrameric immune macroglobulins in three orders of bony fishes. Proc. Natl. Acad. Sci. USA 68: 107-111.

Allenza, P., and T.G. Lessie. 1982. *Pseudomonas cepacia* mutants blocked in the Entner-Doudoroff pathway. J. Bacteriol. 150: 1340-1347.

Almeida, R.A. and R.F. Rosenbusch. 1991. Capsule like surface material of *Mycoplasma dispar* induced by in vitro growth in culture with bovine cells is antigenically related to similar structures expressed in vivo. Infect. Immun. 59: 3119-3125.

Altendorf, K., and F.M. Harold. 1974. Cation transport in bacteria: $K^+$, $Na^+$, and $H^+$. Curr. Top. Membr. Transp. 5: 1-50.

Ambrosius, H., H. Fiebig, and I. Scherbaum. 1982. Phylogenetic aspects of fish immunoglobulins and lymphocyte receptors. Dev. Comp. Immunol. Suppl. 2: 3-13.

Ames, G.F.L. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. 249: 634-644.

Anderson, D.A. 1974. Immunology: Diseases of fishes. Book 4: Fish Immunology. TFH Publications, Neptune City.

Anderson, T.J., and G. Ivanovics. 1967. Isolation and more characterization of haemin dependent mutants of *Bacillus subtilis*. J. Gen. Microbiol. 49: 31-40.

Anraku, Y. 1988. Bacterial electron transport chains. Ann. Rev. Biochem. 57: 101-132.

Aoki, T. 1988. Drug resistance plasmids from fish pathogens. Microbiol. Sci. 5: 219-223.

Aoki, T., T. Kitao, N. Iemura, Y. Mitoma, and T. Nomura. 1983. The susceptibility of *Aeromonas salmonicida* isolated in cultured and wild salmonids to various chemotherapeutics. Bull. Jpn. Soc. Sci. Fish. 49: 17-22.

Aoki, T., Mitoma, Y., and J.H. Crosa. 1986. The characterization of a conjugative R-plasmid isolated from *Aeromonas salmonicida*.. Plasmid 16: 213-218.

Arkoosh, M.R., and S.L. Kaattari. 1991. Development of immunological memory in rainbow trout (*Oncorhynchus mykiss*). I. An immunochemical and cellular analysis of the B cell response. Dev. Comp. Immunol. 15: 279-293.

Ashworth, J.M., and H.L. Kornberg. 1963. Fine control of the glyoxylate cycle by allosteric inhibition of isocitrate lyase. Biochim. Biophys. Acta. 73: 519-522.

Austin, B. and D.A. Austin. 1987. Aeromonads. *In*: Bacterial Fish Pathogens - disease in farmed and wild fish. pp. 111-195. Ellis Horwood, Chichester.

Austin, D.A., D. McIntosh, and B. Austin. 1989. Taxonomy of fish associated *Aeromonas* spp., with the description of *Aeromonas salmonicida* subsp. *smithia* subsp. nov. System. Appl. Microbiol. 11: 277-290.

Barghouthi, S., R. Young, M.O.J. Olson, J.E.L. Arceneaux, L.W. Clem, and B.R. Byers. 1989. Amonabactin, a novel tryptophan- or phenylalanine containing phenolate siderophore in *Aeromonas salmonicida*. J. Bacteriol. 171: 1811-1816.

Belland, R.J., and T.J. Trust. 1987. Cloning of the gene for the surface array protein of *Aeromonas salmonicida* and evidence linking loss of expression with genetic deletion. J. Bacteriol. 169: 4086-4091.

Belland, R.J. and T.J. Trust. 1989. *Aeromonas salmonicida* plasmids: plasmid directed synthesis of proteins *in vitro* and in *Escherichia coli* minicells. J. Gen. Microbiol. 135: 513-524.

Biosca, E.G., and C. Amaro. 1991. Siderophores and related outer membrane proteins in *Vibrio* spp. which are potential pathogens of fish and shellfish. J. Fish Dis. 14: 249-263.

Blake, M.S., K.H. Johnston, G.J. Russel-Jones, and E.C. Gotschlich. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal. Biochem. 136: 175-179.

Bogwald, J., K. Stensvag, J. Hoffman, and T. Jorgensen. 1991. Antibody specificities in Atlantic salmon, *Salmo salar* L., against the fish pathogens *Vibrio salmonicida* and *Vibrio anguillarum*. J. Fish Diseases 14: 79-87.

Bornside, G.H., W.E. Donovan, and M.B. Meyers. 1976. Intracolonic tensions of oxygen and carbon dioxide in germfree, conventional and gnotobiotic rats (39229). Proc. Soc. Exp. Biol. Med. 151: 437-441.

Borthwick, A.C., W.H. Holms, and H.G. Nimmo. 1984. Isolation of active and inactive forms of isocitrate dehydrogenase from *Escherichia coli* ML308. Eur J. Biochem. 141: 393-400.

Botham, J.W., M.F. Grace, and M.J. Manning. 1980. Otogeny of first set and second set alloimmune reactivity in fishes. In: Phylogeny of immunological memory, (Ed. M.J. Manning), pp. 83-92. Elsevier/North-Holland Biomedical Press.

Brown, M.R.W., H. Anwar, and J.W. Costerton. 1988. Surface antigens in vivo: a mirror for vaccine development. Can. J. Microbiol. 34: 491-498.

Brubaker, R.R., and M.J. Surgalla. 1962. Genotypic alteration associated with avirulence in streptomycin-resistant *Pasteurella pestis*. J. Bacteriol. 84: 615-624.

Bruno, D.W., and L.S. Munro. 1986. Observations on *Renibacterium salmoninarum* and the salmonid egg. Dis. Aquat. Org. 1: 83-87.

Bryan, L.E., T. Nicas, B.W. Holloway, and C. Crowther. 1980. Aminoglycoside resistant mutation of *Pseudomonas aeruginosa* defective in cytochrome $c_{552}$ and nitrate reductase. Antimicrob. Agents Chemother. 17: 71-79.

Bryan, L.E., and S. Kwan. 1981. Aminoglycoside resistant mutants of *Pseudomonas aeruginosa* defective in cytochrome *d* and nitrate reductase, and aerobic transport. Antimicrob. Agents Chemother. 19: 958-964.

Buchmeier, N.A. and F. Hefron. 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science. 248: 730-732.

Buck, D., M.E. Spencer, and J.R. Guest. 1985. Primary structure of the succinyl-CoA synthetase of *Escherichia coli*. Biochemistry 24: 6245-6252.

Camprubi, S., M.A. Smith, J.M. Tomas, and P. Williams. 1992. Modulation of surface antigen expression by *Klebsiella pneumoniae* in response to growth environment. Microb. Pathogen. 13: 145-155.

Chamberlain, G.W. 1993. Aquaculture trends and feed projections. World Aquacul. 24: 19-29.

Chart, H., and T.J. Trust. 1983. Aquisition of iron by *Aeromonas salmonicida*. J. Bacteriol. 156: 758-764.

Chart, H., and T.J. Trust. 1984. Characterization of the surface antigens of the marine fish pathogens *Vibrio anguillarum* and *Vibrio ordalii*. Can. J. Microbiol. 30: 703-710.

Chart, H., T.W. Pearson, and T.J. Trust. 1984. Detection of specific fish antibody using an inhibition enzyme-linked immunosorbent assay (inhibition ELISA). J. Immunol. Methods. 68: 19-24.

Chien, M.-S., T.L. Gilbert,, C. Huang, M.L. Landolt, P.J. O'Hara, and J.R. Winton. 1992. Molecular cloning and sequence analysis of the gene coding for the 57-kDa major soluble antigen of the salmonid fish pathogen *Renibacterium salmoninarum*. FEMS Microbiol. Lett. 96: 259-266.

Chu, S., S. Cavaignac, J. Feutrier, B.M. Phipps, M. Kostrzynska, W.W. Kay, and T.J. Trust. 1991. Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida*. J. Biol. Chem. 266: 15258-15265.

Cipriano, R.C. 1981. Assessment of the ability of homeotherm immunoglobulins to confer passive immunity in pokliotherm vertibrates. Fish Health News. 10: vi-vii.

Cipriano, R.C., and S.W. Pyle. 1985. Adjuvant dependant immunity and the agglutinin response of fishes against *Aeromonas salmonicida*, the cause of furunculosis. Can. J. Fish. Aquat. Sci. 42: 1290-1295.

Cochrane, D.M., M.R. Brown, H. Anwar, P.H. Weller, K. Lam, and J.W. Costerton. 1988. Antibody response to *Pseudomonas aeruginosa* surface protein antigens in a rat model of chronic lung infection. J. Med. Microbiol. 27: 255-261.

Corbel, M.J. 1975. The immune response of fish: A review. J. Fish Biol. 7: 539-560.

Courtright, J.B., and U. Henning. 1970. Malate dehydrogenase mutants in *Escherichia coli* K-12. J. Bacteriol. 102: 722-728.

Crosa, J.H., M.H. Shiewe, and S.F. Falkow. 1977. Evidence for plasmid contribution to the virulence of the fish pathogen *Vibrio anguillarum*. Infect. Immun. 18: 509-513.

Crosa, J.H. 1980. A plasmid associated with virulence in the marine fish pathogen Vibrio anguillarum specifies an iron sequestering system. Nature. 284: 566-568.

Daly, J.G. and R.M.W. Stevenson. 1987. Hydrophobic and haemagglutinating properties of *Renibacterium salmoninarum*. J. Gen. Microbiol. 133: 3575-3580.

Daly, J.G. and R.M.W. Stevenson. 1989. Agglutination of salmonid spermatozoa by *Renibacterium salmoninarum*. J. Aquat. Animal Health. 1: 163-164.

Daly, J.G. and R.M.W. Stevenson. 1990. Characterization of the *Renibacterium salmoninarum* haemagglutinin. J. Gen. Microbiol. 136: 949-953.

Darveau, R.P., S. MacIntyre, J.T. Buckley, and R.E.W. Hancock. 1983. Purification and reconstitution of lipid bilayer membranes of an outer membrane, pore-forming protein of *Aeromonas salmonicida*. J. Bacteriol. 155: 1006-1011.

DeLuca, D., M. Wilson, and G.W. Warr. 1983. Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. Eur. J. Immunol. 13: 546-551.

DeMey, J., and M. Moeremans. 1986. In: J.K. Koehler, editor, Advanced Techniques in Biological Electron Microscopy. pp. 229-271. Springer-Verlag, Berlin Heidelberg.

Doig, P., L. Emödy, and T.J. Trust. 1992. Binding of laminin and fibronectin by the trypsin-resistant major structural domain of the crystalline virulence surface array protein of *Aeromonas salmonicida*. J. Biol. Chem. 267: 43-49.

Dooley, J.S.G., H. Engelhardt, W. Baumeister, W.W. Kay, and T.J. Trust. 1989. Three-dimensional structure of an open form of the surface layer from the fish pathogen *Aeromonas salmonicida*. J. Bacteriol. 171: 190-197.

Dorson, M. 1981. Role and characterization of fish antibody. Develop. Biol. Standard. 49: 307-319.

Duff, D.C.B. 1942. The oral immunization of trout against *Bacterium salmonicida*. J. Immunol. 44: 87-94.

Dunn, E.J., A. Polk, D.J. Scarrett, G. Olivier, S. Lall, and M.F.A. Goosen. 1990. Vaccines in aquaculture: The search for an efficient delivery system. Aquacultural Engineering 9: 23-32.

Egidius, E.C., K. Andersen, E. Clausen, and J. Raa. 1981. Cold water vibriosis or "Hitra-disease" in Norwegian salmonid farming. J. Fish Dis. 4: 353-354.

Elliot, T., and J.R. Roth. 1989. Heme deficient *Salmonella typhimurium*: two genes required for ALA synthesis. Mol. Gen. Genet. 216: 303-314.

Ellis, A.E. 1977. Otogeny of the immune response in *Salmo salar*. Histogenesis of the lymphoid organs and appearance of membrane immunoglobulin and mixed leucocyte reactivity. In: Developmental Immunobiology, (J.B. Solomon and J.D. Horton eds.). pp. 225-231. Elsevier/North Holland, Biomedical Press.

Ellis, A.E. 1980. Antigen trapping in the spleen and kidney of the plaice, *Pleuronectes platessa*. J. Fish Dis. 3: 413-426.

Ellis, A.E., 1988a. Ontogeny of the immune system in teleost fish. In: Fish Vaccination. (A.E. Ellis ed.) pp. 20-31. Academic Press.

Ellis, A.E., 1988b. Vaccination against enteric redmouth (ERM). In: Fish Vaccination. (A.E. Ellis ed.) pp. 85-92. Academic Press.

Ellis, A.E. 1991. An appraisal of the extracellular toxins of *Aeromonas salmonicida* ssp. *salmonicida*. J. Fish Dis. 14: 265-277.

Ellis, A.E., T.S. Hastings, and A.L.S. Munro. 1981. The role of *Aeromonas salmonicida* extracellular products in the pathology of furunculosis. J. Fish Disease. 4: 41-51.

Ellis, A.E., A.S. Burrows, T.S. Hastings, and K.J. Stapleton. 1988. Identification of *Aeromonas salmonicida* extracellular protease as a protective antigen against furunculosis by passive immunization. Aquaculture. 70: 207-218.

Ellis, A.E., I.R. Bricknell, and A.L.S. Munro. 1992. Current status of a Scottish furunculosis vaccine. Bull Aquacul. Asso. Can. 92-1: 11-15.

Enger, Ø., and B.K. Thorsen. 1992. Possible ecological implications of the high cell surface hydrophobicity of the fish pathogen *Aeromonas salmonicida*. Can. J. Microbiol. 38: 1048-1052.

Evelyn, T.P.T., 1971. First records of vibriosis in Pacific salmon cultured in Canada, and taxonomic status of the responsible bacterium *V. anguillarum*. J. Fish. Res. Board of Canada. 28: 517-525.

Evelyn, T.P.T., L. Prosperi-Porta, and J.E. Ketcheson. 1986a. Persistence of the kidney-disease bacterium, *Renibacterium salmoninarum*, in coho salmon, *Oncorhynchus kisutch* (Walbaum), eggs treated during and after water-hardening with povidone-iodine. J. Fish Dis. 9: 461-464.

Evelyn, T.P.T., L. Prosperi-Porta, and J.E. Ketcheson. 1986b. Experimental intra-ovum infection of salmonid eggs with *Renibacterium salmoninarum* and vertical transmission of the pathogen with such eggs despite their treatment with erythromycin. Dis. Aquat. Org. 1: 197-202.

Filip, C., G. Fletcher, J.L. Wulff, and C.F. Earhart. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. 115: 717-722

Folch, J., M. Lees, and G.H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226: 497-509.

Fraenkel, D.G., and R.T. Vinopal. 1973. Carbohydrate metabolism in bacteria. Ann. Rev. Microbiol. 27: 69-100.

Fryer, J.L., and J.E. Sanders. 1981. Bacterial kidney disease of salmonid fish. Ann. Rev. Microbiol. 35: 273-298.

Fyfe, L., G. Coleman, and A.L.S. Munro. 1987. Identification of major common extracellular proteins secreted by *Aeromonas salmonicida* strains isolated from diseased fish. Appl. Environ. Microbiol. 53: 722-726.

Garduño, R.A., J.C. Thornton, and W.W. Kay. 1993a. Fate of the fish pathogen *Aeromonas salmonicida* in the peritoneal cavity of rainbow trout. Can. J. Microbiol. 39: 1051-1058.

Garduño, R.A., J.C. Thornton, and W.W. Kay. 1993b. *Aeromonas salmonicida* grown in vivo. Infect. Immun. 61: 3854-3862.

Garduño, R.A., B.M. Phipps, W. Baumeister, and W.W. Kay. 1992. Novel structural patterns in divalent cation-depleted surface layers of *Aeromonas salmonicida* . J. Struct. Biol. In press.

Garduño, R.A., E. Lee, and W.W. Kay. 1992. S-layer mediated association of *Aeromonas salmonicida* with murine macrophages. Infect. Immun. 60: 4373-4382.

Garduño, R.A., and W.W. Kay. 1992. Interaction of the fish pathogen *Aeromonas salmonicida* with rainbow trout macrophages. Infect. Immun. 60: 4612-4620.

Garrote, A., R. Bonet, S. Merino, M.D. Simon-Pujol, and F. Congregado. 1992. Occurence of a capsule in *Aeromonas salmonicida*. FEMS Microbiol. Lett. 95: 127-132.

Getchell, R.G., J.S. Rohovec, and J.L. Fryer. 1985. Comparison of *Renibacterium salmoninarum* isolates by antigenic analysis. Fish Pathol. 20: 149-159.

Goldes, S.A., H.W. Ferguson, P.-Y. Daoust, and R.D. Moccia. 1986. Phagocytosis of the inert suspended clay kaolin by the gills of rainbow trout, *Salmo gairdneri*, Richardson. J. Fish Dis. 9: 147-151.

Gottschalk, G. 1986. Bacterial Metabolism, (2nd Edition). Springer Verlag, New York.

Grieco, M.H. 1982. Resistance to aminoglycoside-aminocyclitol antibiotics. Antimicrob. Therap. 66: 32-37.

Griffiths, E., P. Stevenson, R. Thorpe, and H. Chart. 1985. Naturally occuring antibodies in human sera that react with iron regulated outer membrane proteins of *Escherichia coli*. Infect. Immun. 47: 808-813.

Harrell, L.W., H.M. Etlinger, and H.O. Hodgins. 1975. Humoral factors important in resistance of salmonid fishes to bacterial disease. I. Serum antibody protection of rainbow trout (*Salmo gairdneri*) against vibriosis. Aquacul. 6: 211-219.

Hastings, T.S. 1988. Furunculosis vaccines. In: Fish vaccination. (A.E. Ellis ed.) pp. 93-111. Academic Press.

Hastings, T.S., and A.E. Ellis. 1988. The humoral immune response of rainbow trout and rabbits to extracellular products of *Aeromonas salmonicida*. J. Fish Dis. 11: 147-160.

Hastings, T.S., and A.E. Ellis. 1990. Detection of antibodies induced in rainbow trout by different *Aeromonas salmonicida* vaccine preparations. J. Aquat. Animal Health. 2: 135-140.

Hirst, I.D., T.S. Hastings, and A.E. Ellis. 1991. Siderophore production by *Aeromonas salmonicida*. J. Gen. Microbiol. 137: 1185-1192.

Holm, K.O., E. Strom, K. Stensvag, J. Raa, and T. Jorgensen. 1985. Characteristics of a *Vibrio* sp. associated with the 'Hitra disease' of Atlantic salmon in Norwegian fish farms. Fish Pathology 20: 125-129.

Holm, K.O., and T. Jorgensen. 1987. A successful vaccination of Atlantic salmon, *Salmo salar* L., against 'Hitra disease' or cold water vibriosis. J. Fish Dis. 10: 85-90.

Ishiguro, E.E., W.W. Kay, T. Ainsworth, J.B. Chamberlain, R.A. Austen, J.T. Buckley, and T.J. Trust. 1981. Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. J. Bacteriol. 148: 333-340.

Ishiguro, E.E., T. Ainsworth, T.J. Trust, and W.W. Kay. 1985. Congo red agar, a differential medium for *Aeromonas salmonicida*, detects the presence of the cell surface protein array involved in virulence. J. Bacteriol. 164: 1233-1237.

Ingram, G.A. 1980. Substances involved in the natural resistance of fish to infection - A review. J. Fish Biol. 16: 23-60.

Jacobsen, M.D. 1989. Withdrawal times of freshwater rainbow trout, *Salmo gairdneri* Richardson, after treatment with oxalinic acid, oxytetracyline and trimetoprim. J.Fish Dis. 12:29-36.

Jacobsen, P. and L. Berglind. 1988. Persistence of oxytetracycline in sediments from fish farms. Aquaculture. 70:365-370.

Jalal, M.A.F., M.B. Hossain, D. van der Helm, J. Sanders-Lochr, L.A. Actis, and J.H. Crosa. 1989. Structure of anguibactin, a unique plasmid related bacterial siderophore from the fish pathogen *Vibrio anguillarum*. J. Am. Chem Soc. 111: 292-296.

Johne, B., J. Jarp, and L.R. Hanson. 1989. *Staphylococcus aureus* exopolysaccharide in vivo demonstrated by immunomagnetic separation and electron microscopy. J. Clin. Microbiol. 27: 1631-1635.

Johnson, K.A., and D.F. Amend. 1983. Comparison of efficacy of several delivery methods using *Yersinia ruckeri* bacterin on rainbow trout, *Salmo gairdneri*, Richardson. J. Fish Disease 6: 331-336.

Johnson, K.A., J.K. Flynn, and D.F. Amend. 1982. Duration of immunity in salmonids vaccinated by direct immersion with *Yersinia ruckeri* and *Vibrio anguillarum* bacterins. J. Fish Dis. 5: 207-213.

Karakawa, W.W., A. Sutton, R. Schneerson, A. Kapas, and W.F. Vann. 1988. Capsular antibodies induce type specific phagocytosis of capsulated *Staphylococcus aureus* by human polymorphonuclear leukocytes. Infect. Immun. 56: 1090-1095.

Karczewski, J.M., G.J.E. Sharp, and C.J. Secombes. 1991. Susceptibility of strains of *Aeromonas salmonicida* to killing by cell-free generated superoxide anion. J. Fish Dis. 14: 367-373.

Kay, W.W. 1972. Genetic control of the metabolism of propionate by *Escherichia coli* K-12. Biochim. Biophys. Acta 264: 508-521.

Kay, W.W., J.T. Buckley, E.E. Ishiguro, B.M. Phipps, J.P.L. Monette, and T.J. Trust. 1981. Purification and disposition of a surface protein associated with virulence of *Aeromonas salmonicida*. J. Bacteriol. 147: 1077-1084.

Kay, W.W., B.M. Phipps, E.E Ishiguro, and T.J. Trust. 1985. Porphyrin binding by the surface array virulence protein of *Aeromonas salmonicida*. J. Bacteriol. 164: 1332-1336.

Kay, W.W., B.M. Phipps, R.A. Garduño, and T.J. Trust. 1988. The A-layer of *Aeromonas salmonicida*: Organization and functions., p.44-50. In: Crystalline bacterial cell surface layers. (U.B. Sleytr, P. Messner, D. Pum, and M. Sára, eds.) Springer-Verlag, Berlin.

Kay, W.W., and T.J. Trust. 1991. Form and functions of the regular surface array (S-layer) of *Aeromonas salmonicida*. Experientia 47: 412-414.

Keppie, J., H. Smith, and P.W. Harris-Smith. 1955. The chemical basis of the virulence of *Bacillus anthracis*. III. The role of the terminal bacteraemia in death of guinea pigs from anthrax. British J. Exp. Pathol. 36: 315-322.

Kitao, T., T. Aoki, M. Fukodome, K. Kawano, Y. Wada, and Y. Mizuno. 1983. Serotyping of *Vibrio anguillarum* isolated from freshwater fish in Japan. J. Fish Dis. 6: 175-181.

Klontz, G.W., and D.F. Anderson. 1970. Oral immunization of salmonids: a review. In: Symposium on Diseases of Fish and Shellfishes (S.F. Snieszko, ed.). pp. 16-20. Special Publication, No. 5. Am. Fish. Soc., Wash. D.C.

Kobayashi, K., A. Hara, K. Takano, and H. Hirai. 1982. Studies on subunit components of immunoglobulin M from a bony fish, the chum salmon (*Oncorhynchus nerka*). Mol. Immunol. 19: 90-95

Kodama, H., M. Moustafa, S. Ishiguro, T. Mikami, and H. Izawa. 1984. Extracellular virulence factors of fish vibrio: Relationships between toxic materials, hemolysins, and proteolytic enzyme. Amer. J. Vet. Research 45: 2203-2207.

Kotlarz, D., and H. Buc. 1982. Phosphofructokinases from *Escherichia coli*. Methods Enzymol. 90: 60-70.

Lamers, C.H.J. 1985. The reaction of the immune system of fish to vaccination. Ph.D. Thesis, Dept. of Exp. Animal Morph. and Cell Biol., Agricultural University, Wageninen, The Netherlands.

Lee, C.A., and S. Falkow. 1990. The ability of *Salmonella* to enter mammalian cells is affected by the bacterial growth state. Proc.Natl.Acad.Sci. USA. 87: 4304-4308.

Lee, K.-K., and A.E. Ellis. 1989. The quantitative relationship of lethality between extracellular protease and extracellular haemolysin of *Aeromonas salmonicida* in Atlantic salmon (*Salmo salar* L.). FEMS Microbiol. Lett. 61: 127-132.

Lee, K. -K., and A.E. Ellis. 1990. Glycerophospholipid:cholesterol acyltransferase complexed with lipopolysaccharide (LPS) is a major lethal exotoxin and cytolysin of *Aeromonas salmonicida*: LPS stabilizes and enhances toxicity of the enzyme. J. Bacteriol. 172: 5382-5393.

Lee, K.-K. and A.E. Ellis. 1991. The role of the lethal extracellular cytolysin of *Aeromonas salmonicida* in the pathology of furunculosis. J. Fish Dis. 14: 453-460.

Lee, L.E.J., and N.C. Bols. 1991. Identification of fibronectin in rainbow trout and influence of cortisol on fibronectin accumulation by rainbow trout cell cultures. J. Aq. Animal Health 3: 31-40.

Lemos, M.L., P.C. Salinas, A.E. Torazano, J.L. Barja, and J.H. Crosa. 1988. Chromosome mediated iron uptake system in pathogenic strains of *Vibrio anguillarum*. J. Bacteriol. 170: 1920-1925.

Lessie, T.G. and P.V. Phibbs, Jr. 1984. Alternative pathways of carbohydrate util zation in *Pseudomonas*. Ann. Rev. Microbiol. 38: 359-387.

Lund, V., T. Jørgensen, K.O. Holm, and G. Eggset. 1991. Humoral immune response in Atlantic salmon, *Salmo salar* L., to cellular and extracellular antigens of *Aeromonas salmonicida*. J. Fish Dis. 14: 443-452.

McCarthy, D.H., D.F. Amend, K.A. Johnson, and J.A. Bloom. 1983. *Aeromonas salmonicida*: determination of an antigen associated with protective immunity and evaluation of an experimental bacterin. J. Fish. Dis.1983; 6:155-174.

MacIntyre, S., T.J. Trust, and J.T. Buckley. 1979. Distribution of glycerophospholipid-cholesterol acyltransferase in selected bacterial species. J. Bacteriol. 139: 132-136.

Mackie, C. and T.H. Birkbeck. 1992. Siderophores produced by *Vibrio anguillarum in vitro* and in infected rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 15: 37-45.

Mandrell, R.E. 1992. Further antigenic similarities of *Neisseria gonorrhoeae* lipooligosaccharides and human glycosphingolipids. Infect. Immun. 60: 3017-3020.

Markwell, M.A.K., S.M. Haas, L.L. Biaber, and N.E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87: 206-210.

Marquis, H., and R. Lallier. 1989. Efficacy of passive immunization against *Aeromonas salmonicida* infection in brook trout, *Salvelinus fontinalis* (Mitchill). J. Fish Dis. 12: 233-240.

Michel, C. 1979. Furunculosis of salmonids: vaccination attempts in rainbow trout (*Salmo gairdneri*) by formalin killed germs. Ann. Rech. Vet. 10: 33-40.

Morck, D.W., B.D. Ellis, P.D.G. Domingue, M.E. Olson, and W.J. Costerton. 1991. In vivo expression of iron regulated outer-membrane proteins in *Pasteurella haemolytica*-A1. Microbial. Pathog. 11: 373-378.

Munn, C.B., E.E. Ishiguro, W.W. Kay, and T.J. Trust. 1982. Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. Infect. Immun. 36: 1069-1075.

Munro, A.L.S., D.W. Bruno. 1988. Vaccination against bacterial kidney disease. In: Fish Vaccination. (A.E. Ellis ed.) pp. 124-134. Academic Press.

Newman, S. 1983. *Aeromonas hydrophila*: A review with emphasis on its role in fish disease. In: *Antigens of Fish Pathogens*. pp. 87-117. Collection Fondation Marcel Merieux.

Newman, S.G. 1993. Bacterial vaccines for fish. Ann. Rev. of Fish Dis. 3: 145-185.

Nichols, W.W., and S.N. Young. 1985. Respiration dependent uptake of dihydrostreptomycin by *Escherichia coli*. Biochem. J. 228: 505-512.

Norqvist, A., B. Norman, and H. Wolf-Watz. 1990. Identification and characterization of a zinc metalloprotease associated with invasion by the fish pathogen *Vibrio anguillarum*. Infec. Immunity. 58: 3731-3736.

Olivier, G., T.P.T. Evelyn, and R. Lallier. 1985. Immunogenicity of vaccines from a virulent and an avirulent strain of *Aeromonas salmonicida*. J. Fish Dis. 8: 43-55.

Olivier, G., C.A. Eaton, and N. Campbell. 1986. Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). Vet. Immunol. Immunopathol. 12: 223-234.

Panigrahi, P., G. Losonsky, L.J. DeTolla, and J.G. Morris, Jr. 1992. Human immune response to *Campylobacter jejuni* proteins expressed in vivo. Infect. Immun. 60: 4938-4944.

Paterson, W.D., and J.L. Fryer. 1974. Immune response of juvenile coho salmon (*Onchorhynchus kisutch*) to *Aeromonas salmonicida* cells administered intraperitoneally in Freund's complete adjuvant. J. Fish. Res. Board Canada. 31: 1751-1755.

Penner, P.E., and L.H. Cohen. 1969. Effects of adenosine triphosphate and magnesium ions on the fumarase reaction. J. Biol. Chem. 244: 1070-1075.

Phipps, B.M., and W.W. Kay. 1988. Immunoglobulin binding by the regular surface array of *Aeromonas salmonicida*. J. Biol. Chem. 263: 9298-9303.

Post, G. 1966. Response of rainbow trout (*Salmo gairdneri*) to antigens of *Aeromonas hydrophila*. J. Fish Res. Board Can. 23: 1487-1494.

Postma, P.W. 1987. Phosphotransferase system for glucose and other sugars. In: *Escherichia coli* and *Salmonella typhimurium*. Cellular and molecular biology. (F.C. Neidhardt ed.). American Society for Microbiology. Washington, D.C.

Powers, D.A. 1989. Fish as model systems. Science 246: 352-358.

Reed, L.J., and H. Muench. 1938. A simple method for estimating fifty percent end points. J. Hygiene. 27: 493-497.

Rijpkema, S.G.T., E.M. Bik, W.H. Jansen, H. Gielen, L.F. Versluis, A.H. Stouthamer, P.A.M. Guinee, and F.R. Mooi. 1993. Construction and analysis of a *Vibrio cholerae* ∂-amino levulinic acid auxotroph which confers protective immunity in a rabbit model. Infect. Immun. 60: 2188-2193.

Rottenberg, H. 1979. The measurement of membrane potential and ΔpH in cells, organelles, and vesicles. Meth. Enzymol. 55: 547-569.

Sakai, D.K. 1985. The significance of extracellular protease for growth of a heterotrophic bacterium, *Aeromonas salmonicida*. Appl. Environ. Microbiol. 50:1031-1037.

Sakai, D.K. 1987. Adhesion of *Aeromonas salmonicida* strains associated with net electrostatic charges of host tissue cells. Infect. Immun. 55: 704-710.

Salte, R., Norberg, K., Odegaard, O.R. 1991. Do extracellular products of *Aeromonas salmonicida* induce thrombosis by entering the fish coagulation system at factor X? J. Fish Dis. 14: 401-406.

Salte, R., R. Norberg, J.A. Arnesen, O.R. Odegaard, and G. Eggset. 1992. Serine protease and glycerophospholipid: cholesterol acyltransferase of *Aeromonas salmonicida* work in concert in thrombus formation; in vitro the process is counteracted by plasma antithrombin and alpha$_2$-macroglobulin. J. Fish Dis. 15: 215-227.

Schwyn, B. and J.B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160: 47-56.

Sciortino, C.V., and R.A. Finkelstein. 1983. *Vibrio cholerae* expresses iron regulated outer-membrane proteins in vivo. Infect. Immun. 42: 990-996.

Shand, G.H., H. Anwar, J.J. Kadurugamuwa, M.R.W. Brown, S.H. Silverman, and J. Melling. 1985. In vivo evidence that bacteria in urinary tract infection grow under iron restricted conditions. Infect. Immun. 48: 35-39.

Shieh, H.S. and B.H. Reddy. 1972. Arginine catabolism in the causative agent of furunculosis, *Aeromonas salmonicida*. Int. J. Biochem. 3: 705-708.

Shieh, H.S., and J.R. Maclean. 1975. Purification and properties of an extracellular protease of *Aeromonas salmonicida*, the causative agent of furunculosis. Int. J. Biochem. 6: 653-656.

Shiewe, M.H., T.J. Trust, and J.H. Crosa. 1981. *Vibrio ordalii* sp. nov.: A causative agent of vibriosis in fish. Current Microb. 6: 343-348.

Sigwart, D.F., B.A.D. Stocker, and J.D. Clements. 1989. Effect of a *purA* mutation on efficacy of*Salmonella* live-vaccine vectors. Infect. Immun. 57:1858-1861.

Smibert, R.M., and N.R. Krieg. 1981. *In* Manual of methods for general bacteriology. pp. 409-443. ASM, Washington, D.C.

Smith, H., J. Keppie, and J.L. Stanley. 1953. The chemical basis of the virulence of *Bacillus anthracis*. I. Properties of bacteria grown in vivo and preparation of extracts. British J. Exp. Pathol. 34: 477-485.

Smith, H. 1990. Pathogenicity and the microbe in vivo. J. Gen. Microbiol. 136: 377-383.

Smith, M.W., and F.C. Neidhardt. 1983. 2-Oxoacid dehydrogenase complexes of *Escherichia coli*: Cellular amounts and patterns of synthesis. J. Bacteriol. 156: 81-88

Smith, P.D. 1982. Analysis of the hyperosmotic and bath methods for fish vaccination - comparison of uptake of particulate and non-particulate antigens. Dev. Comp. Immunol. Sup. 2: 181-186.

Smith, P.D. 1988. Vaccination against vibriosis. In: Fish Vaccination. (Ellis, A.E. ed.) Academic Press.

Smith, D.G.E., M.E. Wilcox, P. Williams, R.G. Finch, and S.P. Denyer. 1991. Characterization of cell envelope proteins of *Staphylococcus epidermidis* cultured in human peritoneal dialysate. Infect. Immun. 59: 617-624.

Somers, J.M., G.D. Sweet, and W.W. Kay. 1981. Fluorocitrate resistant tricarboxylate transport mutants of *Salmonella typhimurium*. Mol. Gen. Genet. 181: 338-345.

Sørensen, U.B.S., and J.L. Larsen. 1986. Serotyping of *Vibrio anguillarum*. Appl. Environ. Microbiol. 51: 593-597.

Stamm, J.M. 1989. In vitro resistance by fish pathogens to aquacultural antibacterials, including the quinolones difloxacin (A-56619) and sarafloxacin (A-56620). J. Aquat. Anim. Health. 1: 135-141

Stevenson, R.M.W. 1988. Vaccination against *Aeromonas hydrophila*. In: Fish Vaccination (A.E. Ellis ed.) pp.112-123. Academic Press.

Stevenson, R.M. W., and D.E. Airdrie. 1984. Serological variation among *Yersinia ruckeri* strains. J. Fish Dis. 7: 247-254.

Stolen, J.S., D.P. Anderson, and W.B. Muiswinkel. 1986. Fish immunology. Elsevier, New York.

Sweetman, A.J. and D.E. Griffiths. 1971. Studies on energy linked reactions. Energy linked reduction of oxidized nicotinamine adenine dinucleotide by succinate in *Escherichia coli*. Biochem. J. 121: 117-121.

Taber, H.W., J.P. Mueller, P.F. Miller, and A.S. Arrow. 1987. Bacterial uptake of aminoglycoside antibiotics. 51: 439-457.``

Tatner, M.F. 1986. The ontogeny of humoral immunity in rainbow trout, *Salmo gairdneri*. Vet. Immunol. Immunopathol. 12: 93-105.

Tatner, M. 1987. The quantitative relationship between vaccine dilution, length of immersion time and antigen uptake, using a radiolabelled *Aeromonas salmonicida* bath in direct immersion experiments with rainbow trout, *Salmo gairdneri*. Aquaculture. 62: 173-185.

Tatner, M.F. 1991. Modified extracellular product antigens of *Aeromonas salmonicida* as potential vaccines for the control of furunculosis in Atlantic salmon, *Salmo salar* L. J. Fish Dis. 14: 395-400.

Tatner, M.F., and M.J. Manning. 1983. Growth of the lymphoid organs in rainbow trout, *Salmo gairdneri*, from 1 to 15 months of age. J. Zool. Lond. 199: 503-520.

Tatner, M.F., and M.J. Manning. 1985. The ontogenetic development of the reticuloenddothelial system in the rainbow trout, *Salmo gairdneri* Richardson. J. Fish Dis. 8: 35-41.

Thornton, J.C., S.P. Howard, and J.T. Buckley. 1988. Molecular cloning of a phospholipid: cholesterol acyltransferase from *Aeromonas hydrophila*. Sequence homologies with lecithin:cholesterol acyltransferase and other lipases. Biochim. Biophys. Acta. 959:153-159.

Torazano, A.E., J.L. Barja, R.R. Colwell, and F.M. Hetrick. 1983. Characterization of plasmids in bacterial fish pathogens. Infection and Immunity. 39: 184-192.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 74: 4350-4354.

Trust, T.J. 1986. Pathogenesis of infectious disease of fish. Ann. Rev. Microbiol. 40: 479-502.

Trust, T.J., W.W. Kay, E.E. Ishiguro, J.T. Buckley, and T.W. Pearson. 1982. Properties of A-protein, a virulence factor on the surface of *Aeromonas salmonicida*. pp. 178-180 In: Immunology and Immunization of Fish (W.B. van Muiswinkel and E.L. Cooper eds.). Dev. Comp. Immunol. Suppl. 2.

Trust, T.J., W.W. Kay, and E.E. Ishiguro. 1983. Cell surface hydrophobicity and macrophage association of *Aeromonas salmonicida*. Curr. Microbiol. 9: 315-318.

Tsai, C.M., and C.E. Frasch. .1982. A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Anal. Biochem. 119: 115-119.

Vallejo, A.N., N.W. Miller, and L.W. Clem. 1992. Antigen processing and presentation in teleost immune responses. Ann. Rev. Fish Dis. 2: 73-79.

Viele, P., T.H. Kerstetter, and J. Sullivan. 1980. Adoptive transfer of immunity against *Vibrio anguillarum* in rainbow trout, *Salmo gairdneri* Richardson, vaccinated by the immersion method. J. Fish Biol. 17: 379-386.

Wetzler, L.M., K. Barry, M.S. Blake, and E.C. Gotschlich. 1992. Gonococcal lipooligosaccharide sialylation prevents complement-dependent killing by immune sera. Infect. Immun. 60: 39-43.

Whitfield, C., J.C. Richards, M.B. Perry, B.R. Clarke, and L.L. MacLean. 1991. Expression of two structurally distinct D-galactan O antigens in the lipopolysaccharide of *Klebsiella pneumoniae* serotype 01. J. Bacteriol. 173: 1420-1431.

Wiens, G.D., and S.L. Kaattari. 1991. Monoclonal antibody characterization of a leukoagglutinin produced by *Renibacterium salmoninarum.* Infect. Immun. 59: 631-637.

Williams, P. 1988. Role of the cell envelope in bacterial adaptation to growth in vivo in infections. Biochimie. 70: 987-1011.

Willis, R.C., R.G. Morris, C. Cirakoglu, G.D. Schellenberg, G.H. Gerber, C.E. Furlong. 1974. Preparation of the periplasmic binding proteins from *Salmonella typhimurium* and *Escherichia coli.* Arch. Biochem. Biophys. 161: 64-75.

Zapata, A.G., M. Torroba, F. Alvarez, D.P. Anderson, O.W. Dixon, and M. Wisniewski. 1987. Electron microscopic examination of antigen uptake by salmonid gills after bath immunization with a bacterin. J. Fish Biol. 31: 209-217.

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## Publications:

Full Papers in Refereed Journals:

Thornton, J.C., R.A. Garduño, and William W. Kay. 1993. Live vaccine stains of *Aeromonas salmonicida* lacking A-layer and O-antigen. J. Fish Diseases. In Press. Manuscript number MS 1109. 12 pages; 2 figures; 2 tables. Date of acceptance: September, 1993.

Garduño, R.A., J.C. Thornton, and W.W. Kay. 1993. Fate of the fish pathogen *Aeromonas salmonicida* in the peritoneal cavity of rainbow trout. Can.J. Microbiol. **39**: 1051-1058.

Thornton, J.C., R.A. Garduño, S.J. Carlos, and W.W. Kay. 1993. Novel Antigens Expressed by *Aeromonas salmonicida* Grown In Vivo. Infect. Immun. **61**:4582-4589

Garduño, Rafael A., J.C. Thornton, and William W. Kay. 1993. *Aeromonas salmonicida* Grown *In Vivo*. Infect. Immun. **61**:3854-3862.

Thornton, J.C., R.A. Garduño, S.G. Newman and W.W. Kay. 1991. Surface disorganized, attenuated mutants of *Aeromonas salmonicida* as furunculosis live vaccines. Microbial Pathogenesis. **11**:85-99.

Thornton, J.C., S. P. Howard, and J.T. Buckley. 1988. Molecular Cloning of a Phospholipid: Cholesterol Acyltransferase from *Aeromonas hydrophila*. Sequence Homologies with Lecithin:Cholesterol Acyltransferase and other Lipases. Biochim. Biophys. Acta **959**:153-159.

Non-refereed Journal Publications:

Thornton, J.C., R.A. Garduño, and W.W. Kay. 1992. Potential for Live Vaccines in Aquaculture: An Attenuated Mutant of *Aeromonas salmonicida* as a Live Vaccine for Salmonid Furunculosis. Bull Aquacul. Asso. Can. **91**-3:53-58.

Book Chapters:

Thornton, J.C., Garduño, R.A., and W.W. Kay. 1992. Molecular Biology of Bacterial Fish Diseases. **In**: Molecular biology of fishes. (Mommesen and Hochachka eds) Vol 2: 160-189.

Kay, William W., Julian C. Thornton, and Rafael A. Garduño. 1993. Structure-Function Aspects of the *Aeromonas salmonicida* A-Layer. **In:** Advances in Bacterial Paracrystalline Surface Layers. (T.J. Beveridge and S.F. Koval eds.). pp. 151-158.

Garduño, Rafael A., Julian C. Thornton, and William W. Kay. 1993. Does the S-Layer of *Aeromonas salmonicida* Exist in More than One Functional Organizational State? **In:** Advances in Bacterial Paracrystalline Surface Layers. (T.J. Beveridge and S.F. Koval eds.). pp. 285-288.

Patents pending:

Principal Inventors: J.C. Thornton and W.W. Kay. Patent holder: University of Victoria. Title: Attenuated strains of *Aeromonas salmonicida* useful as fish vaccines. Applied for in: US, Can., Norway, Great Britain (Scotland, England and Ireland) Chile, and Japan.

Other Publications:

J.C. Thornton. 1989. Thesis: M.Sc., Cloning, Sequence, and Expression of the Glycerophospholipid:Cholesterol Acyltransferase gene from *Aeromonas hydrophila*. University of Victoria.

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Title of Dissertation: The Development of a Live Attenuated Vaccine for the Control of Salmonid Furunculosis.

Author: _____

JULIAN CHARLES THORNTON

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