AN ABSTRACT OF THE THESIS OF

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Renibacterium salmoninarum was established as a unique genus and species by sequencing and comparison of its 16S rRNA to those of seventeen genera. The resulting sequence of 1475 nucleotides (97% complete) identified *R. salmoninarum* as a member of the actinomycetes subdivision with a similarity of $\geq$0.89 to other high G+C gram-positive bacteria. A phylogenetic tree diagrammed its evolutionary relationship to other bacteria.

Examination of its virulence factors, following infection of cells derived from rainbow trout anterior kidney, revealed that *R. salmoninarum*, whether live or formalin-killed, was rapidly phagocytosed (in <4.5 h) by mononuclear phagocytes (MP). The long-term intracellular persistence of *R. salmoninarum* was facilitated by its durable cell wall and ability to escape from the phagosome into the cytoplasm. The live bacterium also absorbed to and occasionally penetrated cells resembling lymphocytes.

The live bacterium reduced viability of the MP; however, the MP remained active in vitro, and phagocytosed external bacteria. The MP damaged and destroyed *R. salmoninarum* although bacterial numbers did not decrease significantly until 96 h after infection when dying MP limited bacterial habitat and exposed released bacteria to antibiotics in the medium.

Liposome-encapsulated erythromycin (EL) was efficacious in protecting rainbow trout against *R. salmoninarum* when injected one or seven days post infection and reduced mortality from 82 to 14%. Compared with erythromycin alone, treatment with EL reduced the number of low level infections seen at 92 days by 43-75%. In fish given liposome-encapsulated saline (control liposomes, CL), there was a significant enhancement of disease progression which shortened the mean day to death; however, overall mortality was not increased when compared to fish not given liposomes. The inclusion of erythromycin in the liposomes contraindicated this adverse effect but the data suggests that a different liposome composition might be more effective. The effect of CL on disease progression indicates that lipids may affect MP performance and reduce their ability to kill intracellular *R. salmoninarum*.

Phylogeny and Intracellular Survival of *Renibacterium salmoninarum*

by

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Chapter 1

Introduction

Renibacterium salmoninarum, causative agent of the bacterial kidney disease (BKD), is a pathogen of salmon, trout, and char and is a major problem in today's management of salmonids. Both anadromous and nonanadromous salmonid stocks are susceptible to the disease and BKD is found in fish throughout North America, Chile, Europe, Iceland, and Japan

Once infected, a fish is likely to have the disease throughout its life cycle and death can occur at any time. The chronic disease impairs the ability of smolts to acclimate to salt water and reduces ocean survival. Another concern is that infected hatchery fish can transmit the disease to wild stocks of salmonids.

The control of BKD is problematic. The causative organism, *Renibacterium salmoninarum*, is persistent and often survives the treatments used to control it. Not only is the bacteria horizontally transmitted by fecal matter, abrasions, and mucus secretions, but more critically, it resides within the eggs, ensuring infection of the next generation. As a result, BKD is a continually escalating problem in light of increased emphasis on aquaculture and shipment of infected eggs to and from hatcheries across the world.

Efforts to combat BKD are complicated by the persistence of *R. salmoninarum* and its prevalence in salmonid stocks. Total destruction of brood stocks and thorough disinfection of hatchery facilities have been suggested as a means to control BKD; however, these methods are economically infeasible. The prevailing treatments used to control BKD only slow the growth of the bacteria. Iodine, commonly used to disinfect eggs, does not reach the internalized bacteria, and current vaccines and antibiotics do not effectively control the disease in smolts or adult fish. Once therapy is terminated, the bacterium is likely to continue multiplying.

A better understanding of the BKD organism would facilitate treatment of the disease. It is hypothesized that *R. salmoninarum* survives and replicates within white blood cells, specifically mononuclear phagocytes (MP), and thus is protected against antibiotic treatment. This may explain why the antibiotics erythromycin and oxytetracycline effectively kill the bacterium when grown in culture, but are ineffective in killing the bacterium within fish.

Elimination of BKD requires a new approach. In mammalian research, intracellular bacteria have been killed by using antibiotics encapsulated in microscopic phospholipid liposomes. When introduced into the animal, the antibiotic liposomes are recognized as foreign and are phagocytosed by the MP. Once engulfed, enzymes of the MP lyse the phospholipid coating and

expose the antibiotic which can then directly kill intracellular bacteria.

The research described in this thesis examines three aspects of *R. salmoninarum*: The goals were to: 1. Sequence and compare the rRNA structure of *R. salmoninarum* to those of other intracellular pathogens and related bacteria. 2. Determine if and how the bacterium survives and replicates within salmonid phagocytes. 3. Ascertain the in vivo efficacy of liposome-encapsulated antibiotic in reducing or eliminating intracellularly-surviving *R. salmoninarum*.

Chapter 2

Literature Review

Introduction. *Renibacterium salmoninarum* causes bacterial kidney disease (BKD), a chronic infection affecting wild and hatchery salmonids around the world. First described in Scotland in 1930, the disease was called "Dee" for the river in which infected Atlantic salmon (*Salmo salar*) were dying (Mackie et al., 1933). Five years later, the disease was found in brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*) at a hatchery in Massachusetts (Belding and Merrill, 1935). Now considered one of the most prevalent and difficult of bacterial diseases to control, BKD appears more widespread in the Pacific Northwest than ever before (Fryer and Lannan, in press). Although mortalities can be high among hatchery stocks, the chronic nature of the disease and the anadromous life cycle of salmonids discourages true estimation of the overall numbers of death. Migration of Pacific salmon from fresh to salt water may eliminate many infected fish as studies confirm that as much as 45% mortality occurs after exposure to salt water (Banner et al., 1983 and 1986; Bell, 1961; Fryer and Sanders, 1981; Sanders et al., 1992).

There is little gross pathology in the early stages of BKD. Even in the terminal stages of disease, external pathological changes can be minimal, with hemorrhage, exophthalmos,

abdominal distension, blisters and deep abcesses variously present. Internally, ascites, emptied gastrointestinal systems, swollen and/or discolored kidneys, and splenomegaly may signal onset of fatal infection. The characteristic white granulomatous lesions containing large numbers of the bacterium and leucocytes develop later, notably in the kidney. As the disease progresses, the number of lesions increase until the kidney becomes grayish-white, necrotic, and swollen. Focal lesions are also found in the spleen, liver, and most other organs. An opaque pseudomembrane covering the internal organs is sometimes noted in infected fish (Bruno, 1986; Ferguson, 1989a; Fryer and Sanders, 1981; Smith, 1964). Histopathological studies concluded that BKD resembles mammalian glomerulonephritis and nephrotic syndrome based on the ultrastructural changes in the glomeruli and renal tubules (Young and Chapman, 1978). Obliteration of normal kidney and liver function by large granulomatous lesions, heart failure due to myocardial invasion by infected phagocytes, and tissue damage from products released by *R. salmoninarum* and/or disrupted macrophages are all hypothesized to cause death (Bruno, 1986).

All ages of salmonids are susceptible to this chronic disease which can be transmitted horizontally or vertically. Often, fish are infected at hatching because the bacterium is transmitted from the ovarian fluid of the dam into the ovum (Bullock et al., 1978; Evelyn et al., 1984; Evelyn et al., 1986b, c). The bacterium is also transmitted horizontally which promotes transmission of

BKD within hatchery or netpen-reared salmonid populations or from hatchery to wild salmonids (Fryer and Sanders, 1981; Mitchum et al., 1981; Murray et al., 1992). Although fish die from acute infections of *R. salmoninarum*, many appear healthy for years until a sublethal infection is aggravated by environmental stressors. High or low water temperatures, secondary infections, crowding, or ocean migration have all been implicated in activating a latent infection and causing mortalities (Austin and Austin, 1987; Banner et al., 1986; Fryer and Sanders, 1981; Mitchum et al., 1979).

Renibacterium salmoninarum, a fastidious, obligate pathogen, has an absolute requirement for cysteine and grows best at 15-18°C, with slower growth at 4 and 22°C, and no growth at 37°C (Fryer and Sanders, 1981). Even under optimum conditions, *R. salmoninarum* requires $\geq$18 hours to replicate, a process that is delayed in initial isolation from diseased fish (Fryer and Sanders, 1981; Gutenberger, unpublished data). When BKD is suspected, many laboratories continue incubation of inoculated plates for 3 months before declaring the absence of *R. salmoninarum* (Benediktsdottir et al., 1991). Its slow growth on primary isolation and fastidious nature preordained the failure of early attempts to culture the small gram-positive diplobacillus found in lesions (Belding and Merrill, 1935). Thirty-two years after its discovery, the bacterium was finally cultivated in suspensions of minced chick embryo tissues. The first artificial agar medium to support growth was Dorset's supplemented with

seawater. On this media, bacterial growth was observed after two months incubation at 15°C (Earp et al., 1953). Further modifications of this medium, including the addition of cysteine and human blood, significantly enhanced growth of the bacterium and decreased incubation time to seven to ten days (Ordal and Earp, 1956). This formulation, called cysteine blood agar, finally permitted fulfillment of Koch's postulates (Fryer and Sanders, 1981; Ordal and Earp, 1956). Several changes, including the substitution of bovine serum for human blood, led to the improved kidney disease medium (KDM-2) that is commonly used today (Evelyn, 1977; Fryer and Sanders, 1981).

Taxonomy and Phylogeny. The problems in bacterial taxonomy were obvious in the initial attempts to classify *R. salmoninarum*. From microscopic observations of infected tissues and laboratory cultures, the bacterium was described as pleomorphic, with Chinese letter formations produced by a snapping type replication. This, and its gram-positive nature, lack of endospores, proteolysis, and catalase positive characteristics caused investigators to classify the bacterium with the *Corynebacteria* and for years it was called kidney disease *Corynebacterium* (Ordal and Earp, 1956; Smith, 1964). There were other suggestions concerning classification of the bacterium that noted it resembled members in *Listeria*. One species, *Listeria monocytogenes* which is morphologically similar to *Corynebacterium,* had been isolated from trout (Bullock et al.,

1975). Young and Chapman (1978) studied the bacterium's morphology and manner of division and found no evidence of the postfission snapping process that is characteristic of many corynebacteria. They commented on the nonclassified status of the bacterium and emphasized that morphology is a poor criterion to establish taxonomic relationships.

As evidenced by *R. salmoninarum*, bacterial classification is problematic when based on morphology and phenotype. Characteristics that distinguish the more complex organisms are too imprecise for the limited physiologies of bacteria. Genotypic characteristics, including DNA base ratios, nucleic acid hybridization, cell wall construction, and protein sequences prove better indicators for taxonomic classification of bacteria (Woese, 1987). These tools formed the basis for classification of the "kidney disease *Corynebacterium*", establishing a new genus and species. Sanders and Fryer (1980) characterized the DNA base ratios and cell wall composition of three bacterial isolates from chinook (*Oncorhynchus tshawytscha*) and coho salmon (*O. kisutch*) from Oregon. A guanine plus cytosine (G+C) content of 53% and a cell wall constructed of alternating glucosamine and muramic acid units in the glycan moiety with substantial amounts of lysine (principal diamino acid), glycine, and alanine (suggested as the interpeptide bridges between the peptidoglycan subunits) were found in all three isolates. These observations and an earlier report that mycolic acids were absent (Goodfellow et al., 1976) were convincing evidence that this

bacterium did not belong in the genus *Corynebacterium*. With the cell wall information, unique pathogenesis, and optimum growth temperature, the bacterium was systematically excluded from the genera of *Listeria*, *Erysipelothrix*, *Arthrobacter* and found uniquely different from the *Corynebacterium* species, *C. haemolyticum* and *C. pyrogenes*, which it most resembled (Sanders and Fryer, 1980). Thus in 1980, the bacterium was renamed as a new and separate genus with one species, *Renibacterium salmoninarum* (Sanders and Fryer, 1980). The later finding of an unusual amino sugar in the cell wall, N-acetyl-fucosamine (Kusser and Fiedler, 1983), and fatty acid analysis (Collins, 1982) further warranted the new genus classification. Years later, there is still only one species in the genus as isolates from different salmonids worldwide have remarkably similar biochemical and physiological attributes (Goodfellow et al., 1985).

Despite these differences, *Renibacterium* was considered to be most closely related to members of the Coryneform group (Sanders and Fryer, 1980). Goodfellow et al. (1985) tried to clarify the phylogenetic relationship of *R. salmoninarum* to other bacteria with a numerical taxonomic technique based upon 86 physiological attributes. Forty-four strains of *R. salmoninarum* formed a distinct, tight cluster with a loose relationship to *Micrococcus luteus*, *Rothia dentocariosa*, and *Actinomyces viscosus*. *Arthrobacter* species (including *A. globiformis*) along with *Listeria denitrificans* (now *Jonesia denitrificans*) and *Microbacterium* formed a second cluster and *Lactobacillus*, the

third. Unfortunately, no comparisons were made to members in *Corynebacterium.*

Advances in molecular biology spurred taxonomists and phylogeneticists to examine the nucleic acids of bacteria to resolve familial relationships. For this purpose, the early molecular approaches provided limited phylogenetic relationships. DNA/DNA hybridization was useful only for determining relationships within genera and DNA/ribosomal RNA hybridization allowed only for revision of existing taxonomic structure (Woese, 1987). Protein sequences were essentially useless for phylogenetic purposes (Woese, 1987). The G+C content analysis provided a general classification tool for the division of gram-positive bacteria into valid low (<50%) and high (≥55%) G+C subdivisions, but only permitted identification of obviously misclassified species. The methodology for determining nucleotide sequences revolutionized molecular biology (Sanger and Coulson, 1975) and was rapidly adapted as a more reliable estimate for bacterial classification and evolution (Wayne et al., 1987). The differences between stable genomic sequences provide a means for quantifiable comparisons rather than attempting quantification of phenotypic characteristics which are influenced by varying environmental conditions (Sneath, 1989; Woese, 1987).

The ribosomal RNA (rRNA) is currently the most useful nucleic acid sequence for phylogeny. It is a molecular chronometer, i.e., because of its critical cellular function in

biology, changes in its sequence occur slowly yet randomly in time, allowing observation of long-term evolutionary changes. It occurs in all organisms, and use of the large rRNA (16S and 23S) provide a statistically valid basis for sequence comparison. In fulfilling the requirements as a molecular chronometer, the rRNA has hypervariable regions subject to relatively rapid change interpersed between large highly conserved domains (Woese, 1987).

Fox et al. (1977) used oligonucleotide cataloging of 16S rRNA (a collection of partial sequences of $\leq$20 nucleotides produced by digestion of 16S rRNA with ribonuclease T_1) in a molecular approach to prokaryotic systematics. Many taxonomists followed suit, including Stackebrandt et al. (1988) who provided the first genomic relationship of *R. salmoninarum* to 50 other genera. They determined that *R. salmoninarum* was most closely related to the actinomycetes in the high G+C subdivision of gram-positive bacteria. This supported the wall and lipid component analysis, reliable indicators of actinomycete and coryneform systematics (Collins et al., 1988) and removed the bacterium from its hypothetical association with *Corynebacterium*. This study firmly established a close relationship between *R. salmoninarum* and the *Arthrobacter-Micrococcus* sublines, with a particularly close relationship to *A. globiformis*. The anomaly of the low 53% G+C content of *R. salmoninarum* was the only disparate characteristic for its

inclusion with the actinomycetes group whose members have high G+C contents (≥55%).

Although rRNA cataloging defines major bacterial phyla, it does not resolve branching orders among them or among their subdivisions. The sequencing of the entire 16S rRNA by reverse transcriptase (Lane et al., 1985; Lane et al., 1988) greatly increases the resolving power of the rRNA chronometer and provides the capability to determine both evolutionary relationships and taxonomic relationships (Woese, 1987). A host of papers redefining the phylogeny and taxonomic relationships of bacteria are based on this technology (Charfreitag et al., 1988; Collins et al., 1988; Collins et al., 1989; Dewhirst et al., 1989; Paster et al., 1988; Stackebrandt and Charfreitag, 1990; Weisburg et al., 1989; Williams et al., 1990). Gutenberger et al. (1991) produced a nearly complete sequence (97%) of 1475 nucleotides of the 16S rRNA of *R. salmoninarum*. After comparison of *R. salmoninarum* to 17 genera from three gram-positive subdivisions, the similarity of its sequence to the high G+C bacteria (actinomycetes) averaged 0.91 in contrast to 0.81 for the low G+C (*Clostridium-Bacillus*) bacteria and *Heliobacterium chlorum* (the third subdivision representing bacteria lacking gram-positive walls). This substantiated the report by Stackebrandt et al. (1988) and induced the publication of a new, more accurate G+C content (55.5±0.43%) for *R. salmoninarum* (Banner et al., 1991). A phylogenetic tree depicting the relationship to ten actinomycetes placed *R. salmoninarum* in a cluster containing *Terrabacter*

tumescens, *Streptomyces coelicolor*, and *Nocardiodes luteus*, all bacteria isolated from soil. The pathogenic actinomycetes (*Mycobacterium bovis* and *Nocardia asteroides*) which, like *R. salmoninarum* are intracellular pathogens, were located in another branch, indicating a more distant evolutionary relationship. Unpublished results from this study revealed that *A. globiformis*, a bacterium of soil and silage had the greatest similarity to *R. salmoninarum*; of 1475 nucleotide bases, the sequences differed by only 69 bases. A phylogenetic tree revealed that *R. salmoninarum* and *A. globiformis* formed a branch apart from *T. tumescens*, with *R. salmoninarum* evolving most recently.

The 16S rRNA data are verified by the number of characteristics shared by *R. salmoninarum* and *A. globiformis*. Phenotypic similarities include shape (pleomorphic coccoid-rod), lack of motility, non-sporeforming, lack of acid production from sugars, catalase positive, and growth at 15°C (Fryer and Sanders, 1980; Goodfellow et al., 1985; Sawai et al., 1976). Of more interest, the two bacteria share similar cell wall construction and lipid composition, two important features for taxonomic description (Jones and Krieg, 1986). The primary structure of the cell wall, or peptidoglycan, is controlled by multiple gene actions which are conserved with few mutational changes (Schleifer and Kandler, 1972). And unlike the gram-negative bacteria, the composition and structure of the peptidoglycans of the gram-positive bacteria vary greatly. Both *R. salmoninarum* and *A. globiformis* have

lysine as the principal amino acid and an A3α peptidoglycan (Keddie et al., 1986; Kusser and Fiedler, 1983). Fatty acid analysis of both bacteria reveals a lack of mycolic acids, the long chain 3-hydroxy 2-branched acids found only in certain taxa as *Corynebacterium*, *Mycobacterium*, and *Nocardia* (Goodfellow et al., 1976; Jones and Krieg, 1986; Keddie et al., 1986). The close relationship and evolution of a fastidious pathogen from a soil bacterium may seem obtuse at first examination. However, the finding of both soil saprophytes and animal pathogens within the same genera or even the same species, as *Nocardia asteroides*, is not unusual (Lechevalier, 1986; Wayne and Kubica, 1986). As more chemotaxonomic information is obtained, other similarities between the two bacteria are likely to be found.

Pathogenic Mechanisms. As early as 1953, *R. salmoninarum* was hypothesized to be an intracellular pathogen based on its size, frequent intracytoplasmic location in mononuclear phagocytes, and the difficulty with culture (Earp et al., 1953). Before *R. salmoninarum* was cultured on agar, it was believed to be the rickettsial species (*Neorickettsia helmintheca*) that causes salmon poisoning in dogs because the metacercarial stage of the fluke vector (*Troglotrema salminicola*) was found in the 1946-47 outbreaks of BKD in salmon (Earp et al., 1953). By 1956, *R. salmoninarum* had been cultivated on non-living media and the association with Rickettsiales was dropped (Ordal and Earp, 1956). Other researchers noted the similarity of *R.*

salmoninarum to *L. monocytogenes*, another gram-positive bacterium that survives intracellularly and which causes disease in numerous animals (Ajmal and Hobbs, 1967; Bullock et al., 1975; Farber and Peterkin, 1991). There was recorded evidence for the intracellular nature of *R. salmoninarum* from numerous studies (Hendricks and Leek, 1975; Wolf and Dunbar, 1959; Yamamoto, 1975) but Young and Chapman (1978) produced the first electron microscopic evidence showing the bacterium inside macrophages. They noted that cells resembling neutrophils, macrophages, and pigment cells phagocytosed the bacterium. A later study by Bruno (1986) recorded the chronological gross pathology, supported by light microscopy studies of selected organs in laboratory-infected rainbow trout (*Onchorhynchus mykiss* Richardson) and Atlantic salmon (*Salmo salar* L.). He found *R. salmoninarum* in phagocytes of the kidney and spleen within 45 minutes after intraperitoneal injection. Between six to ten days, low numbers of the bacterium were detected inside the mononuclear phagocytes and at 14 days, there were many intra- and extra-cellular *R. salmoninarum*. By 21 days, macrophages appeared to have burst and released large numbers of the bacterium. Melanin, from the melanomacrophages and melanocytes, was dispersed throughout the tissues. The bacterium was also found in macrophages in the pancreas, pyloric caeca, stomach, urinary tract, and peritoneal fluid. In addition, epithelial cells appeared to phagocytose *R. salmoninarum*.

That *R. salmoninarum* appeared to survive within macrophages is not a contraindication of the killing efficacy of the piscine phagocytic system. The mononuclear phagocytes (MP) in fish include circulating and tissue-associated macrophages (termed monocytes and macrophages, respectively) and, like their mammalian counterparts, are capable of phagocytosing, killing, and degrading most microorganisms (Avtalion and Shahrabani, 1975; Chung and Secombes, 1988; Rowley et al., 1988; Zelikoff et al., 1991). The melanomacrophages, the third component in the piscine MP system, are macrophages containing melanin derived from the phagocytosis of melanocytes (the storage depots of melanin) or loose melanin granules and may serve a protective role (Ellis, 1982). Unlike mammals, the neutrophils in fish have no or limited phagocytic activity despite a full complement of enzymes (Ferguson, 1989b). This suggests that bactericidal activity occurs extracellularly, resulting in extensive tissue damage. The melanomacrophages may have evolved to quench and protect against free-radicals produced by neutrophils and MP. The melanin may also protect against damage to the melanomacrophages themselves.

As the primary defense against pathogens, piscine MP are avidly phagocytic and well armed for the killing and degradation of bacteria. Foreign objects, as microorganisms, are either absorbed to the MP membrane or actively phagocytosed, processes that are generally enhanced in the presence of complement and/or specific antibody and an indication that C3b

and immunoglobulin (Ig) membrane receptors are present on piscine MP (Blazer, 1991; Rowley et al., 1988). The foreign objects are then internalized by invagination of the membrane to form phagosomes. Stimulation of the MP membrane by soluble or insoluble antigens (as bacteria) triggers respiratory burst, the first defensive mechanism of the MP. It is the respiratory burst, a release of reactive oxygen radicals (as O_2^-, H_2O_2), which is responsible for the killing of most microorganisms, whether intra- or extra-cellularly located (Chung and Secombes, 1987 and 1988).

The second phase of MP defense involves the fusion of the phagosome and lysosomes to form a phagolysosome. The lysosomes contain hydrolytic proteins and enzymes necessary to continue killing and degradation of phagocytosed materials (Ghadially, 1988; Male and Roitt, 1989). Research on this phase has been essentially ignored in fish MP, but it is likely similar to that in mammals. Piscine MP have been implicated in antigen presentation, a process occuring only after phagocytosis and degradation of internalized microorganisms (Rowley et al., 1988).

Activation by immunostimulants, bacteria, or bacterial cell wall proteins increases chemotaxic and phagocytic activity as well as enhancing the antimicrobial and antitumor effects of MP (Chung and Secombes, 1987; Secombes, 1986; Zelikoff et al., 1991). Studies on the MP from rainbow trout have provided much of this evidence. Live *Streptococcus sp.* was killed within 72 hours by MP from trout previously stimulated with formalin-killed

bacteria 25 days prior to challenge (Sakai et al., 1989). In another study, immunization also effectively increased killing of helminth parasites by activated MP, reducing the number of metacercarieae by 70-80%. Without specific antisera, the killing activity of activated and non-activated MP were equivalent (Whyte et al., 1990). An immunomodulant from abalone given five days prior to challenge enhanced the resistance of rainbow trout to *Vibrio anguillarum* with 73% surviving compared with 14% in the non-stimulated fish. This was attributed to activation of MP based on the enhanced phagocytosis and respiratory burst seen in these cells (Sakai et al., 1991). In vitro research with peritoneal or kidney MP showed that avirulent *A. salmonicida* were killed by non-activated and activated MP, especially if the bacteria were opsonized by complement (Olivier et al. 1986; Graham et al., 1988). The virulent *A. salmonicida* was less susceptible to killing by non-activated MP even when opsonized with complement although activated MP readily killed non-opsonized bacteria (Olivier et al., 1986; Graham et al., 1988).

Most pathogens of salmonids do not survive lengthy periods within MP, although specific virulence factors, as the A layer of *Aeromonas salmonicida*, may allow them to resist phagocytosis (Olivier et al., 1986) or kill the MP (Gutenberger, unpublished data). However, survival and replication of microorganisms within phagocytic cells of vertebrates is not uncommon and representatives from many genera exist, including *Shigella* (Clerc et al., 1987), *Salmonella* (Buchmeier and

Heffron, 1991), *Mycobacterium* (Sibley et al., 1987), *Nocardia* (Davis-Scibienski and Beaman, 1980), *Campylobacter* (Kiehlbauch et al., 1985), *Listeria* (Farber and Peterkin, 1991), *Legionella* (Dowling et al., 1992), *Brucella* (Riley and Robertson, 1984), *Paracoccidioides* (Brummer et al., 1990), *Leishmania* (Alving and Swartz, 1984) and *Toxoplasma* (Jones et al., 1972). The ability of these microorganisms to resist or inhibit intraleukocytic killing systems relies upon different mechanisms. *Mycobacterium lepraemurium* (Armstrong and Hart, 1971), *Toxoplasma gondii* (Jones et al., 1972), *Salmonella typhimurium* (Buckmeier & Heffron, 1991), and *Nocardia asteroides* (Davis-Scibienski and Beaman, 1980) remain in the phagosome. This method of survival entails the ability to prevent fusion of the phagosome and lysosomes or withstand the harsh enzymatic environment formed after fusion of the two organelles. Some *Legionella* spp. interupt phagocyte activation and prevent subsequent production of the bactericidal metabolites from respiratory burst (Dowling et al., 1992). Other microorganisms, as *Listeria monocytogenes* and *Shigella flexneri*, escape from the phagosome into the cytoplasm, thus avoiding the enzymes in the phagolysosome (Farber and Peterkin, 1991; Sibley et al., 1987). This mechanism involves lysis of the phagosome membrane and bacteria are found in the cytoplasm as quickly as 15 minutes after infection.

Reports of intracellular survival mechanisms for some bacterial species are contradictory and may reflect differences in

experimental techniques or strains. For instance, both *Mycobacterium tuberculosis* (nee *M. bovis*) and *Mycobacterium leprae* are reported to survive intracellularly by remaining in the phagosome and preventing phagosome-lysosome fusion (Sibley et al., 1987; Armstrong & Hart, 1971) or by escaping into the cytoplasm (Myrvik et al., 1988; Evans & Levy, 1972). This confusion may be attributable to differing virulence mechanisms existing between strains or length of passage in specific laboratory animals. As an example, only one strain of *Legionella pneumophila* appears to inhibit phagosome-lysosome fusion (Dowling et al., 1992; Horwitz, 1983).

The microorganisms that survive intracellularly also replicate intracellularly, the rate and effect on the MP dependent upon bacterial virulence or other factors. Multiplication of some pathogens, as *S. flexneri* and *Legionella spp.*, can be so extensive that the entire MP fills with bacteria, resulting in rapid host cell death (Clerc et al., 1987; Dowling et al., 1992). *Legionella pneumophila* multiplies one to two $\log_{10}$ within 24 hours in human MP (Dowling et al., 1992). *Mycobacterium tuberculosis* replicates slowly but can subsist intracellularly for years which may account for the chronic nature and reoccurrence of disease. In human MP, *M. tuberculosis* requires 23 ± 2 hours to double and in seven days one tubercle bacillus generates over 60 progeny, enough to kill the MP (Crowle, 1988). Another less virulent mycobacteria, *M. avis*, multiplies extensively inside MP with up to 10^3 organisms found within a MP (Crowle, 1988).

The ability of MP to limit intracellular replication is compromised under various conditions and normal serum factors appear to directly affect this. Gamma interferon accelerates replication of intracellular *M. tuberculosis* in vitro, possibly due to stimulation of the MP with subsequent production of mycobacterial growth-enhancing factors (Crowle, 1988). This might explain why secondary infections induce active disease process from a latent infection. Vitamin D has the opposite effect and in combination with normal serum seems to prime the MP response to anti-tuberculosis immune lymphokine (Crowle, 1988).

Individual genetic inheritance also dictates intracellular killing by MP. In mammalian systems, the resistance or susceptibility of an animal to intracellular organisms is profoundly affected by a dominant/recessive allele (*Bcg*) which may regulate MP activation (Solbach et al., 1991). Just as some animals are more resistant to tuberculosis, some salmonid species and individuals are more resistant to BKD (Withler and Evelyn, 1990). In this respect, the differential species/stock and individual susceptibility of salmonids to renibacteria may be related to the inherent ability of their MP to respond quickly to bacterial pathogens.

The cell surface components of many bacteria correlate with their ability to enter and endure within MP. The hemolysin (listeriolysin O) of *L. monocytogenes*, the first virulence factor described as crucial for intracellular survival and growth, is responsible for phagosome membrane lysis and bacterial entry

into the cytoplasm. The β-hemolysin is a sulfhydryl-activated cytolysin of 57-58 kilodaltons (kDa) and is produced and released in large quantities in culture supernatants (Farber and Peterkin, 1991). A cell surface protein (Mip) of *Legionella* is vital to efficient invasion of MP although the mechanism inducing uptake is unknown (Dowling et al., 1992). It is the lipids, not proteins, which are suspected to cause the adherence of mycobacteria to the phagosome membrane resulting in fragmentation and disintegration of the phagosome and subsequent cytoplasmic entry. The cord factor (trehalose 6,6'-dimycolate) and sulfolipids have toxic properties and/or are associated with virulence although efforts to correlate this with phagosome disruption have proven fruitless (Myrvik et al., 1988). Notably, alveolar macrophages from rabbits vaccinated with the BCG strain of *M. tuberculosis* were resistant to phagosome disruption by this same strain. No single protein has been implicated in the virulence of *S. flexneri*, but an associated plasmid appears responsible for its phagocytosis and lysis of the phagosome membrane (Clerc et al., 1987).

For *R. salmoninarum* to survive intracellularly, it must withstand or somehow inactivate or utilize the bactericidal mechanisms of MP. *Renibacterium salmoninarum* produces excess quantities of a soluble, heat-stable extracellular protein (up to one mg/ml in moribund fish tissues and sera, and bacterial culture supernatants) that likely plays a role in its pathogenicity (Bruno, 1990; Daly and Stevenson, 1987; Getchell et al., 1985;

Turaga et al., 1987). This protein, variously called antigen F, soluble antigen (Getchell et al., 1985), or haemagglutinin (Daly and Stevenson, 1987), is also termed p57 in deference to its primary component, a 57-58 kDa protein doublet (Rockey et al., 1991b; Wiens and Kaattari, 1989 and 1991). In vitro studies of p57 reveal that it agglutinates mammalian erythrocytes (Daly and Stevenson, 1987), salmonid leukocytes and spermatozoa (Daly and Stevenson, 1989; Wiens and Kaattari, 1991), and suppresses antibody formation (Rockey et al., 1991b; Turaga et al., 1987). It is also responsible for the hydrophobic nature of the bacterium, a factor that enhances uptake by MP (Bruno, 1988; Dubreuil et al., 1990). Aggregates of the p57 molecule may protrude as tiny fimbriae through a polysaccharide capsule and account for the autoagglutination of the bacterium (Dubreuil et al., 1990). Monoclonal antibodies (Mab) against different epitopes of p57 define regions related to the function and structure of the protein (Wiens and Kaattari, 1991). The Mab binding the region proximal to the amino terminus of the protein blocks hemagglutinating activity, suggesting that this area is a functional domain. This research also indicated that the outer amino terminus and midportion of the protein are the antigenic binding sites to which antisera recognizing *R. salmoninarum* react, the innermost region being occluded when attached to the bacterial cell. Breakdown products of p57 of 45, 36, 34, 25 and 20 kDa all react with different Mab against p57 and apparently result from digestion by a serine proteinase, also a component of the extracellular

proteins elaborated by *R. salmoninarum*. This proteinase digests p57 after several days incubation at bacterial physiological temperatures and immediately at 37°C, resulting in a loss of the p57 biological activities (Rockey et al., 1991b). The proteinase is inactive against other substrates such as bovine serum albumin, ovalbumin, carbonic anhydrase and trout antibody unless denaturants or reducing agents are added. The autologous specificity of the proteinase might be responsible for activation/regulation of an enzyme or gene expression and tightly coordinated to p57 biological activity. Without further research on the proteinase's activity against other substrates, the role played in pathogenicity or its regulation remains unknown.

Other characteristics of *R. salmoninarum* further suggest its evolution as an intracellular parasite. It has a durable cell wall which is resistant to proteinase K, high pressure, and lysozyme (Gutenberger et al., 1991; Fryer and Sanders, 1981). It also produces catalase which can reduce H_2O_2 into water and oxygen, is acid tolerant, and has a hydrophobic cell surface amenable to phagocytic uptake. That *R. salmoninarum* replicates slowly, with a possible tendency towards dormancy, also suggests the integral adoption of an intracellular mode of survival. Advantages of slow growth include a longer intracellular residence by avoiding destruction of its host cell, reduced exposure of replicating bacteria to MP enzymes, and decreased susceptibility to antibiotics that affect actively growing bacteria.

The only other bacteria in salmonids that might claim status as an intracellular pathogen is the unspeciated mycobacteria which was responsible for the chronic fish tuberculosis, a disease unexpectedly present in hatchery salmonids returning from the ocean in the early 1950's. The disease, and hence research, was eliminated by the discontinuation of feeding raw, unpasteurized fish carcasses to young salmonids (Austin and Austin, 1987; Fryer and Sanders, 1981) but the generic relationship of this pathogenic mycobacteria to those in mammalian disease suggest possible intracellular habitation.

Transmission. *Renibacterium salmoninarum* has proven more resilient than the mycobacterial species and transmittance occurs both horizontally and vertically. Horizontal transmission of the bacterium is possible through skin abrasions (Wolf and Dunbar, 1959) and cannibalism of infected fish (Wood and Wallis, 1955); however, the more convincing and natural transmission may occur in water holding infected fish. Mitchum and Sherman (1981) found that BKD-negative brook, brown, and rainbow trout (*Salvelinus fontenalis, Salmo trutta,* and *O. mykiss,* respectively) became infected and died from BKD within nine months after introduction into a lake holding infected wild brook trout. In the laboratory, the exposure of chinook salmon (*O. tshawytscha*) to waterborne challenges (10^4 to 10^6 CFU/ml of water for 15 or 30 minutes) or fish infected with *R. salmoninarum* also resulted in

death after five to seven months (Murray et al., 1992). Similarly, detectable BKD in brook trout was noted six weeks after exposure to 10^6 CFU/ml of water (Elliott et al., 1991). Fecal material from infected fish can harbor the bacterium up to 21 days in fresh water (Bell et al., 1980; Austin and Rayment, 1985), possibly the route by which healthy sockeye salmon became infected after cohabitation with artificially infected sockeye salmon (Bell et al., 1984). The oral-fecal route of infection was also suspected as the cause for disease transmission in farmed salmon held in sea water because the bacterium survives up to seven days in salt water. Oral intubation of infected feces into young coho salmon demonstrated that this was a feasible route of infection (Balfry et al., 1991).

Vertical transmission is likely the most important means for continued persistence of the bacterium in salmonid populations. Unlike many vertically transmitted diseases in fish, *R. salmoninarum* is internalized within the ovum (Evelyn et al., 1984). Consequently, disinfection of infected eggs with iodophor and antibiotics does not prevent transmission and the offspring are infected prior to hatching (Bruno and Munro, 1986; Evelyn et al., 1986a, b, and c). Disinfection with iodine (250 ppm) for two hours also failed to remove all viable surface-bound *R. salmoninarum* (Evelyn et al., 1986c). Evelyn et al. (1986a) found that trout and salmon eggs could be internally infected by immersion in infected coelomic (ovarian) fluid prior to fertilization and water-hardening. Bruno and Munro (1986)

suggested that bacteria enter by the micropyle and/or the pore canals prior to water hardening and fertilization after observing the bacterium entering developing oogonia within the ovary. The bacterium may survive inside the yolk, rather than the perivitelline fluid, because the small molecules of iodine should pass through the egg membrane (Evelyn et al., 1984; Evelyn et al., 1986c). Cells of the bacterium occur in very low numbers, possibly one or two per egg (Evelyn et al., 1986c). The dam has been exclusively implicated in vertical transmission (Evelyn et al., 1986b) even though *R. salmoninarum* avidly binds to, and is carried on the tails of spermatozoa (Daly and Stevenson, 1989). Given that only the head of the spermatozoan enters the micropyle, it is thought that entry of flagella-bound bacteria into the egg is unlikely.

Control. The traditional approaches to treatment of BKD are marginally successful if temporary disease control is deemed sufficient and total failures if elimination from salmonid populations is the final goal. The only effective method of eradicating the bacterium is by destruction of infected fish, a proposition that is unreasonable given the worldwide distribution of the disease and the improbability of finding many sources of bacteria-free eggs. Recently, the segregation and fertilization of eggs from brood dams with low levels of *R. salmoninarum* seems to reduce the mortality and enhance return rates of progeny (Pascho et al., 1991; Warren, 1991).

Identification and use of genetically resistant genotypes of salmonids may also merit some attention (Bruno and Johnstone, 1990; Suzumoto et al., 1977; Winter et al., 1980; Withler and Evelyn, 1990). Diet composition has periodically attracted interest as a means for reducing BKD, but various combinations of minerals and vitamins as dietary supplements have generally proven ineffective, although, the addition of fluorine and iodine reduced disease severity and/or mortality by BKD in several studies (Elliott et al., 1989).

Numerous attempts in developing effective vaccines against *R. salmoninarum* have been unsuccessful (Elliott et al., 1989). The bacterium induces an antibody response in fish (Evelyn, 1971; Paterson et al., 1981; Weber and Zwicker, 1979;) which is not protective and fails to correlate with infection levels (Baudin Laurencin et al., 1977; Bruno, 1987; Hsu et al., 1991; Paterson et al., 1981). Vaccination attempts have included administration of killed, whole *R. salmoninarum* or its proteins (with or without adjuvant) by intraperitoneal injection, feeding, immersion, or hyperosmotic infiltration. Other than elevations in specific antibody and, in some cases, a reduction in BKD lesions, the immune response developed by vaccination is insufficient to confer protection (Elliott et al., 1989). Indeed, an increase in antibody production in combination with infection may serve only to induce the formation and subsequent deposition of soluble antigen-antibody complexes on the glomerular capillary wall, resulting in glomerulonephritis (Kaattari et al., 1988; Sami et

al., 1992). Also, opsonization of the bacterium with antibody may contribute to enhanced phagocytosis by the MP (Sakai et al., 1989b), a probable refuge from the immune system. As has been noted for other intracellular pathogens, coating of intracellularly surviving bacteria with specific antibody and complement promotes efficient phagocytosis but not killing and intracellular replication is still possible (Horwitz, 1984).

Chemotherapeutants and antibiotics still provide the most convenient, albeit incomplete, protection against *R. salmoninarum*. The 1950's abounded with research detailing the therapeutic value of the sulfa drugs in controlling BKD. Any of a variety of sulfonamides (sulfadiazine, sulfamethazine, sulfamerizine, sulfanilamide, Gantrisin, sulfathiazole) fed at a rate of 8-12 g/100 lbs of fish per day for 20 to 49 days (with possible additional weekly supplementation each month during the summer months) were tested in fish either naturally or artificially infected. The results were similar: there was a definitive prophylactic effect in arresting disease progress, but treatment merely delayed death (Allison, 1958; Earp et al., 1953; Rucker et al., 1951; Rucker et al., 1954; Snieszko and Griffin, 1955; Wood and Wallis, 1955). In deference to its proposed kinship to Rickettsiales, Snieszko (1953) noted that the sulfonamides were inadequate against the intracellular bacterium. The antibiotics, chloramphenicol, oxytetracycline, and aureomycin, were tested (3.4 g per 100 lb of body weight) with less or equivalent results (Snieszko and Griffin, 1955). Wolf and Dunbar (1959) performed a

more expansive study of 34 chemotherapeutants including penicillin G, erythromycin, and tetracycline on 16 strains of *R. salmoninarum*. Of these, erythromycin (oral dose of 100 mg antibiotic/kg fish for 21 days) appeared most promising and mortality was reduced to 47% at 207 days after therapy, with only two of the remaining 51 fish positive as detected by Gram staining and by inoculation of media. This was a considerable improvement over the other chemotherapeutants tested and they concluded that erythromycin eliminated BKD in about half of the fish. Despite the efficacy of erythromycin, Wolf and Dunbar recognised that the bacterium's intracellular location protected it against therapy with reactivation of disease possible. Sixteen years later, the same treatment was still deemed best for treatment of BKD, although it was evident that the drug did not provide complete control in either laboratory or field conditions (Bullock et al., 1975).

A study evaluated over 70 antimicrobial compounds in vitro against 40 strains of *R. salmoninarum*, with an emphasis on those proven effective against gram-positive bacteria (Austin, 1985). All strains of the bacterium showed a remarkable uniformity in their susceptibility or resistance to the antimicrobials. Forty-five chemotherapeutants were fed prophylactically in doses of 1, 5, 10, 25, 50, 75, 100, and 125 mg antibiotic/kg of fish/day for 10 days prior to infection with *R. salmoninarum*. Erythromycin (the salts thiocyanate, and phosphate), penicillin G, clindamycin HCL, spiramycin, and

rifampicin were most effective in reducing mortality, giving at least 60% protection at dosages ≤75 mg antibiotic/kg of fish. Of these, all except rifampicin, were efficacious at ≤100 mg antibiotic/kg fish when given ten days after the start of an infection in which 100% mortality was recorded for the untreated fish by the end of the 56 day observation period.

Some routes and timing of antibiotic administration seem to prevent death in the adults and reduce transmission to progeny. DeCew (1972) found that penicillin G procaine (15,000-30,000 international units/kg), dihydrostreptomycin sulfate (19-38 mg/kg) or oxytetracycline (4-8 mg/kg) enhanced adult survival 24 to 160% and egg production by 30 to 250% after three subcutaneous injections given at 21 day intervals. Mandible and fin teratogenesis occurred in progeny from injected adults but this was alleviated by giving the last injection at 32 days prior to spawning. Masu salmon (*Oncorhynchus masou*) injected intraperitoneally with erythromycin (10 mg/kg) 20 days before spawning had 0.5% mortality due to BKD compared with 2.3% in uninjected fish. No pathological damage through the alevin stage was noted (Sakai et al., 1986). Evelyn et al. (1986a) determined that brood stock injected with erythromycin (20 mg antibiotic/kg fish) 30 to 56 days prior to spawning yielded eggs with a bactericidal concentration of antibiotic in the yolk. This was significant because the alternate treatment, immersion of eggs in an erythromycin bath, permitted accumulation of the drug only in the perivitelline fluid of the egg and not in the yolk where the

bacterium is thought to occur (Evelyn et al., 1986b). Also the erythromycin leached rapidly from the eggs, preventing bactericidal effect. Brown et al. (1990) further experimented with pre-spawning injections of antibiotics and found that an injection of any of five antibiotics (erythromycin phosphate, penicillin G, oxytetracycline, cephradrine, or rifampicin) into the dorsal sinus of the dam significantly reduced the number of infected progeny at the alevin stage. The antibiotics were deposited in the yolk of the egg and at the alevin stage, *R. salmoninarum* was found in only 14-30% of the progeny from the treated dams compared with 93% from the untreated dams.

There is no lack of efficacious drugs for control of *R. salmoninarum*. In an in vitro study of 21 drugs and 11 strains of the bacterium, Bandin et al. (1991) found erythromycin (5.5-21.9 mg/l) and oxytetracycline (<0.6-11 mg/l) best for bactericidal control of all strains. The compounds cefazolin and tiamulin were also bactericidal for 90% of the strains at 21.9 and 11 mg/l, respectively. Quinolones were not effective against *R. salmoninarum* although this class of antibiotic concentrates intracellularly. Gutenberger et al. (1989) tested over 100 unnamed, experimental chemotherapeutants against four strains of *R. salmoninarum* in vitro. All strains were susceptible to 51 of the compounds, with 19 of these effectively inhibiting growth at 1 mg/l or less. Except for sarafloxacin, a fluoroquinolone which is a promising broad-spectrum antibiotic for veterinary use (although its efficacy against *R. salmoninarum* is mediocre), none

of these drugs were tested in vivo. This is primarily due to the recognition of the expense involved in obtaining approval by the U.S. Food and Drug Administration (FDA) for food animal use. This underscores the necessity for improving the therapeutic effects of the only antibiotics currently allowed, erythromycin and oxytetracycline. Erythromycin, despite its widespread use in hatcheries and sea farms, is designated as an experimental drug and has yet to be registered for treatment of BKD, although the necessary experimentation to fulfill the FDA requirements is ongoing (Moffitt, 1990).

Development and Use of Liposomes. Antibiotic treatment can control the disease to a certain extent, but, unexpectedly, and often coincidental with environmental stresses such as elevated water temperatures, BKD will flourish in salmonid populations previously considered free of the disease. With the limited antibiotic choices and the intractability of *R. salmoninarum*, development of another method of treatment is salient and feasible with the advent of liposomes.

In the early 1960's, Alec Bangham developed microscopic spheres of phospholipid with water enclosed inside which he called liposomes (Lasic, 1992). By the late 1970's, the potential of these liposomes as carriers of antibiotics, enzymes, nucleic acids, vitamins or other substances for pharmaceutical or medical application was recognised (Gregoriadis, 1979; Lasic, 1992). Liposomes are avidly consumed and their contents released upon

lysis by the enzymes of MP. Thus liposomes provide a means for targeting intracellular parasites (Gregoriadis, 1979; Lasic, 1992) and this translates into reduced levels of drug needed in treatment of a disease concomittant with a decrease in drug toxicity.

Liposomes are created by evaporating a film of a phospholipid mixture onto a flask wall and adding an aqueous solution (swelling solution) containing the compound of interest. Primarily, phosphatidylcholine and cholesterol, often with an electrically charged lipid to improve stability, are used to form liposomes but there are many permutations to suit a particular need (Gregoriadis, 1984). With vigorous agitation, large multilamellar vesicles (MLV) of up to several fractions of a millimeter are formed which can be modified into small or large unilamellar vesicles (SUV, 20-50 nm or LUV, $\geq$60 nm, respectively) (Chapman, 1984; Lasic, 1992; Rao, 1984). Each of these, MLV, SUV, or LUV, has advantages. The multiple layers of MLV provide more area for incorporation of the aqueous solution. The MLV are easily prepared and sized by selective filtration, are relatively stable in storage and in the presence of blood components, and are cleared rapidly by the reticuloendothelial system. However, the MLV have low and/or variable degree of drug encapsulation; this may not be critical for lipophilic drugs which are incorporated into the bilayer phase of the liposomes (Rao, 1984). The SUV, prepared under high pressure through a French press or by ultrasonication, are

insensitive to osmotic changes and have a longer half-life in circulation which promotes prolonged drug release and targeting to nonreticuloendothelial cells and tissues. There is, however, limited encapsulation efficiency. The LUV are mostly unimembranous and their formation results in a high trapping efficiency of the swelling solution. This type of liposome has an intermediate clearance rate between the SUV and MLV, but are probably most suited for drug delivery to MP rather than for prolonged drug release in the circulation (Rao, 1984). After formation of liposomes, nonencapsulated drug is removed by centrifugation, dialysis, or gel filtration (Rao, 1984).

Efficiency of drug encapsulation varies with the drug and method used to create liposomes. From <1 to 50% of the swelling solution can be entrapped (Alving and Swartz, 1984; Bermudez et al., 1987; MacLeod and Prescott, 1988; Rao, 1984). Either hydophobic or polar drugs can be incorporated into liposomes, the former by dissolution into the chloroform lipid/cholesterol mixture prior to evaporation into a film and the latter by dissolution into the aqueous buffer used to form the liposomes (Chapman, 1984). After entrapment, the drug is liberated only by leakage through the liposome bilayer or by destruction of the vesicle. Storage of liposomes in the swelling solution at 4°C prevents leakage from MLV (Alving and Swartz, 1984).

In mammals, antibiotics incorporated into liposomes have been used successfully to kill surviving intracellular bacteria. The MP of mice infected in vivo with *L. monocytogenes* were

treated in vitro with liposome-encapsulated ampicillin. The ampicillin liposomes enhanced the therapeutic activity by 90-fold and killed 99% of the intracellular bacteria compared with nonencapsulated ampicillin which had no antibacterial effect. The authors attributed this dramatic effect to an increased delivery to sites of infection (Bakker-Woudenberg et al., 1986) in the liver and spleen where MP are concentrated (New et al., 1978). *Brucella* species can infect most mammals and as few as ten bacteria may result in long-term chronic disease. Two injections of liposomes containing ten milligrams of either dihydrostreptomycin, gentamicin, kanamycin, or streptomycin/kg of body weight at seven and ten days after infection successfully eliminated brucellosis in mice (Fountain et al., 1985). The double dosage was deemed necessary because a single injection of streptomycin liposomes reduced infection by approximately 100-fold but did not kill all bacteria. In comparison, an equivalent amount of free drug reduced bacterial numbers by only one log (Fountain et al., 1985). Killing of other intracellular bacteria as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium* has been enhanced by the encapsulation of chemotherapeutants in liposomes (Bonventre and Gregoriadis, 1978; Desiderio and Campbell, 1983; Fountain et al., 1981; Stevenson et al., 1983).

The increased efficacy of liposome-encapsulated chemotherapeutant reduces the amount of drug needed to eliminate an infection as aptly demonstrated in the treatment of

leishmaniasis. This disease is treated with antimony, a drug which is very effective against *Leishmania*, an obligate intracellular protozoan parasite of MP, but also highly toxic at the dose and prolonged admininistration required for cure. Mice injected with amastigotes of *L. donovani* (visceral leishmaniasis) and treated with one or three injections of 20 mg/kg of free antimony at 10 days post infection remained infected, with no parasite reduction as determined by light microscopic examination of 200 liver cell nuclei in an impression smear. In contrast, an equivalent amount of antimony entrapped in liposomes completely eliminated the infection. The antimony liposomes had a half life of about one hour in the plasma and were found in the liver at this time, but the free drug was not detectable 15 minutes after injection (New et al., 1978). Alving et al. (1978) had similar success in treating leishmaniasis infections in hamsters. Multilamellar liposomes containing antimony were 330 to 640 times more effective in reducing mortality than was the free drug. A dose of 2.5 mg of liposome-encapsulated antimony/kg virtually eliminated mortality in the fourth and fifth weeks after infection. In comparison, 832 mg/kg of unencapsulated antimony was required to achieve the same effect. They suggested that as disease progressed, liposome-encapsulated drug would be the more effective treatment, cautioning that beyond a certain point, extensive tissue destruction and secondary infections limit efficacy and the animals die despite elimination of the parasites. Treatment with

antimony liposomes provides another advantage: prevention of resistant parasites that develop with treatment failures after an unsuccessful balancing of toxic versus therapeutic effects.

Often the efficacy of a drug in treating an intracellular infection is related to its proficiency in penetrating the membranes of phagocytes. Encapsulation of such a therapeutant into liposomes appears to enhance its bactericidal effect. Aminoglycosides, such as rifapentine and amikacin, are known to concentrate intracellularly and as much as 50 to 70% of the serum concentration may penetrate neutrophils. Human macrophages infected in vitro with the *Mycobacterium avium* complex (organisms frequently responsible for death in patients with HIV) were given liposomes containing 20 mg of amikacin at 24 hours or at 24 and 72 hours after infection. The liposome treatments were associated with 92 to 99% killing of the bacteria within 24 hours of treatment, a significant improvement over that obtained by amikacin alone. Unencapsulated rifapentine killed 65% of the intracellular bacteria but bacterial growth resumed within 48 hours after antibiotic addition (Bermudez et al., 1987).

The composition of liposomes has a significant effect on its target cells, the MP, and can inhibit microbicidal activity or internalization of microorganisms. Some natural phospholipids (specifically, the phospholipid head groups of phophatidylserine, phosphatidylethanolamine, phosphatidic acid or diphosphatidyl glycerol) prevented the induction of MP microbicidal activity by

interferon-γ against *Leishmania major* in an in vitro study using resident peritoneal MP from mice (Gilbreath et al., 1989). The degree of inhibition for the 'bad' phospholipids was directly influenced by the number of unsaturated bonds in the phospholipid fatty acids attached to the phospholipid head group. With phosphatidylethanolamine as the head group, fatty acid chains with three unsaturated bonds (di-18:3) greatly inhibited the ability of MP to kill *L. major* in comparison with fatty acid chains having one unsaturated bond (di-18:1). The authors hypothesized that some phospholipids interfere with the induction of activation (MP-interferon interaction) by presenting optimal substrates for phospholipase A2 activity, thus inhibiting lymphokine activity. Other potential components used in the formulation of liposomes can be blatantly toxic: 60 μM of stearylamine in liposomes killed 50% of a murine macrophage cell line (Kronberg et al., 1990). Cholesterol in liposome composition is used to prevent leakage of internal solutes in the presence of serum by decreasing the mobility of the lipid chains (Kronberg et al., 1990) but it, too, may affect MP. Mukherjee et al. (1988) demonstrated that the microviscosity of the murine macrophage membrane was altered by addition of liposomes containing cholesterol:phospholipid and attributed this to the cholesterol content. This, in turn, had a significant effect on endocytosis of *L. donovani*. Alving and Swartz (1984) found in treating leishmaniasis that liposome compositions of dipalmitoyl phosphatidylcholine (highly saturated long chain phospholipid)

and cholesterol were most efficacious and liposomes containing phosphatidylserine were the least effective. As a cautionary note, when liposomes containing only saline were injected, there was a greater mortality due to leishmaniasis than in those animals injected with saline alone indicating that liposome therapy might aggravate certain pathologic conditions.

The liposome composition also can affect the rate of intracellular killing. In one study, mouse peritoneal MP were infected with *L. monocytogenes* and the effects of ampicillin in two different liposome compositions were tested (Bakker-Woudenberg et al., 1988). Fluid liposomes of cholesterol, phosphatidylcholine, and phosphatidylserine were more susceptible to intracellular degradation, and released most of their ampicillin within two hours, resulting in 90% and 97% killing (25 and 50 mg ampicillin, respectively) of *Listeria monocytogenes* by six hours. Solid liposomes composed of cholesterol, distearoylphosphatidylcholine and dipalmitoylphosphatidylglycerol remained intact inside MP a longer time and by two hours were only 25% degraded. Although this liposome type reduced the multiplication of intracellular bacteria compared with the controls, there was no bacterial killing with 25 mg and only 70% killing with 50 mg of ampicillin. Free ampicillin (50 mg) plus empty liposomes of both types did not kill the bacteria. In another study, liposomes of phosphatidylcholine were avidly phagocytosed by mouse peritoneal MP in vitro but were not completely degraded after

two hours even though electron microscopy revealed apparent ongoing degradation (Mattenberger-Kreber et al., 1976). With this knowledge, a controlled release of antibiotic might be achieved by mixing two or more liposome compositions for both immediate and long-term killing of intracellular organisms, a factor of significance for microorganisms that replicate slowly.

Liposome use in fish has potential for delivery of chemotherapeutants and vaccines via immersion, injection or oral routes. In fish, MP are found primarily in the spleen, anterior kidney, and to a lesser extent in the posterior kidney. Power et al. (1990) compared the uptake and distribution of two preparations of liposomes in rainbow trout. Liposomes of phophatidylcholine and ^{3}H-cholesterol in the form of LUV (250 nm) and MLV (1-5 μm) were injected intraperitoneally into fish and 24 hours later, tissues removed and examined. Uptake of liposomes was greatest by the spleen, anterior and posterior kidney, with lesser amounts reported in the liver>gill>heart>fat>muscle. The uptake of LUV was significantly greater than that of MLV, particularly in the spleen and anterior kidney, indicating that the smaller liposomes may pass more readily into circulation from the peritoneal cavity. The larger MLV may remain in the peritoneum longer with phagocytosis by resident macrophages likely.

Liposomes have already been tested as therapeutic delivery devices in rainbow trout. Rodgers (1990) prepared a vaccine against *Aeromonas salmonicida*, the causative agent of

furunculosis, and compared the efficacy of liposome-encapsulated vaccine (approximately 30% of vaccine components contained in phosphatidylcholine liposomes) with non-encapsulated vaccine in all-female trout fry at a field site which annually experienced furunculosis. Both vaccines, with and without liposomes, reduced total mortality from 37 to 15% or less; however, the liposome formulation was significantly better than that without liposomes, and produced larger fish.

The results from another study on liposomes in rainbow trout were disappointing. Hypothesizing that lipid would augment the clinical benefits of antibiotic by acting as an adjuvant, Austin (1985) fed phosphatidylcholine liposomes containing clindamycin at a rate of 100 mg/kg/day for ten days to rainbow trout infected with *R. salmoninarum*. Although unencapsulated clindamycin alone reduced mortality from 100 to 28%, the liposome-encapsulated clindamycin provided no protection. The use of Tweens (Sigma Chem. Co.), which are compositions of saturated fatty acids, in combination with clindamycin resulted in equally poor survival. The lack of protection in combination with the apparent debilitating effect of orally fed lipids on disease progression caused the author to suggest that fish should be fed low fat diets although details on the total lipid amounts fed to fish were not mentioned.

Despite the negative results from Austin's study, treatment of BKD with liposome-encapsulated antibiotic is warranted, given the propensity of *R. salmoninarum* to survive within MP. If

entrapped within liposomes, erythromycin may be more efficacious, as is another intracellular penetrant, amikacin, against the slow growing bacterium. In addition, the use of liposome-encapsulated antibiotic may prevent development of antibiotic-resistant forms of *R. salmoninarum* (Bell et al., 1988) and reduce the amount, and hence, toxicity and cost of antibiotic given. Furthermore, direct targeting of drug into MP may reduce the number of fish with low level infections.

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Chapter 3

A phylogenetic comparison of the 16S rRNA sequence of the fish pathogen, *Renibacterium salmoninarum*, to gram-positive bacteria

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Abstract

The 16S rRNA of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonids, was sequenced by reverse transcriptase to produce a nearly complete sequence (97%) of 1475 nucleotides. Phylogenetic comparisons to seventeen genera and signature sequence analysis indicated that *R. salmoninarum* was a member of the high G+C gram-positive eubacterial subdivision although the reported G+C value is only 53%. A phylogenetic tree details the relationship of *R. salmoninarum* to ten actinomycetes from diverse environments.

Introduction

Renibacterium salmoninarum is the cause of a chronic, debilitating bacterial disease in salmon, trout, and char. An obligate pathogen of fish, *R. salmoninarum* has a unique cell wall that can tolerate high levels of lysozyme and which may permit survival and possible replication within the macrophage (Fryer and Sanders, 1981). Although the bacterium has been isolated from salmonids world-wide, only one species of *Renibacterium* is known.

Based on phenotypic characteristics, the classification and phylogeny of *Renibacterium* have been problematic. The bacterium was originally believed to belong to the genus *Corynebacterium*; however, analyses of the cell wall and fatty

acids indicated a unique genus (Sanders and Fryer, 1980; Collins, 1982; Kusser and Fiedler, 1983). Although the physiological and chemical properties of *R. salmoninarum* resemble the arthrobacters (Jones and Collins, 1986), its guanine plus cytosine (G+C) content (53%) is low relative to these organisms (59-70%). A numerical phenetic analysis based on 86 physiological attributes demonstrated that the strains of *Renibacterium* formed a distinct cluster, separate from 12 other bacteria which included species of *Arthrobacter*, *Micrococcus*, *Jonesia* (*Listeria denitrificans*) and *Actinomyces* (Goodfellow et al., 1985). A study comparing the 16S rRNA catalogs of over 165 gram-positive organisms revealed that *R. salmoninarum* is a member of the *Arthrobacter-Micrococcus* subline of the actinomycetes (Stackebrandt et al., 1988b). The present study, which is based on the nearly complete sequence of 16S rRNA rather than oligonucleotide fragments from T_1 digestion, confirms the relationship of *R. salmoninarum* to the high G+C bacteria in the actinomycetes subdivision.

Materials and Methods

Renibacterium salmoninarum (type strain ATCC 33209) was grown to log phase (0.52 OD at 520 nm, 2 g wet weight) in two, one liter batches of kidney disease medium with charcoal. The bacterial cells were washed twice with distilled water and resuspended in 5 ml of buffer. After the addition of 1 mg of lysozyme, the solution was passed through a French pressure cell

at 20,000 psi. To complete cell lysis, 0.5 ml of 20% SDS and 1 mg of proteinase K were added and incubated at 50°C for 5 min. The nucleic acids were purified as described by Lane et al. (1988). Large quantities of protein in the preparation necessitated ten extractions with phenol and chloroform to obtain a clean interface.

After ethanol precipitation, the nucleic acid extract was dissolved in buffer, mixed with cesium trifluoroacetate to a density of 1.65 g/ml and centrifuged (300,000 x *g* in a vertical tube rotor) to obtain the RNA fraction. The resulting RNA band was re-extracted once each with phenol and chloroform to remove residual protein, dissolved in buffer and quantified by spectrophotometric absorbance at 260 and 280 nm. An unknown component(s) extracted concurrently with the RNA produced high absorbance values at 260 nm; hence, the presence and quantity of rRNA was verified by electrophoresis of the extract on a 3% acrylamide gel.

The rRNA was sequenced using the reverse transcriptase/dideoxy-nucleotide chain terminated method Lane et al., 1988) and nucleotidyl transferase (DeBorde et al., 1986). Seven oligonucleotide primers, each specific to a different conserved region of eubacterial 16S rRNA, were used to construct the nucleotide sequence of 1475 bases, which represents 97% of the complete 16S rRNA. Long stretches of manually aligned sequence (111-182, 221-453, 480-838, 849-1005, 1042-1133, and 1139-1449, based on *Escherichia coli* numeration (Brosius et al., 1978) were compared with the sequences of 17 genera (Table

3.1). Phylogenetic trees were calculated by a distance matrix method, omitting regions of ambiguous sequence alignment (Fitch and Margoliash, 1967; Jukes and Cantor, 1969; Olsen, 1988).

Results and Discussion

The sequence of *R. salmoninarum* (Fig. 3.1; EMBL gene bank, No. X51601) was compared to the sequences of 17 bacteria (Table 3.1) from three gram-positive subdivisions. From the two best characterized subdivisions, the high G+C (> 55%) and the low G+C (< 50%), ten and six bacteria, respectively, were selected. The third subdivision (bacteria lacking gram-positive staining walls), was represented by *Heliobacterium chlorum* (Woese, 1987). The comparison was limited to representative bacteria with published 16S rRNA sequences that are nearly complete.

The comparison of a 1224 nucleotide sequence confirmed that *R. salmoninarum* is most similar to the high G+C subdivision (Table 3.1). The similarity of *R. salmoninarum* to the high G+C group averaged 0.91 $\pm$ 0.02, whereas its similarity to the low G+C was only 0.81 $\pm$ 0.01. The relationship of *R. salmoninarum* to the high G+C gram-positive bacteria was also confirmed by the signature sequences (positions 168, 906, 955, 998, 1116, 1167, and 1410) which distinguish the gram-positive subdivisions from each other (Woese, 1987). In addition, *R. salmoninarum* shared other changes with the high G+C bacteria that were not seen in the low G+C bacteria (Table 3.2). We also noted five unique changes at

Table 3.1. List of the gram-positive bacteria compared to *Renibacterium salmoninarum* for analysis by 16S rRNA sequencing.

Organism (Strain[a])	Reference	mol%G+C[b]	Similarity[c]
High G+C			
Terrabacter tumescens (NCIB 8914) (*Pimelobacter*)	Collins et al., 1989b	69-73	0.937
Corynebacterium variabilis (NCIB 9455^T)	Collins et al., 1989c	60-67	0.909
Streptomyces coelicolor A3	Baylis and Bibb, 1987	59	0.907
Rhodococcus erythropolis (DSM 43188)	Stackebrandt et al., 1988a	67-71	0.906
Tsukamurella paurometabolum (DSM 20162^T) (*Corynebacterium*)	Collins et al., 1988	67-68	0.905
Mycobacterium bovis BCG	Suzuki et al., 1988	62-70	0.900
Nocardiodes luteus (NCIB 11455)	Collins et al., 1989b	68	0.896
Saccharomonospora viridis (ATCC 15386)	Embley et al., 1988	74-75	0.892
Actinomyces viscosus (DSM 43027)	Stackebrandt and Charfreitag, 1990	59-70	0.891
Nocardia asteroides (DSM 43005)	Stackebrandt et al., 1989a	63-70	0.891
Low G+C			
Bacillus subtilis	Green et al., 1985	43	0.822
Vagococcus fluvialis (NCDO 2497)	Collins et al., 1989a	34	0.803
Lactococcus raffinolactis (NCDO 617^T)	Collins et al., 1989a	40-42	0.798
Clostridium innocuum (ATCC 14501)	Weisberg et al., 1989	43-44	0.794
Mycoplasma capricolum	Iwami et al., 1984	24	0.789
Acholeplasma laidlawii (JA1)	Weisberg et al., 1989	31-36	0.784
Other			
Heliobacterium chlorum	Woese et al., 1985	52	0.817

[a] Superscript T denotes type strain.

[b] %G+C for the species.

[c] Values derived from comparison of 16S rRNA sequences (1224 nucleotides) to *R. salmoninarum*. Sequence regions of ambiguous alignment were omitted from the analysis.

Fig. 3.1. The 16S rRNA sequence for *Renibacterium salmoninarum* as determined by reverse transcriptase sequencing. The first and last nucleotides in the sequence are analogous to positions 1 and 1475 of the *Escherichia coli* 16S rRNA sequence (Brosius et al., 1986). N is an undetermined nucleotide and the letters W, H, Y, D, and S follow IUCC convention.

UUUUACGGAG	AGUUU-AUUC	UGGCUCAGGA	UGAACGCUGG	CGGCGUGCUU	AACACAUGCA	60
AGUCGAACGA	UGAAGCGGUC	GUUGCGCCGU	GGAUUAGUGG	CGAACGGGUG	AGUAAUACGU	120
GAGUAACCUG	CCCUUGACUU	CGGGAUAAGC	CUGGGAAACU	GGGUCUAAUA	CUGGAUACGA	180
CCUAUCACCG	CAUGGUGUGU	AGGUGGAAAG	UUUUUGCGGU	UUUGGAUGGA	CUCGCGGCCU	240
AUCAGCUUGU	UGGUGAGGUA	AUAGCUAACC	AAGGCGACGA	CGGGUAGCCG	GCCUGAGAGG	300
GUGACCGGCC	ACACUGGGAC	UGAGACACGG	CCCAGACUCC	UACGGGAGGC	AGCAGUGGGG	360
AAUAUUGCAC	AAUGGGCGAA	AGCCUGAUGC	AGCGACGCCG	CGUGAGGGAU	GACGGCCUUC	420
GGGUUGUAAA	CCUCUUUCAG	UAGGGAACAA	GACAUCAUUU	UUGUGGUGUU	GAGGGU-CUU	480
GCAGAAGAAG	CACCGGCUAA	CUACGUGCCA	GCAGCGGCGG	UAAUGCGUAG	GGUGCAAGCG	540
UUAU-CGGAA	UuAUUGGGCG	UAA-GAGCUC	GUAGGCGGUU	UGUCGCGUCU	GCCGUGAAAG	600
UCcGGGGCUC	aACUCCGGAU	CUGCGGUGGG	UACGGGCAGA	CUAGAGUGAU	GUAGGGGAGA	660
CUGGAAUUCC	UGGUGUAGCG	GUGaAAUGCG	CAGAUAUCAG	GAGGAACACC	GAUGGCGAAG	720
GCAGGUCUCU	GGGCAUUAAC	UGACGCUGAG	GAGCGAAAGC	AUGGGGAGCG	AACAGGAUUA	780
GAUACCCUGG	UAGUCCAUGC	CGUAAACGUU	GGGCACUAGG	UGUGGGGGAC	AUUCCACGUU	840
CUCCGCGCCG	UAGCUAACGC	AUUAAGUGCC	CCGCCUGGGG	AGUACGGCCG	CAAGGCUHAA	900
ACUCAAAGNA	AUUGACGGGG	G-CCGCACAA	GCGGCGGAGC	AUGCGGAUUA	AUUCGAUgCA	960
ACGCGAAGAA	CCUUACCAAG	GCUUGACAUG	GAUUAGAAAA	GUGCAGAAAU	GUACUCCCCM	1020
UUUUGGGUUG	GUUCACAGGU	GGHGCAUGGU	UGUCGUCAGC	UCGUGUCGUG	AGAUGUUGGG	1080
UUAAGUCCCG	CAACGAGCGC	AACCCUSGUU	CUAUGUUGCC	AGCCGUUAUG	GUGGGGACUC	1140
AUAGGAGACU	GCCGGGGUNA	ACUCGGAGGA	AGGUGGGGAU	GACGUCAAAU	CAUCAUGCCC	1200
CUUAUGUCUU	GGGCUUCACG	CAUGCUACAA	UGGCCGGUAC	AAAGGGUUGC	GAUACUGUGA	1260
GGUGGAGCUA	AUCCCAAAAA	GCCNGUCGUA	GUUCGGAUUG	GGGUCUGCAA	CUCGACCCCA	1320
UGAAGUCGGA	AUCGCUAGUA	AUCGCAGAUC	AGYADCGCUG	CGGUGAAUAC	GUUCCCGGGC	1380
CUUGUACACA	CCGCCCGUCA	AGUCACGAAA	GUUGGUAACA	CCCGAAGH-G	GUGGCCUAAC	1440
CCWUUUUGGG	AWGGGAACGU	cGAuGGUNGG				1470

Figure 3.1

Table 3.2. Changes in the consensus nucleotides of the 16S rRNA of *Renibacterium salmoninarum* and the high G+C bacteria are shown in comparison to the low G+C bacteria and *Heliobacterium chlorum*. Some unusual pairings (as G:G at 595:644) are justified by secondary structure.

Position	Change[a]	Bacteria[b]											
		Rs	Tt	Nl	Av	Cv	Re	Mb	Na	Tp	Sv	Sc	low
Unpaired	**nucleotides:**												
153	C*--> G	+	-	-	-	+	+	+	+	+	+	+	-
450	G*--> C	+	-	-	-	-	-	-	-	-	-	-	-
483	C*--> G	+	-	-	-	-	-	-	-	-	-	-	-
508	U*--> C	-	-	+	-	-	-	+	-	+	-	-	-
620	C*--> U	-	-	+	+	+	-	+	-	+	G	+	-[1]
701	U*--> C	+	+	+	+	+	+	+	+	+	+	+	-
819	A*--> U	+	+	+	+	G	G	G	G	G	G	G	-
1167	A*--> U	+	+	+	+	+	+	+	+	+	+	+	-
Paired	**nucleotides:**												
444:	G*--> A	+	+	+	+	C	+	+	+	+	C	+	+[2]
490	U	+	+	+	+	G	+	+	+	+	G	+	+[2]
445:	G	+	+	C	+	A	+	U	+	+	+	+	+
489	C*--> U	+	-	G	-	+	-	G	-	-	-	-	-
595:	A*--> G	+	+	+	+	+	+	+	+	-	+	-	-
644	U*--> G	+	+	+	+	+	+	+	+	+	+	+	-
601:	G*--> U	-	-	-	C	+	+	+	A	+	-	-	-
637	U	+	+	+	+	G	G	G	+	G	+	+	+
822:	U*--> G	+	+	+	+	+	+	+	+	+	+	+	A
878	C	+	+	+	+	+	+	+	+	+	+	+	U
950:	U*	+	+	+	+	+	+	+	+	+	+	+	+
1231	G*--> A	+	+	+	+	+	+	+	+	+	+	-	-
952:	U*--> C	+	+	+	+	-	-	-	-	-	-	-	-
1229	A*--> G	+	+	+	+	-	-	-	-	-	-	-	-
955:	U*--> A	+	+	+	+	+	+	+	+	+	+	C	-
1225	A*--> U	+	+	+	+	+	+	+	+	+	G	g	-
1003:	G*--> A	+	-	-	-	-	-	-	-	-	-	-	-[3]
1037	C*--> U	+	-	-	-	-	-	-	-	-	-	-	-[3]
1440:	U	+	+	+	+	+	+	+	+	+	+	+	+
1461	G*--> A	+	-	-	-	-	-	-	-	-	-	-	-

[a] Symbols: (*) = conserved nucleotide; (+) = change to indicated base; (-) = no change from conserved base.

[b] Rs = *R. salmoninarum*; Tt = *T. tumescens*; Nl = *N. luteus*; Av = *A. viscosus*; Cv = *C. variabilis*; Re = *R. erythropolis*; Mb = *M. bovis*; Na = *N. asteroides*; Tp = *T. paurometabolum*; Sv = *S. viridis*; Sc = *S. coelicolor*; low = all low G+C from Table 3.1 and *H. chlorum*.

[1] *A. laidlawii* has an U; [2] excepting *H. chlorum* and *A. laidlawii*; [3] *C. innocuum* has an A:G pair.

conserved positions in the sequence of *R. salmoninarum* (positions 450, 483, 1003, 1037 and 1461) not seen in the other gram positive bacteria (Table 3.2).

The alliance of *R. salmoninarum* with the high G+C bacteria, despite its low G+C value of 53% (Sanders and Fryer, 1980), is a taxonomic problem noted by Stackebrandt et al. (1988b). C.R. Banner (Department of Microbiology, Oregon State University, Corvallis, OR, USA), has completed studies which indicate the mean G+C content of seven strains (including the type strain ATCC 33209) of *R. salmoninarum* is 55.5 $\pm$ 0.43%. These values would place *R. salmoninarum* in the high G+C group.

Phylogenetic analysis revealed the position of *R. salmoninarum* in the high G+C group (Fig. 3.2). *Terrabacter tumescens*, with a similarity of 0.937, branched most closely to *R. salmoninarum*. *Streptomyces coelicolor* (0.907) and *Nocardiodes luteus* (0.896) also appeared in the same cluster. The other high G+C bacteria with similarities of 0.897 to 0.909 formed another branch.

In this study, *R. salmoninarum* is most closely related to *T. tumescens*, although the two share few important chemotaxonomic characteristics (cell wall, lipid, and menaquinone contents) currently used to classify the gram-positive bacteria. Some *Arthrobacter* and *Micrococcus* species share important features with *R. salmoninarum*, including an A3α peptidoglycan, lysine as the diamino acid in the interpeptide bridge, and the lack of mycolic acids (Jones and Collins, 1986; Schleifer, 1986). These

Fig. 3.2 Unrooted phylogenetic tree depicting the relationship of *Renibacterium salmoninarum* (ATCC 33209) to closely related high G+C gram-positive bacteria. A total of 1224 positions in the 16S sequence of the rRNA were used for analysis and construction of the tree by a distance matrix method (Fitch and Margoliash, 1967; Jukes and Cantor, 1969; Olsen, 1988). *Escherichia coli* and *Pseudomonas testosteroni* served as outgroups.

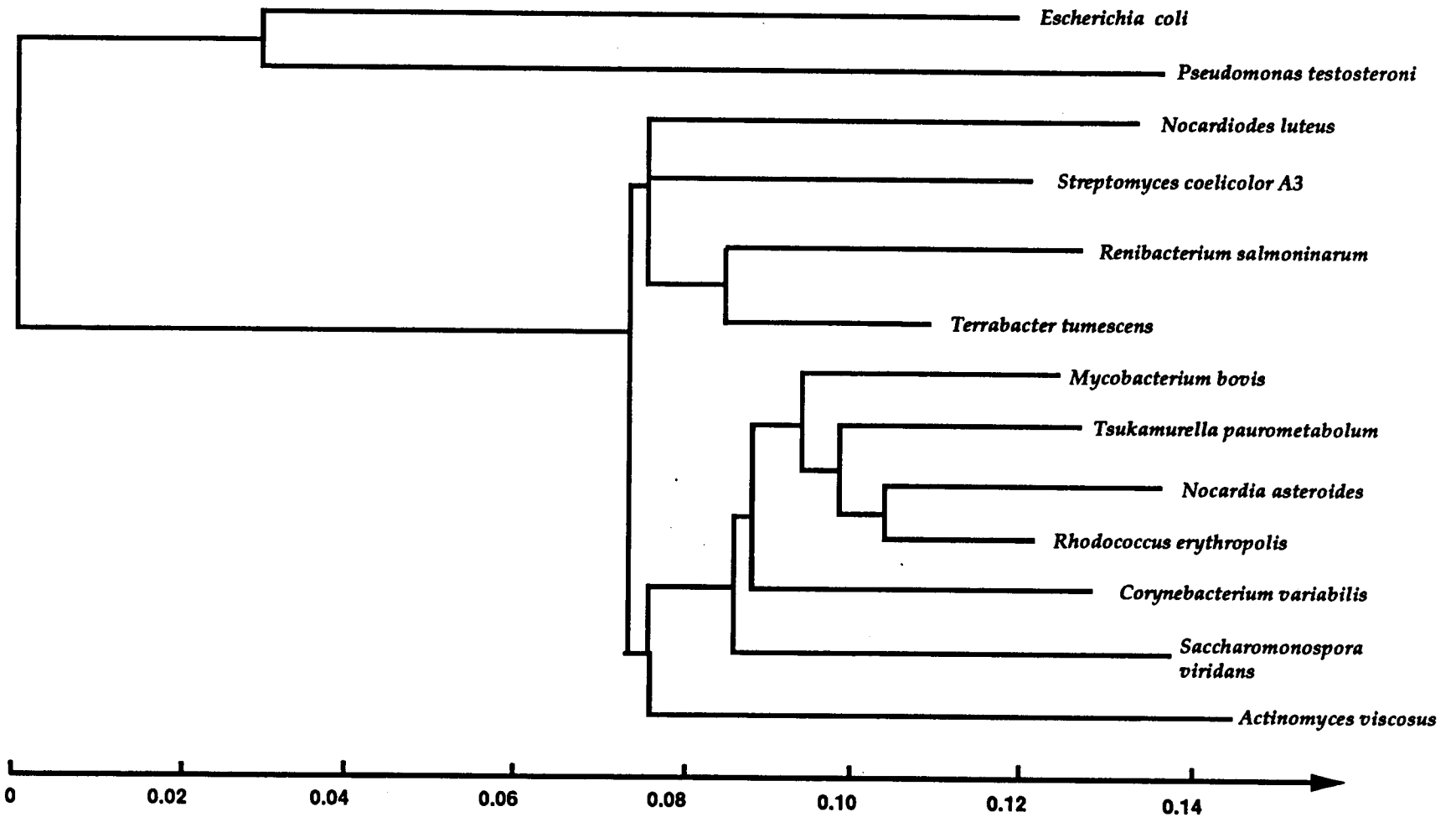
Escherichia coli
Pseudomonas testosteroni
Nocardiodes luteus
Streptomyces coelicolor A3
Renibacterium salmoninarum
Terrabacter tumescens
Mycobacterium bovis
Tsukamurella paurometabolum
Nocardia asteroides
Rhodococcus erythropolis
Corynebacterium variabilis
Saccharomonospora viridans
Actinomyces viscosus
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Fixed point mutations per sequence position

Figure 3.2

characteristics correlate with the close genetic relationship between these genera and *R. salmoninarum* as evidenced by oligonucleotide cataloging (Stackebrandt et al., 1988b). Moreover, both *R. salmoninarum* and *Arthrobacter globiformis* contain a rare amino sugar, N-acetyl-fucosamine, in their cell wall polysaccharide (Kusser and Fiedler, 1983; Vinogradov et al., 1988) and have a similarity comparable to more than 95% (Stackebrandt et al., 1988b; Woese, 1987).

Renibacterium salmoninarum, as an obligate pathogen of salmonids, is unique within this group. The *Arthrobacter* and *Micrococcus* genera contain species primarily found in soil and water (Jones and Collins, 1986; Schleifer, 1986). *Renibacterium salmoninarum* also differs from its relatives in being fastidious, with a requirement for cysteine. The bacterium grows slowly, even at an optimum temperature of 15°C in vitro (range 5-20°C). In salmon, it causes mortality (78-100%) at 6.5-12°C (Fryer and Sanders, 1981).

The relationship of *R. salmoninarum* to the genera of *Arthrobacter* and *Micrococcus* exemplifies the problem of correlating phenotypic and genotypic characteristics for taxonomic classification. *Renibacterium salmoninarum* has evolved into a very effective parasite which is singularly different from its closest relatives. Despite similarities in rRNA and some chemotaxonomic characteristics to *Arthrobacter*, the unique phenotypic characteristics in *R. salmoninarum* warrant its classification as a separate genus.

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Chapter 4

Intracellular Survival of *Renibacterium salmoninarum* in Trout Mononuclear Phagocytes

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Abstract

In vitro infection of anterior kidney cells from rainbow trout (*Oncorhynchus mykiss*) revealed that *Renibacterium salmoninarum* survived and replicated slowly within the mononuclear phagocytes (MP). Intracellular survival of the bacterium, based on quantitative analysis of transmission electron micrographs, partially depended on its ability to disrupt the enclosing phagosomal membrane, permitting bacterial entry into the cytoplasm. At 4.5 h, 67% of the bacterial cells, both intact and damaged, were found in the cytoplasm. A factor associated with the bacterial cell wall is believed responsible for the localized dissolution of the phagosomal membrane as formalin-killed *R. salmoninarum* also escaped into the cytoplasm, albeit at a slower rate and after sustaining greater cell wall damage. The bacterium also embedded in and penetrated the plasma membrane of lymphocytes, an event attributed to the factor causing phagosome disruption. The durability of its cell wall further enhanced survival within MP and significant bacterial losses occurred only after 96 h as the MP died, exposing bacteria to antibiotics in the media. Despite the toxic effects of live *R. salmoninarum*, the MP were able to destroy limited numbers of the bacterium and this may account for the difficulty in distinguishing bacterial replication despite a modest increase at 48 h. However, dividing bacteria were noted through 96 h in the micrographs.

Introduction

Bacterial pathogens in the genera *Mycobacterium*, *Nocardia*, *Listeria*, *Campylobacter*, *Salmonella*, *Shigella*, and *Legionella* survive within macrophages and cause disease in many species including humans, rodents, sheep, and birds (Buchmeier and Heffron, 1989; Davis-Scibienski and Beaman, 1980; Farber and Peterkin, 1991; Frehel et al., 1986; Kiehlbauch et al, 1985). In vivo observations of *Renibacterium salmoninarum*, the coccobacillus responsible for bacterial kidney disease (BKD) in salmonid fish (Sanders and Fryer, 1980), have indicated that it might survive and replicate within macrophages. Like the intracellular pathogens *Mycobacterium bovis* and *Nocardia asteroides*, *R. salmoninarum* is a fastidious gram-positive actinomycete (Gutenberger et al., 1991) that causes a chronic disease not easily controlled with antibiotics (Fryer and Sanders, 1981). The late stages of BKD are characterized by a systemic infection with granulomatous lesions occurring in the kidney, liver, spleen and other organs. Histologically, the disease resembles human glomerulonephritis (Young and Chapman, 1978; Bruno, 1986). Death can result within several months after infection, often facilitated by changes in environmental conditions (Fryer and Sanders, 1981). Death is also hypothesized to occur years later when a latent infection is triggered by secondary infections or environmental stressors (Austin and Austin, 1987).

Some attributes of the fastidious bacterium, including its resistance to lysozyme, slow rate of replication (>18 h), and prodigious production of a soluble hemolysin/agglutinin, suggest that it could live within the macrophage (Bruno and Munro, 1986; Daly and Stevenson, 1990; Fryer and Sanders, 1981). This would help explain the difficulty in eliminating the organism from salmonid populations using antibiotics and the inability, to date, to develop an effective vaccine.

Most intracellular bacteria survive by one of several methods. Some remain within the phagosome and either prevent fusion with lysosomes or resist the enzymes of the phagolysosome (Buchmeier and Heffron, 1991; Davis-Scibienski and Beaman, 1980; Frehel et al., 1986; Kiehlbauch et al, 1985; Sibley et al., 1987). Other intracellular pathogens escape into the cytoplasm to avoid exposure to phagosome-lysosome fusion (Clerc et al., 1987; Farber and Peterkin, 1991; Myrvik et al., 1988). To examine the mechanisms of survival of *R. salmoninarum* and the concomitant reactions of the immune cells, we infected kidney cells from rainbow trout with the bacterium. In fish, the anterior portion of the kidney is the hematopoietic equivalent to bone marrow in mammals and is rich in lymphocytes, macrophages, neutrophils, and other cells important in the immune system. Examination of micrographs from transmission electron microscopy permitted quantification and qualitative scrutiny of the reactions and response of live *R. salmoninarum* within the mononuclear phagocytes (MP) over a period of ten days. As controls, kidney

cells were infected with formalin-killed *R. salmoninarum* or the nonpathogenic *Arthrobacter globiformis* (a related gram-positive bacterium). In addition to electron microscopic analysis, the number of viable intra- and extra-cellular bacteria were determined by assaying colony-forming units. Viable kidney cells were enumerated by trypan blue dye exclusion.

Materials and Methods

Bacteria. The virulent D-6 strain of *R. salmoninarum*, originally isolated from coho salmon in salt water (Craig Banner, Dept. Microbiology, Oregon State University, Corvallis, Ore.) was grown in kidney disease broth with charcoal (Daly and Stevenson, 1985) at 15°C on a shaker until log phase growth was observed (8 days). Killed *R. salmoninarum* were prepared by the addition of formalin (0.3% of total volume) after sampling for counts by the colony forming units (CFU) assay. The formalin-killed bacteria were centrifuged four times with intermediate 3 min vortexings to disperse the pellet. This treatment removed formalin and much of the external cell-associated protein. Prior to infection, the formalin-killed and live *R. salmoninarum* were pelleted by centrifugation and resuspended in 10% L-15 to similar optical densities at 520 nm. The number of live *R. salmoninarum* was determined to be 1.16 x 10^8 CFU/ml by counts on kidney disease medium 2 (KDM-2) (Evelyn, 1977). Numbers of formalin-killed *R.*

salmoninarum were estimated as 1.62 x 10^8 CFU/ml based on counts done prior to formalin treatment.

Arthrobacter globiformis (DSM 20124), a close relative of *R. salmoninarum*, served as a nonpathogenic gram-positive control and was grown for 24 h at 26°C in typtic soy broth (4 g/l) supplemented with yeast extract (2 g/l). The culture was prepared as described for live *R. salmoninarum* to yield an infecting dose of 4.4 x 10^8 CFU/ml.

Collection and cultivation of trout kidney cells. Modified L-15 tissue culture medium with L-glutamine (Flow Laboratories, Inc., McLean, Vir.) was supplemented with glucose (3.95 g/500 ml), 10% heat-inactivated fetal bovine serum, fungisone (5 mg/ml) and buffered with 1 M tris HCl to pH 7.4 (10% L-15; Braun-Nesje et al., 1981).

Eight rainbow trout (*Oncorhynchus mykiss*, Shasta strain), weighing 320-658 g, were euthanized with an overdose of benzocaine and bled from the caudal vein by syringe before aseptically removing the anterior kidneys. All cell preparation was performed in polypropylene tubes at 4°C to prevent loss of adherent cells. The kidney tissue, 13.7 g, was manually homogenized to single cell suspension in 10% L-15 (supplemented with heparin, 10,000 U; penicillin-streptomycin, 500 I.U./ml and 500 mg/ml; and gentamycin, 250 mg/ml; pH 7.4). To provide macrophage variety, four of the eight fish were injected intraperitoneally with 1 ml Freund's incomplete adjuvant emulsified with the extracellular protein of *R. salmoninarum*

(supernatant from broth culture, 1.0 O.D. at 520 nm, lyophilized to dryness and dialysed three times against phosphate buffered saline; injection equivalent to 0.625 ml bacterial culture), three days prior to tissue collection (Mbawuike et al., 1986). Cells collected from peritoneal lavage (primarily from two responding animals) were added to the kidney cell preparation and accounted for 4.5% of the total cell preparation (except where specifically stated, cell counts do not include erythrocytes). Large clumps were removed by centrifugation, approximately 20 x *g*, 1 min, and the supernatant pelleted and washed two times (200 x *g* for 20 min) with 10% L-15 plus antibiotics. Cells were maintained in suspension, rather than in monolayer cultures, to maximize handling ease and counting accuracy. Suspension cultures also permitted retention of important immune cells, i.e. lymphocytes. The cell preparation was diluted to 7.7 x 10^7 cells/ml and kept in polypropylene tubes on a tissue culture rotator (Scientific Industries, Bohemia, NY) overnight at 15°C .

Infection of kidney cells. Prior to infection, cells were washed three times (200 x *g* for 20 min, at 4°C) with 10% L-15 to remove the antibiotic-containing medium, diluted to 2.6 x 10^7 cells/ml, and aliquoted into four tubes. The cells were pelleted and the supernatant replaced with an equivalent volume of bacteria (live or formalin-killed *R. salmoninarum*, or *A. globiformis*) in 10% L-15 or in 10% L-15 alone (control). At this time, designated as 0 h (29 h post-collection of cells), there were 4 to 17 bacteria per cell. Cells were then rotated for 2 h at 15°C.

After incubation, the tubes were centrifuged at 100 x *g*, 5 min, to separate the cells from noningested, extracellular bacteria. This centrifugation step was repeated three times with 10% L-15 containing penicillin G (165 I.U./ml) and gentamycin (250 mg/ml). These antibiotics do not penetrate phagocytic cells (Fountain et al., 1985; Sanchez et al., 1986) but do prevent the extracellular growth of *R. salmoninarum*. The cells from each treatment were aliquoted in 1 ml amounts into 2 ml presiliconized polypropylene microcentrifuge tubes (Slickseal; Island Scientific, Bainbridge Island, Wash.), capped, and stored at a 25° angle at 15°C. Previous work showed that the viability of kidney cells in primary culture (infected or not) is not improved by frequent medium changes so changes were made at 169 h only.

Transmission electron microscopy. At 0, 1, 2, 4.5, 24, 48, 96, 168 and 240 h, one aliquot from each treatment was centrifuged to pellet cells and extracellular bacteria. The pellet was fixed with 2% glutaraldehyde (purified Grade I, Sigma, St. Louis, Mo.) in 0.1 M cacodylate buffer (pH 7.2) at 15°C. After 1 h, the cells were washed with 0.2 M cacodylate buffer (pH 7.3), spun in a microcentrifuge (model 235B, Fisher Scientific, Pittsburgh, Penn.) for 3 min and the supernatant removed. To maximize cell density, each cell pellet was carefully stirred with two drops of cooled 2% agarose (type VII, low gelling temperature <30°C; Sigma, St. Louis, Mo.) (Watanabe et al., 1988). The gel-cell mixture was spun 1.5 min in a microcentrifuge and the agarose pellets allowed to gel on ice before cutting into 1 mm cubes in buffer. Following

two rinses in buffer, the cubes were post-fixed in aqueous 1% osmium tetroxide, dehydrated in acetone and embedded in medcast-araldite resin (Ted Pella, Inc., Redding, Calif.). Tissue blocks were sectioned on a Sorvall ultramicrotome using a sapphire knife (LKB, Bromma, Sweden). To avoid serial sections, at least 20 μm was removed from a tissue block so that sections on each grid were unique. The grids were stained with saturated aqueous uranyl acetate, washed, dried and stained with bismuth subnitrate (Riva, 1974). Micrographs were taken in a Zeiss EM 10/A transmission electron microscope at an accelerating voltage of 60 kV. Consecutive micrographs covering one section/ribbon on a grid permitted unbiased sampling. A low magnification (1,250x) was used to maximize the number of cells viewed per micrograph. Contact prints of each micrograph negative were made and the individual cells numbered, avoiding cells already numbered in another micrograph. Using the numbered cells as a map, selected features for each cell and bacterium were recorded from the micrograph negatives which were magnified (56,000x) on a microfiche reader (Model 4020, Micro Design, Hartford, Wis.). Statistical significance was determined by chi-square analysis of the observations summed in contingency tables.

i. Assessment of cell viability and intracellular bacterial location. The MP were rated by the integrity of their plasma membrane, nucleus, and cytoplasm (Ghadially, 1988) and ranked as viable, intact-nonviable, or nonintact-nonviable (Table 4.1).

Table 4.1. In the electron micrographs, mononuclear phagocytes (MP) were classified according to the integrity of their cellular components (viability; Ghadially, 1988) and the assurance that intracellular *Renibacterium salmoninarum* was associated with the cell (intactness). Unless otherwise noted, only the MP classified as viable or intact-nonviable were used in statistical analysis. Bacteria associated with the nonintact-nonviable MP were considered to be extracellular.

Classification	Integrity of cellular components		
	Plasma membrane (% intact)	Cytoplasm	Nucleus
Viable	>95	contiguous	normal
Intact-nonviable[1]	60-95	contiguous/disrupted	normal/pyknotic
Nonintact-nonviable[2]	<60	skeletal, with scant or no organelles	pyknotic or absent

[1] The presence of one or more abnormalities in cell integrity classified the MP as nonviable. An MP was deemed intact if the cytoplasm and plasma membrane were complete enough to retain intracellular bacteria.

[2] These cells were designated MP if intact phagolysosomes containing bacteria were enclosed within a cytoplasmic skeleton associated with some plasma membrane.

In rating viability, minor allowance was made for artifact from electron microscopy preparation.

Intracellular bacteria were recorded as located in a phagosome when surrounded by electron-lucent or electron dense areas within a phagosome membrane (>50% intact) or in the cytoplasm when they were surrounded by host cell plasma with <50% phagosome membrane contact. The bacteria seen in the nonintact-nonviable MP were considered extracellular.

ii. Assessment of bacterial cell damage. Bacteria were considered intact if the integrity of the shape, cytoplasm, and cell wall (>50%) was retained. The latter assessment was made based on the difficulty of obtaining clean-cut sections of the formidable cell wall of *R. salmoninarum.*

Colony forming units assay. To determine the number of viable intracellular and extracellular *R. salmoninarum* and *A. globiformis* at 24, 48, 96, 168 and 240 h, three tubes (1 ml aliquots of kidney cells) from each treatment were centrifuged at 100 x *g* for 1.5 min to pellet the kidney cells. The supernatant, containing extracellular bacteria, was separated into another tube and the bacteria pelleted in a microcentrifuge. The pellet was washed three times with peptone saline to remove all antibiotic and diluted to 1 ml for CFU determination. To enumerate intracellular bacteria, 1 ml of sterile distilled water was added to the kidney cell pellets to disrupt cellular membranes. This was centrifuged 5 min in a microcentrifuge and the process repeated two times. If cell lysis was incomplete after treatment with

distilled water (determined by light microscopic examination), Triton X-100 (1% total) was added for 10 min. This procedure disrupted the cells but did not reduce the bacterial CFU. The bacterial pellet was then diluted to 1 ml. The intracellular and extracellular bacterial fractions were diluted in peptone saline (10^0-10^7/ml) and inoculated on duplicate plates of the appropriate medium. Bacterial colonies were counted after maximum growth was obtained (6 weeks for *R. salmoninarum* and 5 days for *A. globiformis*).

Cell enumeration and viability. To determine the effect of treatment on the kidney cells, viable and dead cells were enumerated on a hemacytometer using trypan blue dye exclusion. For each treatment, 20 µl samples were collected at 0, 24, 48, 96, 168, and 240 h from each of three tubes designated for this purpose. Cell samples, kept on ice until counting, were diluted to 2 to 8 x 10^5 viable cells/ml with 10% L-15, mixed 1:1 with 0.2% trypan blue in water, and counted immediately in a Spencer hemacytometer at a magnification of 400x. During counting, cells were grouped into five subsets based on size and type (Table 4.2). Data were log transformed and analyzed using univariate repeated-measures analysis of variance.

Table 4.2. Viable cells were enumerated and categorized by size and morphology using trypan blue exclusion and a hemacytometer. Sizes for cell types are based on those from Yasutake and Wales (1983).

Cell subset	Size (μm)	Cell type(s)
Large cells	>7	lymphocytes, neutrophils mononuclear phagocytes,
Small cells	5-7	small lymphocytes, thrombocytes
Erythrocytes	≥7 x 13	red blood cells
Dead cells[1]	NA[2]	various
Other[1]	4	released nuclei

[1] These increased over time and were not included in calculations.
[2] Not applicable.

Results

Kidney cell number and viability (trypan blue exclusion assay). At initiation of infection, 94% of the kidney cell population was viable. Survival of kidney cells differed with treatment. At 96 h, live *R. salmoninarum* significantly reduced the number of viable cells ($P<0.01$) compared with the other three treatments (formalin-killed *R. salmoninarum*, *A. globiformis*, or control). Although the overall cell population decreased in number with time, the individual cell subsets reacted differently to in vitro culture and treatment (Fig. 4.1). Small cells (thrombocytes and small lymphocytes) died rapidly and by 48 h, a 1.0 to 1.5 $\log_{10}$ decrease in number was observed regardless of treatment ($P>0.05$). Treatment also had no effect on the number of erythrocytes and they were relatively resistant to the extended culture time. Large cells (mononuclear phagocytes, neutrophils, plasma cells and others) were affected by the addition of bacteria, whether live or formalin-killed *R. salmoninarum* or the nonpathogenic *A. globiformis*. All bacterial treatments caused a significant reduction in the number of large cells relative to the control ($P\leq0.01$). The effects of live *R. salmoninarum* on the large cells were more severe than either the formalin-killed *R. salmoninarum* or *A. globiformis* ($P\leq0.05$).

Figure 4.1. The effect of treatment and time on the viability of the (A) kidney cell population and its subsets: (B) small cells (small lymphocytes, thrombocytes), (C) erythrocytes, and (D) large cells (mononuclear phagocytes, lymphocytes, neutrophils). Each point represents the mean of three replicates. Live *Renibacterium salmoninarum* (RS) significantly affected the number of viable cells at 96 h ($P<0.01$), primarily attributable to its effect on the large cell subset. The other two bacterial treatments also reduced large cell numbers compared with the noninfected control ($P\leq0.01$).

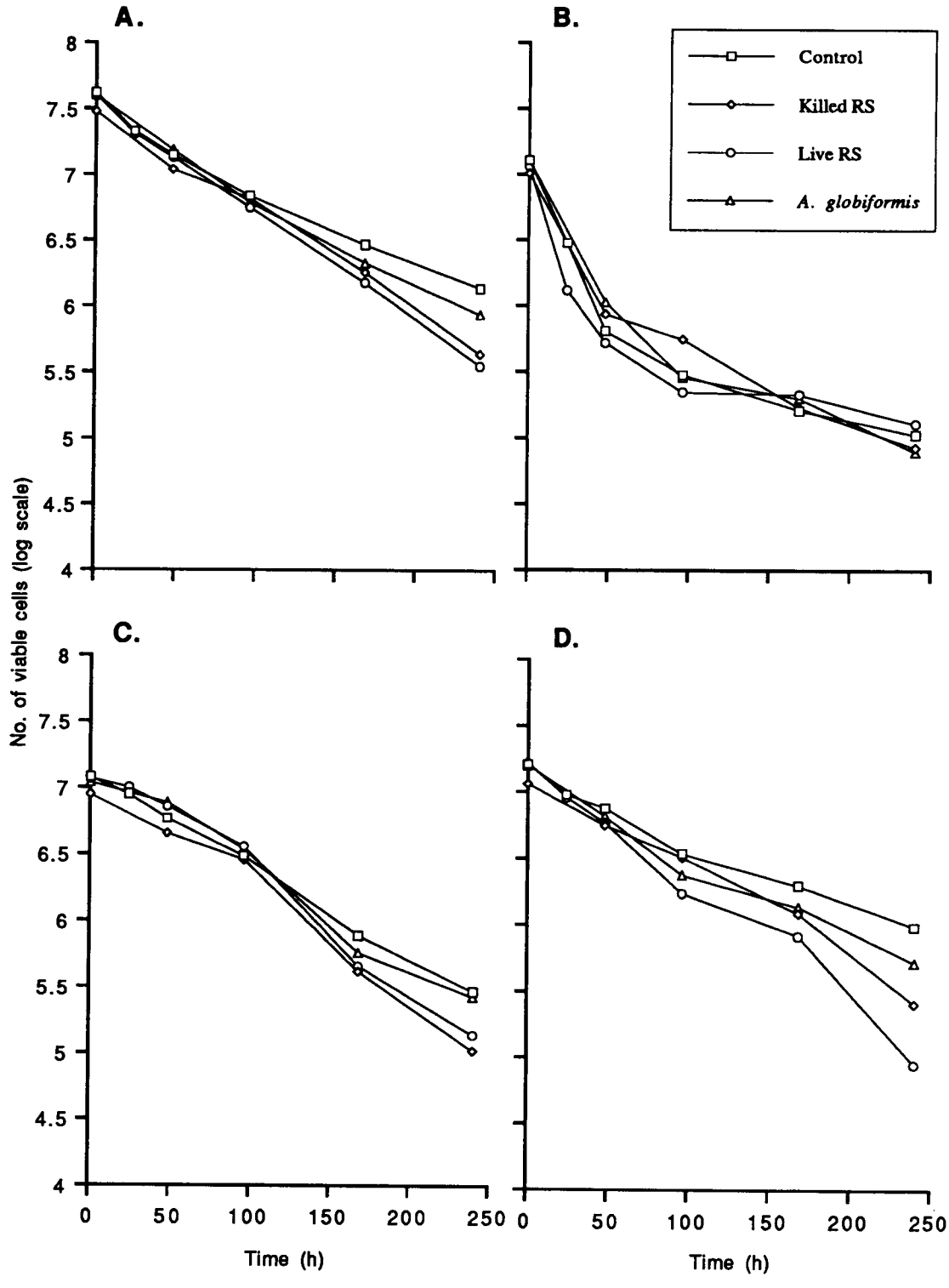

A.
B.
C.
D.
Control
Killed RS
Live RS
A. globiformis
No. of viable cells (log scale)
8
7.5
7
6.5
6
5.5
5
4.5
4
0
50
100
150
200
250
Time (h)

Figure 4.1

Viability of *Renibacterium salmoninarum* in mononuclear phagocytes (CFU assay). *Renibacterium salmoninarum* survived in high numbers through 96 h and more than 94% of the viable bacterial cells were intracellular (Table 4.3). Between 24 and 48 h, the number of viable intracellular *R. salmoninarum* increased 15% concomitant with a mean decrease of 73% in the number of extracellular bacteria, although these changes were statistically insignificant. Beyond 96 h, there is a significant decrease in the number of viable intracellular bacteria perhaps attributable to the decline in viable MP (Fig. 4.2). By 240 h, only 0.06% (2.53×10^4 CFU/ml) of the 24 h total viable *R. salmoninarum* remained, likely a consequence of the release of bacteria from disrupted MP and subsequent exposure to the antibiotic-containing medium.

In contrast, *A. globiformis* was rapidly killed and/or poorly ingested by the MP. By 24 h, there were only 6.9×10^4 CFU/ml viable *A. globiformis* within the MP (Table 4.3) despite an infecting dose nearly four times that of *R. salmoninarum*. Lacking CFU data prior to 24 h, electron micrographs at 1, 2, and 4.5 h revealed that *A. globiformis* was ingested, at least in limited numbers. At 48 h, there was a 31% increase in intracellular bacteria (1.12×10^5 CFU/ml) indicating that active phagocytosis of *A. globiformis* had occurred. Between 48 and 96 h, 94.4% of the intracellular *A. globiformis* died and at 168 h, both the intra- and extra-cellular bacteria were essentially eliminated.

Table 4.3. The number of viable intracellular and extracellular *Renibacterium salmoninarum* and *Arthrobacter globiformis* after infection of mononuclear phagocytes as determined by colony forming units (CFU) assay.

Bacteria	Time (h)	No. viable bacteria (±SD)[1]			% Intracellular bacterial cells
		Intracellular	Extracellular	Total	
R. salmoninarum	24	3918 (±339)	238 (±127)	4156 (±289)	94 (±3)
	48	4485 (±831)	65 (±3)	4550 (±828)	99 (±0.3)
	96	2963 (±20)	98 (±39)	3060 (±44)	97 (±1)
	168	73 *(0)	19 (±3)	95 *(0)	77 *(0)
	240	0.4 (±0.1)	2 (±1)	3 (±1)	16 (±4)
A. globiformis					
	24	7 (±2)	70 (±30)	77 (±31)	10 (±4)
	48	18 (±5)	52 (±9)	70 (±13)	26 (±3)
	96	1 (±0.1)	1 (±1)	2 (±0.5)	32 (±14)
	168	0 (±0)	0.04 (±0.03)	0.04 (±0.03)	0 (±0)

[1]Each value represents the mean CFU x 10^4/ml from three replicates except where starred *(one value).

Figure 4.2. The number of viable intracellular *Renibacterium salmoninarum* (RS) relative to the large cell subset (mononuclear phagocytes (MP), lymphocytes, and neutrophils) is shown. The bacterium is internalized within MP whose numbers were estimated (at 24 and 96 h) from electron micrographs. Bacterial numbers were quantified by the colony forming units assay.

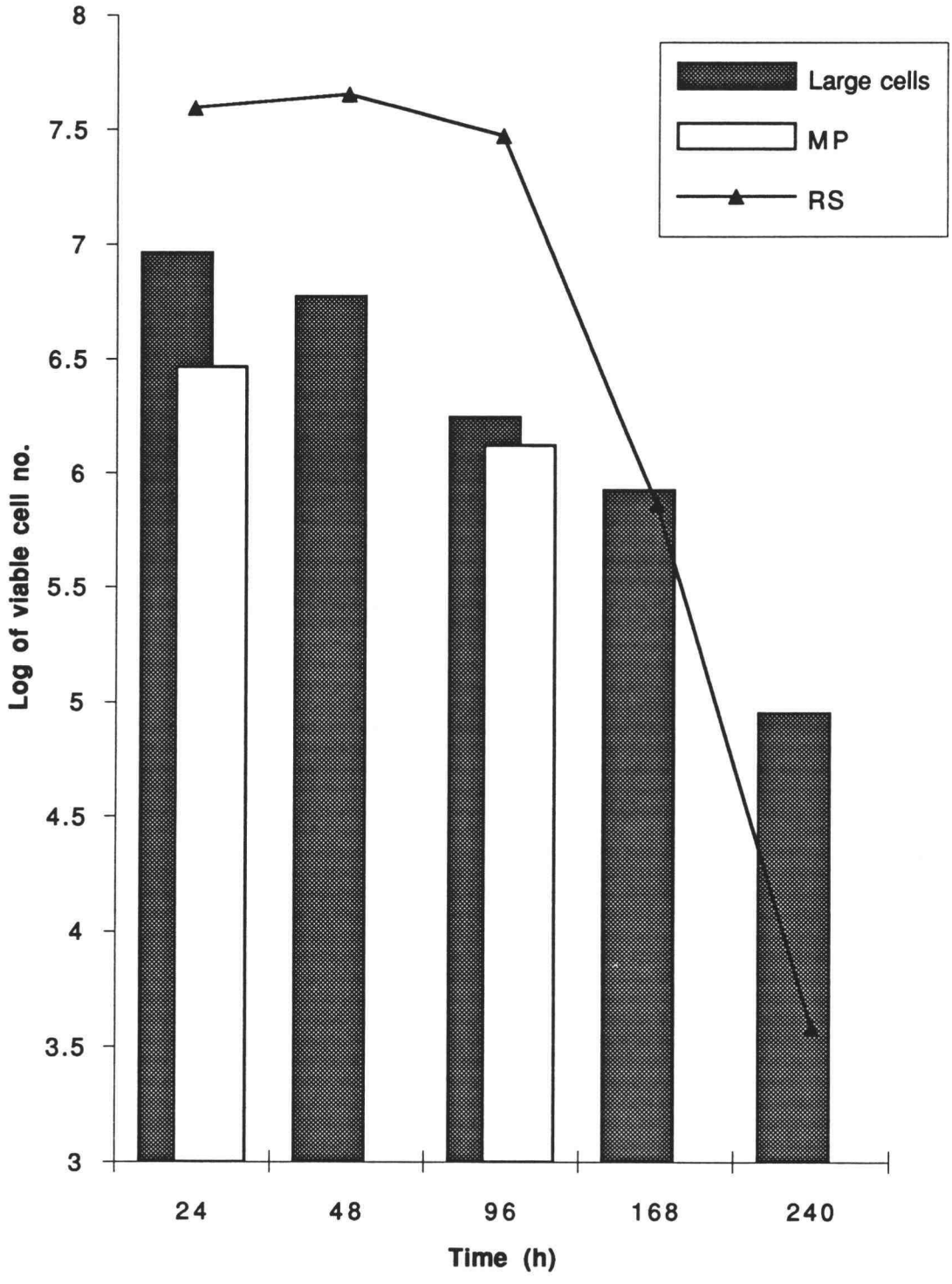

8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
Log of viable cell no.
24
48
96
168
240
Time (h)
Large cells
MP
RS

Figure 4.2

Quantitative and qualitative observations from electron microscopy. (i) Intracellular survival of *R. salmoninarum*. The ability of *R. salmoninarum* to survive within the MP was notable especially compared with the nonpathogenic *A. globiformis*. Examination of several hundred cell profiles from the *A. globiformis* infection at 1, 2, 4.5, 24, and 48 h revealed only four cells with intracellular bacteria and few extracellular bacteria. In contrast, *R. salmoninarum* were common in MP. The numbers of intracellular bacteria increased with time and as many as 9 and 42 bacteria, at 4.5 and 96 h, respectively, could be counted in a cross-section of a MP (Fig. 4.3).

Renibacterium salmoninarum, whether live or formalin killed, was resistant to the destructive mechanisms of the MP. Fifty and sixty-six percent of the bacteria (n=278 and 119 for formalin-killed and live *R. salmoninarum*, respectively) had intact cell walls at 4.5 h and and by 96 h, the numbers decreased by only 7-10% (n=319 and 292). If the MP were able to easily degrade the formalin-killed bacteria, there should have been a decrease in bacterial numbers per MP cross section and an increase in numbers of empty MP. But at 96 h, there was an average of eight bacteria/cross section and 32% empty MP compared with six bacteria/cross section and 37% empty MP in the live *R. salmoninarum* treatment. The resiliency of the bacterial cell wall was also revealed by the numerous bacteria seen at 168 and 240 h in the micrographs. At these intervals, *R. salmoninarum* (live treatment) were found primarily in the culture medium among

Figure 4.3. The distribution of intracellular bacteria in cross sections of mononuclear phagocytes at 4.5 and 96 h after infection with live or killed *Renibacterium salmoninarum*. N = 40-65 MP per treatment and time. Data were obtained from electron micrographs.

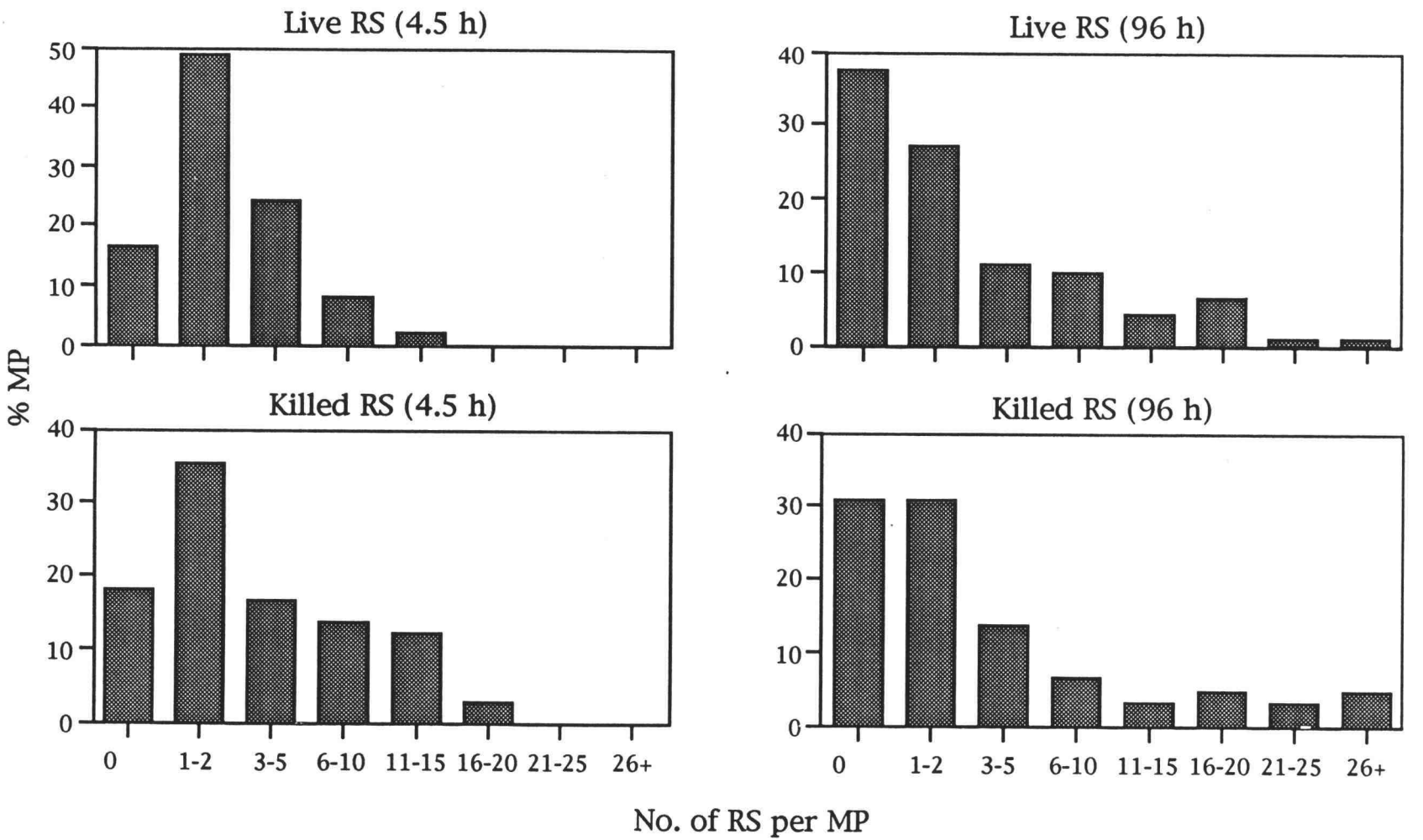

Live RS (4.5 h)
Live RS (96 h)
Killed RS (4.5 h)
Killed RS (96 h)
% MP
No. of RS per MP
0
1-2
3-5
6-10
11-15
16-20
21-25
26+

Figure 4.3

cellular debris and dissociated pyknotic nuclei but the formalin-killed bacteria were found both within intact MP or extracellularly. Many of the live and formalin-killed *R. salmoninarum* were morphologically intact and retained their coccobacillus shape, a relatively complete cell wall and contiguous cytoplasm despite their former or present intracellular residence and antibiotic exposure.

That the MP were able to damage or kill some bacteria explains the necessity for the exit of *R. salmoninarum* from the phagosome into the cytoplasm (Fig. 4.4 and 4.5). The bacteria actively left the phagosomes and this change in location occurred most rapidly for live bacterium. At 4.5 h, most live *R. salmoninarum* were located in the cytoplasm (Table 4.4), many with fragments of phagosome membrane in proximity to the bacteria. In contrast, the formalin-killed *R. salmoninarum* left the phagosome more slowly and at 4.5 h, significantly fewer ($P<0.001$) were found in the cytoplasm compared with the live *R. salmoninarum* treatment. By 96 h a significant increase in the number of formalin-killed *R. salmoninarum* in the cytoplasm was observed ($P<0.002$), equivalent to the number of live *R. salmoninarum* seen at this time. There was no significant change in the number of live *R. salmoninarum* in the cytoplasm between 4.5 and 96 h. This could be attributed to the ongoing phagocytosis of bacteria released from dead MP. In their escape from the phagosome, the bacteria, whether live or formalin-killed, seemed to cause at least partial dissolution and mechanical disruption of

Figure 4.4. At 4.5 hours post infection (A), live *Renibacterium salmoninarum* is found in phagosomes (p1) and in the cytoplasm (*) of mononuclear phagocytes (MP). The bacterium, judged intact based on retaining 50% of its cell wall and a contiguous cytoplasm, appears to lyse the phagosome membrane (arrowhead) which allows access into the cytoplasm. Bacteria in the cytoplasm show evidence of their former phagosome residence as witnessed by fragments of closely associated membranes. Through 96 hours (B), the MP are still active, phagocytosing bacteria (inset) along with cellular debris from dying cells. Although the newly engulfed bacteria (p1) have likely experienced exposure to the killing mechanisms of other MP and media antibiotics, most are intact and retain the ability to lyse the phagosomal membrane (arrowheads). Bacteria are also seen in the cytoplasm (*) of this cell. Melanin, electron-dense and uniformly black, is seen in other phagosomes (p2). Nu = nucleus.

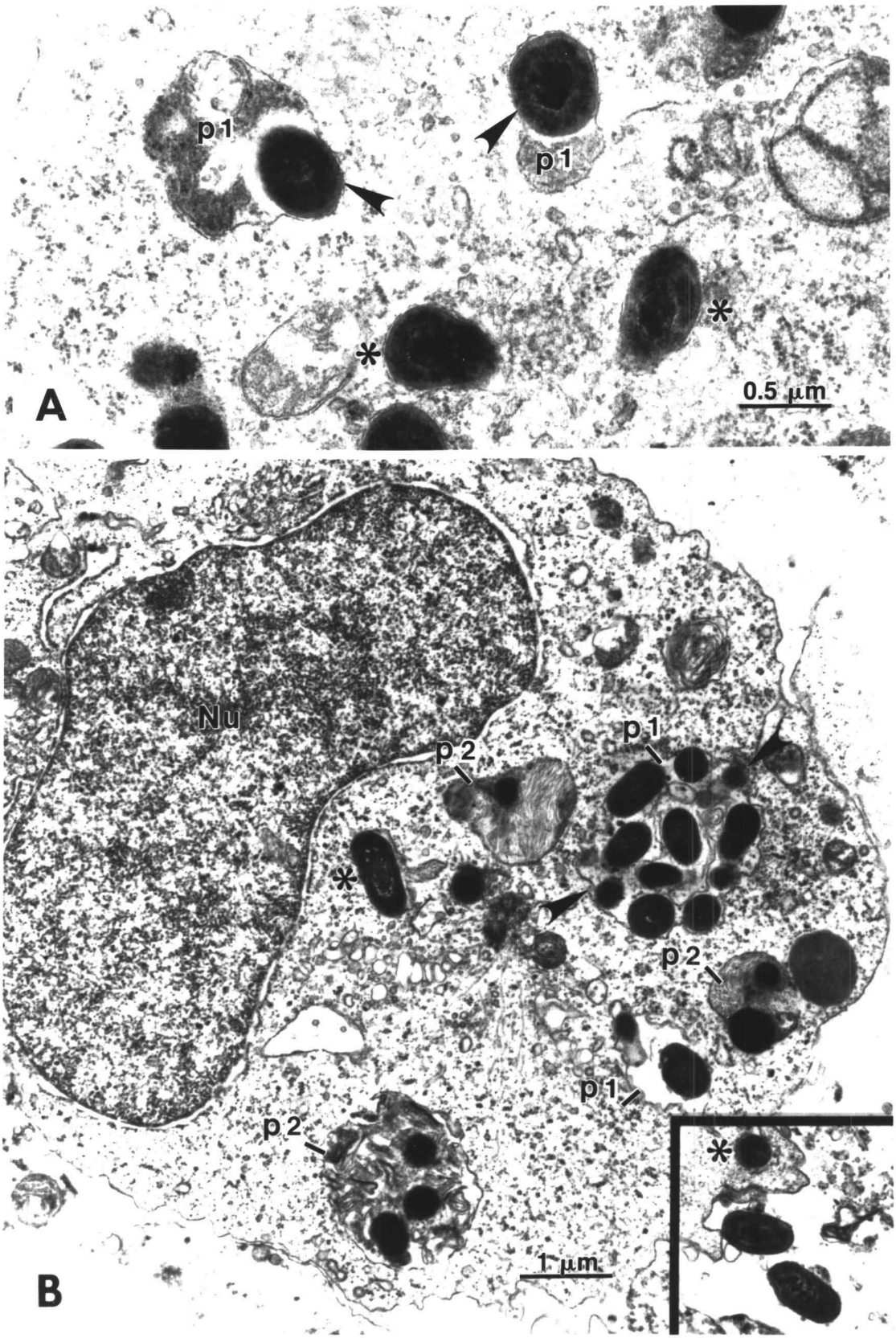
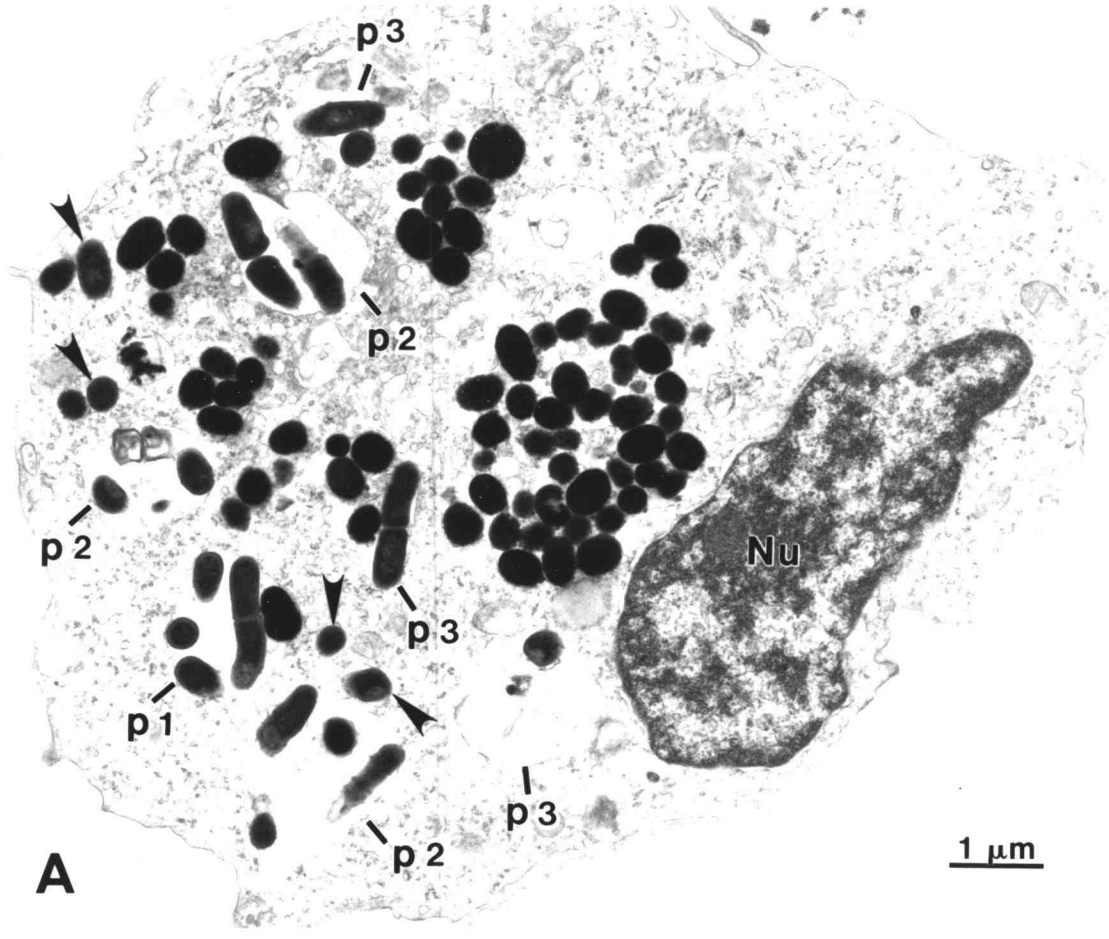

p1
p1
*
*
A
0.5 µm
Nu
p2
p1
*
p2
p1
p2
1 µm
B
*

Figure 4.5. Formalin-killed *Renibacterium salmoninarum*, 96 hours after infection, are resistant to the killing mechanisms of the mononuclear phagocytes (MP). Four bacteria are enclosed in a phagosome with an intact membrane (p1) and eight are in phagosomes with incomplete membranes (p2). Another four bacteria are in transition (p3) between phagosome and cytoplasmic residence as the phagosome membranes have disintegrated, leaving only an empty space around part of the bacteria. Another four bacteria are present in the cytoplasm (arrowheads). The numerous sightings of intact bacterial replicants, preserved by formalin fixation, indicate that their cell wall construction is protective throughout the process of division. Extensive deposits of melanin (intense black granules) are present in the cytoplasm and in two phagosomes, along with bacteria. An enlargement (B) demonstrates bacteria in the cytoplasm (>50% cell wall intact; arrowheads), and in deteriorating phagosomes (p2, p3). Although the transverse cell wall from division is still apparent in one bacterium (*), the exterior wall has been degraded. Melanin (m) is seen in the cytoplasm. N = nucleus.


p 3
p 2
p 2
p 3
p 1
p 3
p 2
Nu
1 µm
A

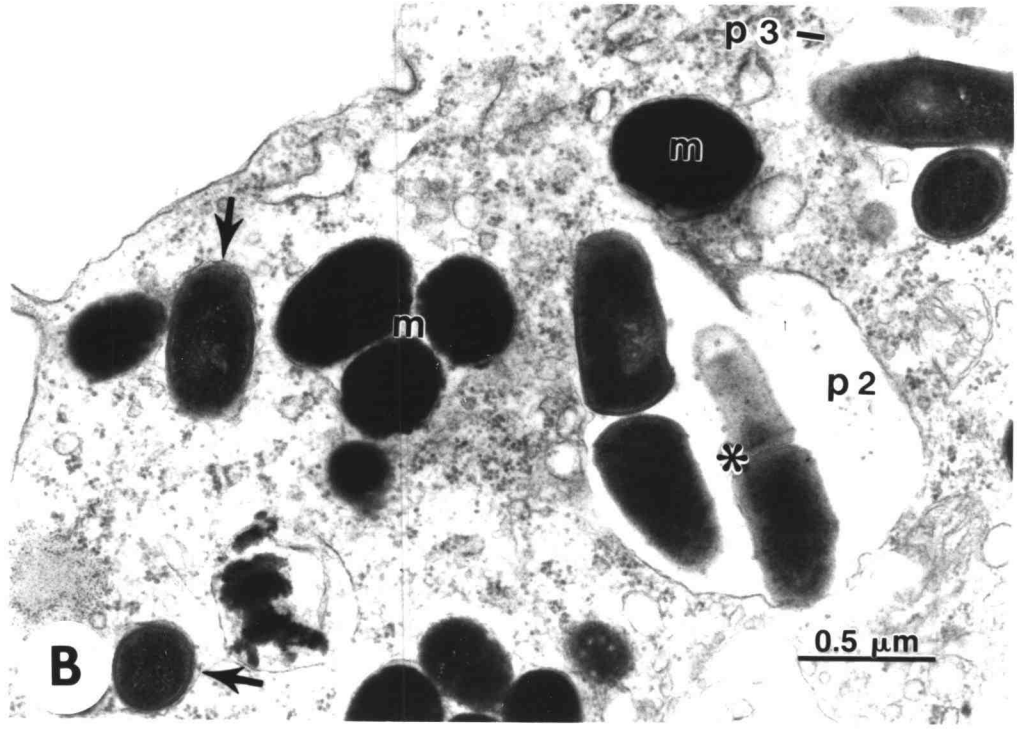

p 3
m
m
p 2
*
0.5 µm
B

Table 4.4. Intracellular location of live and formalin-killed *Renibacterium salmoninarum* (RS) at 4.5 and 96 h post infection of mononuclear phagocytes (MP).

Treatment	Time (h)	No. MP observed	Percent frequency of RS location in MP population[1]			
			phagosome only	≥50% of RS in phagosome	≥50% of RS in cytoplasm	cytoplasm only
Live RS	4.5	40	27.5	7.5	7.5	57.5
	96	53	34.0	5.6	15.1	45.3
Killed RS	4.5	56	55.3	23.2	3.6	17.9
	96	40	37.5	10.0	5.0	47.5

[1]For each mononuclear phagocyte, bacterial location was calculated by (no. bacteria in cytoplasm/total intracellular bacteria) and the observations totaled to determine % frequency.

the enclosing phagosome membrane so that membrane remnants often remained in contact with bacteria located in the cytoplasm. Damaged bacterial cells were not precluded from the locale change (Fig. 4.6). The ratios of damaged to intact bacteria within the phagosome or the cytoplasm were comparable at 4.5 h indicating that bacteria damaged in the phagosome escaped into the cytoplasm.

(ii) Intracellular replication of *Renibacterium salmoninarum*. Intracellular replication of *R. salmoninarum* was not successfully quantified by electron microscopy but replicating forms were present in the electron micrographs through ten days (Table 4.5; Fig. 4.7). At 4.5 h, the frequency of dividing bacteria equaled 5.0% of the total intracellular bacteria and increased two-fold (11.0%) by 24 h. By 96 h, the process of replication was slowed to 3.8% in the intracellular bacterial population. However, these numbers were not significantly different from those of the formalin-killed bacteria which served as a nonliving (and thus, unchanging) measure of replicating forms. Throughout the experiment there were never more than two replicating bacteria per MP profile in the live treatment. In a previous experiment performed under similar conditions, 44% of the intracellular bacteria (n=9) were actively replicating in one MP profile at 72 h, indicating that intracellular replication had occurred. Antibiotics in the culture medium prevented extracellular division of *R. salmoninarum* (unpublished data) which was reflected in the low number of extracellular replicants seen.

Figure 4.6. Total intracellular *Renibacterium salmoninarum*, intact and damaged (<50% cell wall intact), in the phagosome and cytoplasm of mononuclear phagocytes (MP) at 4.5 h. Intracellular bacteria numbered 278 in 51 MP and 119 in 40 MP in the killed and live *R. salmoninarum* treatments, respectively. Data were taken from electron micrographs.

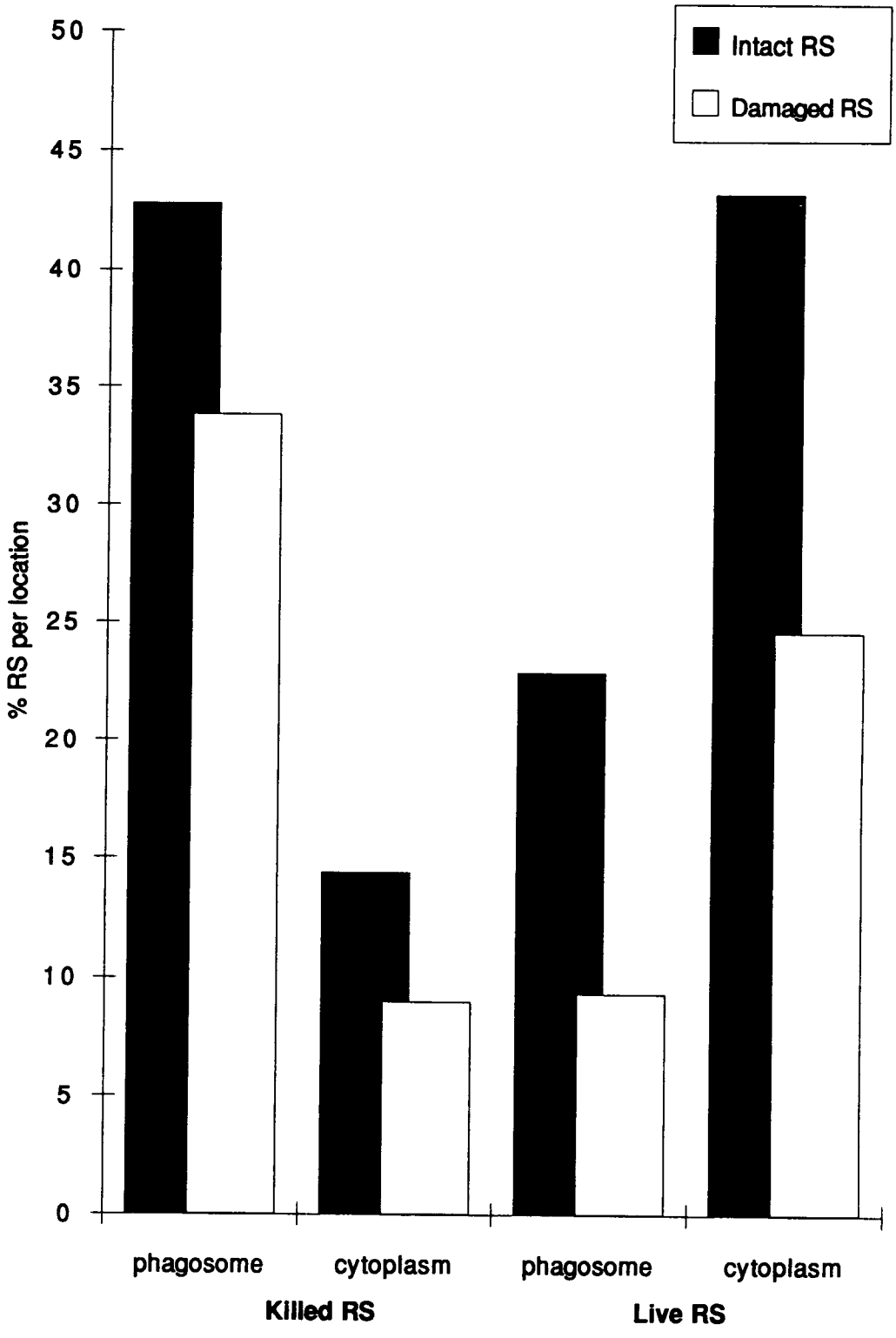

Intact RS
Damaged RS
% RS per location
50
45
40
35
30
25
20
15
10
5
0
phagosome
cytoplasm
phagosome
cytoplasm
Killed RS
Live RS

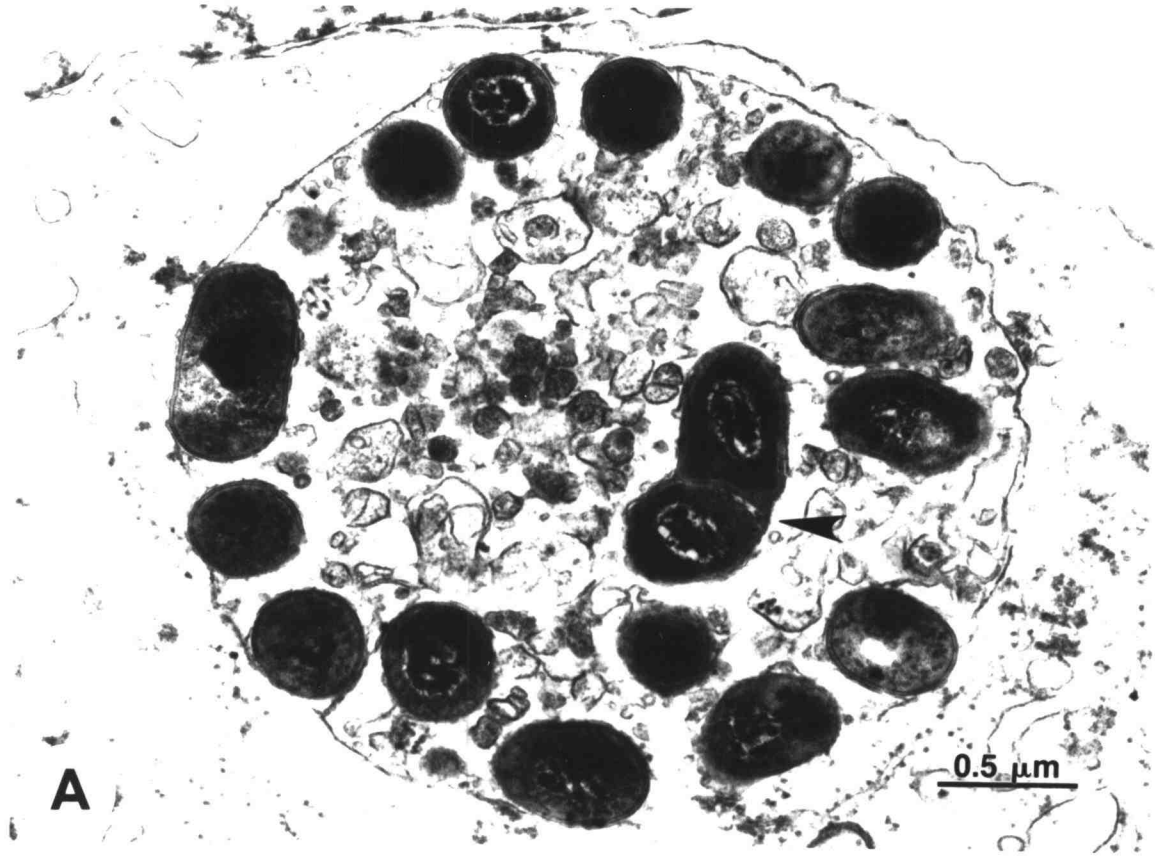
Figure 4.6

Table 4.5 Electron microscopic determination of intra- and extra-cellular replication rates of *Renibacterium salmoninarum* (RS) post infection of mononuclear phagocytes. As a comparison, formalin-killed RS fixed during log phase growth is shown.

Treatment	Time (h)	No. of *R. salmoninarum*[1]			% Dividing RS[1]
		Dividing	Nondividing	Total	
Live RS	4.5	6 (4)	113 (125)	119 (129)	5.0 (3.1)
	24	3 (1)	24 (33)	27 (34)	11.0 (2.9)
	96	11 (37)	280 (1758)	291 (1795)	3.8 (2.1)
Killed RS	4.5	15 (5)	303 (223)	318 (228)	4.7 (2.2)
	24	7 (2)	87 (2)	94 (2)	7.4 (0.0)
	96	16 (20)	303 (619)	319 (639)	5.0 (3.1)

[1]intracellular and (extracellular) *R. salmoninarum*.

Figure 4.7: Replicating *Renibacterium salmoninarum* are seen at 96 h (A) and ten days (B). In (A), a longitudinal section of a dividing bacterium (arrrowhead) is found in a phagosome with other bacteria. Cell debris in the phagosome suggests that the bacteria had previously inhabited another cell which had since died. By 10 days (B), very few intact mononuclear phagocytes exist and this bacterium, lacking completed cell wall construction (white arrowhead), was seen in a clump of cellular debris. Antibiotics in the media prevented extracellular bacterial division, thus the process of replication must have started in a MP.


A
0.5 µm


B
0.2 µm

(iii) Condition of mononuclear phagocytes. Treatment affected MP differently, often recognized more by the severity of damage than by numbers of viable MP. At 4.5 h, identification of MP in the uninfected control was difficult because many had yet to phagocytose extracellular debris. In a comparison of the treatments with live and formalin-killed *R. salmoninarum*, differential damage to the MP was apparent by 4.5 h after infection (Table 4.6). The MP infected with the live bacteria sustained significantly more damage (31%) than those infected with the formalin-killed bacteria (10%) ($P<0.001$). At 96 h, 76% of the MP in the control treatment were viable, significantly higher than in formalin-killed (38%) and live *R. salmoninarum* (23%) treatments ($P<0.0001$). The extensive damage induced by live *R. salmoninarum* increased the total number of nonintact-nonviable MP to 56% by 96 h compared to 30% for formalin-killed *R. salmoninarum* ($P<0.001$).

The MP persisted longer in culture than the other immune cells. At 4.5 h after infection with live or formalin-killed *R. salmoninarum* (33.5 h after culture initiation), the proportion of MP in the kidney cell population was <32% (Table 4.7). By 96 h, the ratio of MP to the other cells was significantly enriched by ≥72% ($P<0.0001$) and equivalent frequencies occurred in both the control and *R. salmoninarum* treatments.

Despite adverse conditions, the MP were active in vitro, and residual bodies, the late forms of phagolysosomes (completed or nearly completed digestion) were obvious in 9-12% of the MP cell

Table 4.6. The effect of treatment on mononuclear phagocyte (MP) viability as described by the frequency of viable, intact-nonviable, or nonintact-nonviable cells.

Treatment[2]	Time (h)	Percent frequency of MP viability[1] (no. observations)		
		Viable	Intact-nonviable	Nonintact-nonviable
Control	4.5	ND[3]	ND	ND
	96	75.7 (28)	10.8 (4)	13.5 (5)
Live RS	4.5	51.8 (42)	17.3 (14)	30.9 (25)
	96	22.9 (43)	21.3 (40)	55.8 (105)
Killed RS	4.5	47.4 (37)	42.3 (33)	10.3 (8)
	96	38.2 (29)	31.6 (24)	30.2 (23)

[1] Viable = intact plasma membrane, contiguous cytoplasm, and normal nuclei; intact-nonviable = disrupted plasma membrane, cytoplasm, or pyknotic nuclei; nonintact-nonviable = disrupted plasma membrane, poorly defined cytoplasm, and pyknotic nuclei.
[2] RS = *Renibacterium salmoninarum*
[3] Not done

Table 4.7. Frequency of mononuclear phagocytes (MP) in cell population as observed from electron micrographs.

Treatment[2]	Time (h)	No. cell profiles	Percent frequency of occurrence[1]		
			MP minus melanin	MP plus melanin	Other cells
Control	96	42	54.7	16.7	28.6
Live RS	4.5	126	23.0	8.7	68.3
	96	40	45.0	30.0	25.0
Killed RS	4.5	106	17.9	10.4	71.7
	96	55	40.0	32.7	27.3

[1]Excludes erythrocytes
[2]RS = *Renibacterium salmoninarum*

profiles at 4.5 h. This increased by 96 h and 48-56% of the MP had residual bodies, indicating that the surviving MP maintained phagolysosomal activity despite the internal presence of *R. salmoninarum*. Residual bodies were also observed in 47% of the MP in the control treatment at 96 h indicating that these formations were not dependent upon the addition of bacteria but were a normal phenomenon associated with the phagocytic and destructive activities of the MP. Efforts to demonstrate enhanced damage to *R. salmoninarum* in MP that contained residual bodies proved inconsequential.

(iv) Melanin containing cells. Melanomacrophages, a subgroup of MP that contain melanin granules, mimicked the actions of other MP (Fig. 4.8). The melanomacrophages were actively phagocytic (Table 4.8) and contained as many as 14 and 22 bacteria/cell cross-section at 4.5 and 96 h, respectively. At 4.5 h, 9-10% of the kidney cell population (excluding erythrocytes) consisted of melanomacrophages, about one third of the total number of MP (Table 4.7). The number of MP that could be classified as melanomacrophages increased with time due to active ingestion of loose melanin granules released from disrupted cells. By 96 h, the number of MP with and without melanin were nearly equivalent. The ability of melanomacrophages to damage *R. salmoninarum* was comparable to the other MP. The role of melanin in these cells was not immediately apparent although in 65-74% of the melanomacrophages at 4.5 h, one or more granules were associated with phagolysosomes (with or without bacteria) in

Figure 4.8. (A). At 96 hours, mononuclear phagocytes (MP) containing melanin actively phagocytosed bacteria (arrowheads). The large deteriorating phagosome (P) contains melanin released from melanocytes or other disrupted melanomacrophages. Two melanin granules (m) and two residual bodies (rb) are seen in the cytoplasm, as is a condensed nucleus (n) from a dead erythrocyte. In (B), a residual body (rb), an old phagosome that has completed degradation of its contents, is a tribute to the activity of the MP. The melanin (clustered around m) eventually disintegrated as noted by a loss of electron density. Although melanin was often seen in areas of degradation, its value to the MP was unclear and it had no apparent effect on closely associated *Renibacterium salmoninarum* (C). Even the formalin-killed bacterium seen here (arrow) appears intact. Nu = nucleus.

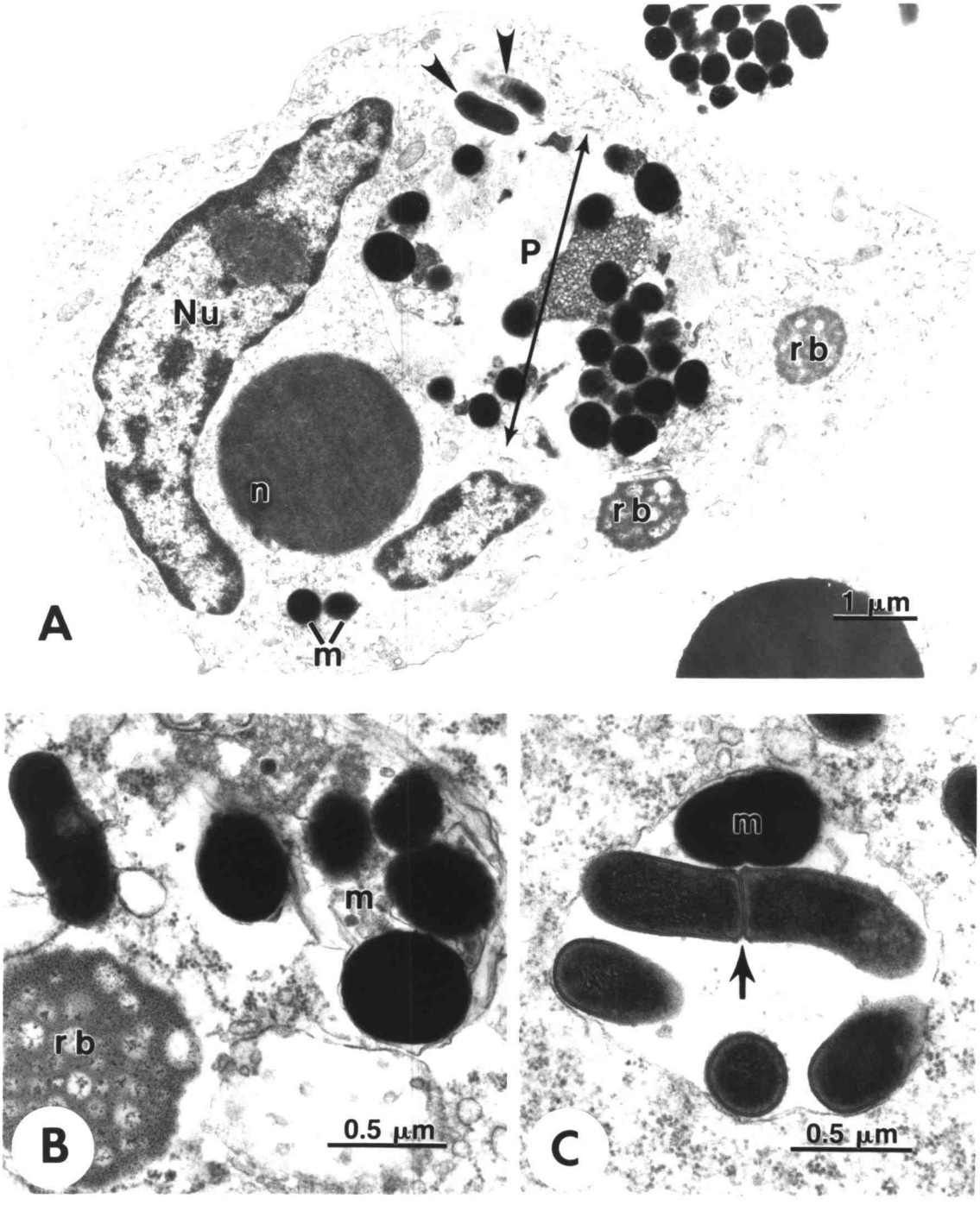

A
Nu
n
m
P
rb
rb
1 µm
B
m
rb
0.5 µm
C
m
0.5 µm

Table 4.8. Phagocytic and melanin activity in melanomacrophages noted in electron micrographs.

Treatment[1]	Time (h)	No. cells	Percent melanomacrophage population	
			Phagocytosis	Melanin activity[2]
Control	96	15	33.3	80.0
Live RS	4.5	17	64.7	64.7
	96	31	83.9	58.1
Killed RS	4.5	27	70.4	74.1
	96	30	76.7	70.0

[1]RS = *Renibacterium salmoninarum*

[2]One or more melanin granules per melanomacrophage were associated with phagolysosomes.

various stages of digestion. Melanin interactions with phagolysosomes did not increase at 96 h nor was there a difference between the live and formalin-killed *R. salmoninarum* treatments.

(v) Infection of other cells by *Renibacterium salmoninarum*. Active phagocytosis of *R. salmoninarum* by cells other than MP was not noted. However, as many as one to nine *R. salmoninarum*, live or formalin-killed, were found embedded in the plasma membrane of cells resembling lymphocytes at 4.5 h (Fig. 4.9). In ten cases, one to five bacteria (per cross section) had penetrated into the cytoplasm after embedding in and disrupting the plasma membrane of these cells. Cells with surface-embedded bacteria were phagocytosed by MP, and this, in addition to the bacterial-induced membrane disruptions, may partially account for the disappearance of this cell type by 96 h. More rarely, *R. salmoninarum* was embedded on the surface of erythrocytes but was never noted in the cytoplasm (Fig. 4.10). The embedding entry (absorption-type) was never noted on the MP surface.

Figure 4.9. At 4.5 hours after infection, *Renibacterium salmoninarum* was found embedded (arrowheads) in the plasma membrane of cells resembling lymphocytes (A). An enlargement of (A) reveals that the live bacterium partially lysed the membrane and entered the cytoplasm (B), a factor which may have played a role in the early demise of these cells. Nu = nucleus.

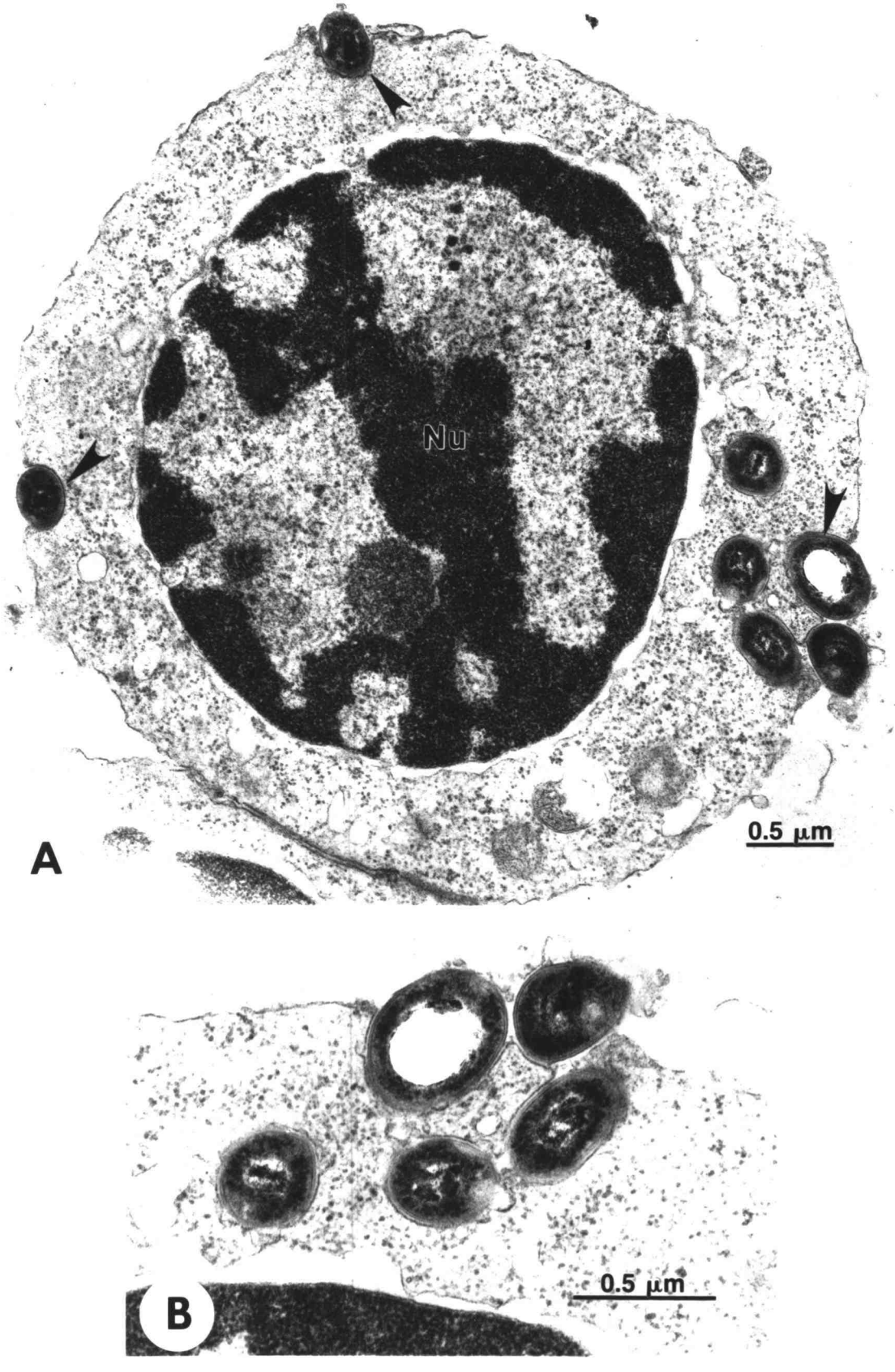
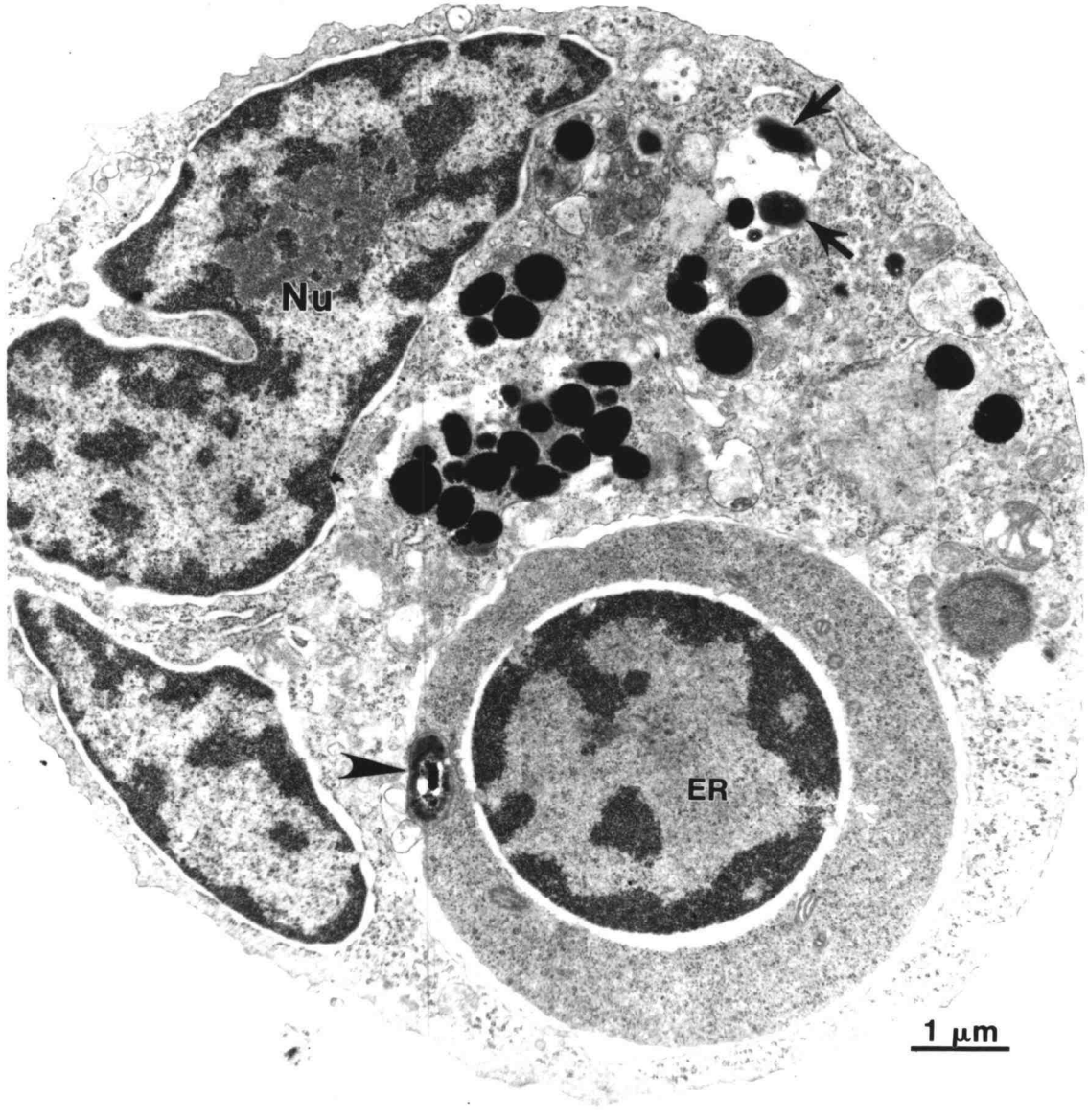

Nu
0.5 µm
A
0.5 µm
B

Figure 4.10. Cell surface-associated bacteria, seemed to induce phagocytosis by the mononuclear phagocytes (MP) as many affected cells were enclosed in phagosomes. An immature erythrocyte (ER) with a membrane-embedded *Renibacterium salmoninarum* (arrowhead) has been phagocytosed by a MP at 4.5 hours. Although erythrocytes were plentiful in the cell population, phagocytosis of these cells by MP, even this active melanomacrophage which has engulfed other bacteria (arrows), was not normally seen. Nu = nucleus.


Nu
ER
1 µm

Discussion

Results from the study indicate that the ability of *R. salmoninarum* to escape from phagosomes and move into the cytoplasm contributed to its lengthy survival within the MP. Live *R. salmoninarum* facilitated the location change most quickly and at 4.5 h, more than three times the number in the phagosome were found in the cytoplasm. Although formalin-killed *R. salmoninarum* was significantly slower in leaving phagosomes, numbers equivalent to the live bacterium were found in the cytoplasm at 96 h suggesting that a cell surface component is responsible for the location transfer. The extracellular protein of *R. salmoninarum* (originally named antigen F; Getchell et al., 1985) is water soluble and cell surface-associated and could be an important virulence factor. It is produced in prodigious quantities in infected fish or culture and in vitro studies have shown it to have immunosuppressive, proteolytic, hemolytic, and agglutinating activities (Bruno and Munro, 1986; Daly and Stevenson, 1990; Evenden et al., 1990; Rockey et al., 1991). Other intracellular bacteria produce extracellular proteins believed responsible for phagosome membrane lysis. The hemolysin (Listeriolysin O) of *Listeria monocytogenes* is responsible for phagosome membrane lysis and descriptions of this protein are not dissimilar to the extracellular proteins of *R. salmoninarum*. Both are produced and released in large quantities into culture supernatants, have similar molecular weights (57-58 kD), and are β-hemolytic (Farber and

Peterkin, 1991; Bruno and Munro, 1986; Rockey et al., 1991). *Shigella flexneri* has a plasmid that is correlated to phagocytosis by macrophages and which permits phagosome membrane lysis followed by rapid multiplication in the cytoplasm (Clerc et al., 1987). Some strains of *Mycobacterium tuberculosis* can escape into the cytoplasm and the success of its transfer is dependent on its virulence (Myrvik et al., 1988). Within five to six h after infection with *M. tuberculosis* H37Rv, the destruction of the phagosome membrane is apparent and by 24 h, 60 to 100% of the bacteria are located in the cytoplasm. In vivo, different strains of *R. salmoninarum* seem to consistently use the phagosome escape mechanism to enhance survival and the bacterium is seen in the cytoplasm of macrophages in electron micrographs (Ferguson, 1989a; Young and Chapman, 1978). It appears that the factor permitting the escape of *R. salmoninarum*, regardless of bacterial viability, eventually results in disruption of the phagosomal membrane and subsequent deposition of the bacterium into the cytoplasm.

In the present study, *R. salmoninarum* often retained a morphologically intact structure in the phagolysosome, even during apparent intensive enzymatic activity. The durability of formalin-killed and live *R. salmoninarum* was equivalent based on the number of MP containing bacteria (68.4 vs 63.5%) at 96 h. Although a greater number of live *R. salmoninarum* were intact, whether in the cytoplasm or phagosome, the durability of the bacterial cell wall was indisputable and formalin-killed *R.*

salmoninarum was found structurally intact inside MP at 240 h. The number of *R. salmoninarum* observed by electron microscopy at 240 h seem at odds with the number of viable *R. salmoninarum* reported by CFU assay and indicate that the cell walls of dead bacteria deteriorate slowly. That many formalin-killed bacteria were present indicates that the MP, although able to damage and/or kill the bacterium, has difficulty in degrading the bacterial cell wall. The extensive measures required to lyse *R. salmoninarum* (lysozyme and pressure of 20,000 psi followed by detergent and proteinase K) (Gutenberger et al., 1991) suggest that a durable cell wall is a factor in its intracellular survival. It is extremely resistant to lysozyme, the endogenous enzyme in the cytoplasm of macrophages, and remains intact after a week of exposure to 1 mg/ml (Fryer and Sanders, 1981).

Renibacterium salmoninarum has a lengthy generation time of $\geq$18 h even in aerated culture medium (unpublished data, D6 strain) and intracellular residence did not decrease generation time. In the MP, the number of bacteria peaked between 24 to 48 h after infection, an indication of ongoing replication. By 96 h, a reduction in bacteria was evident. A number of factors contributed to the difficulty in quantifying intracellular replication. As the MP died and released their intracellular residents, the bacteria were exposed to antibiotics in the media prior to phagocytosis by other MP. This probably slowed the replication rate of *R. salmoninarum.* Some bacteria were also killed by the MP. The appearance of dividing *R. salmoninarum* in

the culture media at ten days might be attributed to attempts to divide prior to release from dead MP. It can be argued that the fluctuations seen in the live bacterial population signify active, ongoing growth that is gradually inhibited by loss of viable MP. A similar pattern has been observed for virulent *M. tuberculosis* whose in vitro replication rates (16 h) in culture medium are similar to *R. salmoninarum* (≥18 h). In human MP, *M. tuberculosis* replicates exponentially at a rate of 23 ± 2.1 h until there is a depletion of habitable MP which have limited survival in vitro (Crowle, 1988).

Apart from the normal attrition of MP in vitro, *R. salmoninarum* had a debilitating effect on the MP. This was most obvious in MP infected with the live bacterium. By 96 h, fragmented cells with vacant cytoplasms were recognizable as MP only by the associated phagolysosomes containing bacteria. Lysis of MP by live *R. salmoninarum* with a subsequent release of intracellular enzymes may have contributed to cell destruction although the reasons for lysis were not obvious. Generally, as numbers of intracellular bacteria increase, death of MP becomes more likely (Clerc et al., 1987; Crowle, 1988; Dowling et al., 1992). In our study, there were never more than 19 live *R. salmoninarum* per viable MP cross section. By extrapolation from TEM, trypan blue exclusion, and CFU data, there was an average of 20 *R. salmoninarum* per viable MP at 96 h. The MP seemed to tolerate higher numbers of formalin-killed *R. salmoninarum* and as many as 42 were observed in viable MP. In the case of *M. tuberculosis*,

30 to 60 bacilli will kill human MP but MP containing up to 1000 *M. avium* remain viable (Crowle, 1988). Although related to virulence, the mechanisms by which intracellular bacteria kill host cells are unclear (Clerc et al.1987; Dowling et al, 1992; Crowle, 1988). In our study, physical evidence, as phagosome membrane lysis or extensive phagolysosome activity, did not denote death of the MP. Some MP retained evidence of active degradation and killing of *R. salmoninarum* at 96 h. However, the lack of phagolysosomal activity or inability to kill bacteria also failed to identify possible "cytocidal" mechanisms by the bacterium.

Despite the debilitating effects of *R. salmoninarum* in culture, the MP (excluding erythrocytes) were the most resistant cell type in the population and were capable of killing the bacterium. Degraded remnants of *R. salmoninarum*, lacking cell walls and internal integrity, were seen in phagolysosomes at 4.5 h. The decrease in viable *R. salmoninarum* at 96 h is attributed to killing by MP, although exposure to culture antibiotic likely enhanced killing of those bacteria recently phagocytosed. After 96 h, there was a significant decrease in macrophage viability and it was impossible to determine whether the MP or antibiotic exposure was killing newly phagocytosed bacteria.

The killing of *R. salmoninarum* by MP may be partially attributable to the presence of lymphocytes and previously activated MP, although these components are not necessarily required for the killing of intracellular pathogens. In our study,

lymphocytes were an integral part of the cell population for at least 24 h post infection, and may have played a role in activating the MP. In addition, some MP may have been activated by the pre-collection exposure to the extracellular proteins of *R. salmoninarum* in Freund's incomplete adjuvant although their numbers contributed less than 4.5% of the total cell population. Other virulent intracellular bacteria are killed by MP, a phenomenon not limited to activated MP. Monkey alveolar macrophages, after infection with *Legionella pneumophila*, killed 60-97% of the bacteria within 30 min. However, the virulent legionellae that survive multiply more than two logs in 96 h (Dowling et al., 1992). Normal, non-activated human MP are able to kill virulent tubercle bacilli, reducing the numbers by approximately 50% (Crowle, 1988). Crowle suggests that some MP in a population are able to kill their intracellular bacteria, although others are not, thus an infection with *M. tuberulosis* is perpetuated in vivo. This may also be true for *R. salmoninarum* in vivo.

A population of the MP, the melanomacrophages, contained melanin granules. The function of the melanin in the melanomacrophages remains obscure. It has been postulated that ingestion of melanin by phagocytes may protect cells during phagocytosis of tissue debris rich in free-radicals released by neutrophils (Ellis, 1982; Ferguson, 1989b; Rowley et al., 1988). In our study, loose melanin granules, released from disrupted melanomacrophages and melanocytes, were ingested by other MP as early as 4.5 h after infection, enriching the numbers of

melanin-containing MP at 96 h. The presence of bacteria was not required to trigger melanin release and uptake as this also occurred in the control treatment. Dissemination of loose melanin in the tissues with subsequent uptake by MP is a notable feature in fish infected with *R. salmoninarum* (Bruno, 1986; Young and Chapman, 1978). From our data, we could not define a function for the internalized melanin. Although melanomacrophages actively phagocytosed bacteria, the inclusion of melanin in the phagosomes did not seem to protect MP from the toxic affects of the bacterium because many melanomacrophages were nonviable. Inside the MP, melanin was often observed in association with bacteria in the phagosome or cytoplasm or in areas of previous or ongoing phagolysosome activity. The morphology of the bacterium in association with melanin did not suggest a toxic effect from the melanin. Melanin granules often disintegrated, usually in association with phagolysosomal activity, until all that remained were tiny fragments in old phagolysosomes or residual bodies.

Renibacterium salmoninarum, in this and other unpublished experiments, was readily phagocytosed by MP in the absence of specific opsonins, an asset shared by other intracellular bacteria (Kielbauch et al., 1985). The heat-inactivated fetal bovine serum in the culture medium did not contribute complement components, nor was any homologous fish serum added to the cultured cells. Rose and Levine (in press) found that opsonization of *R. salmoninarum* with the C3b component of complement was necessary for phagocytosis by trout kidney macrophages. Our use

of suspension, as opposed to adherent, cultures may explain the differences. Complement opsonization of *R. salmoninarum* may promote internalization but other factors, such as the bacterial cell surface, obviously play a role in the phagocytosis and internalization of *R. salmoninarum.*

The extracellular protein that induces the lysis of the phagosome membrane is likely responsible for the ability of *R. salmoninarum* to penetrate cells other than MP. In this study, *R. salmoninarum* was found embedded in the plasma membrane of cells resembling lymphocytes. The bacterium disrupted the membrane and penetrated into the cytoplasm; the efficiency of this process increased if the bacterium was live rather than formalin-killed. The bacterium was also occasionally found embedded on erythrocytes, with no penetration. This might explain the in vitro studies showing that the extracellular protein of *R. salmoninarum* agglutinates piscine leukocytes and mammalian erythrocytes (Daly and Stevenson, 1987; Wiens and Kaattari, 1991) although an in vivo function for this attribute has yet to be delineated. Other pathogenic bacteria are able to induce their own endocytosis to perpetuate their survival, replication and spread in cells other than MP (Farber and Peterkin, 1991; Sansonetti et al., 1986). *Shigella flexneri*, like *R. salmoninarum*, lyses the phagosome membrane and multiplies in the cytoplasm of MP, but will also spontaneously enter HeLa cells (<10%) (Sansonetti et al., 1986). However, the entry of shigellae into these cells appears to be by phagocytosis only and direct penetration with

subsequent disruption of the cell plasma membrane was not observed. This is also true for *Listeria mononcytogenes*, whose ingestion by Caco-2 cells (human colonic carcinoma cell line) is by phagocytosis without disruption of the plasma membrane (Mounier, 1990). However, *R. salmoninarum* did disrupt the plasma membrane of lymphoid cells which presumably resulted in their death and rapid depletion from culture by 48 h. The membrane-embedded bacteria apparently induced the phagocytosis of these cells by the MP, another reason for their disappearance. Although this might be another means of facilitating bacterial entry into the MP, a more functional reason, such as elimination of cells that could potentially activate MP to a more active killing role, is suggested.

Results from this study demonstrate the virulence mechanisms which allow *R. salmoninarum* to perpetuate chronic infections of BKD and resist treatment by antibiotics and vaccines. Given the similarities in cell wall construction, the differences in resistance to enzymatic degradation and/or internalization by MP are striking between *R. salmoninarum* and its closest relative, *A. globiformis*. By virtue of its cell surface components, *R. salmoninarum* is readily phagocytosed by MP and is able to lyse and move from the phagolysosome into the cytoplasm. It is likely, as with other intracellular pathogens, that the virulence of a particular strain is due to the quantity and/or presence or absence of one or more secreted proteins (Farber and Peterkin, 1991). The cell surface protein(s) of *R. salmoninarum* that promotes the lysis

of the phagolysosomal membrane is likely a virulence factor because bacteria lacking the extracellular protein have reduced virulence (Bruno, 1990). This defensive mechanism of the bacterium does not require active growth (or even viability) and in concert with its durable cell wall, *R. salmoninarum* can remain dormant in the cytoplasm of the MP. The slow replication noted within MP also provides advantages. The MP tolerate limited numbers of virulent intracellular *R. salmoninarum* and extensive replication would result in loss of protective, habitable domains. In addition, dormant or slowly growing bacteria are resistant to many antibiotics (beta-lactams, or penicillins, and other cell wall inhibitors) whose effects are directed towards actively replicating bacteria (Tuomanen et al., 1986). Nongrowing bacteria thus represent a reservoir of viable pathogens after the antibiotic is removed or falls below minimal inhibitory concentrations.

The tenuous balance between the MP and internalized *R. salmoninarum* that allows some infected animals to survive many years is probably upset by secondary infections, substandard or changing environmental conditions, or reproductive stress which provide opportunities for bacterial growth. Most infections of tuberculosis begin with one to two bacteria (Myrvik et al., 1988). It could be suggested that BKD begins similarly in salmonids. Spawning, with its concomitant stress, allows the bacterium to gain entrance into the ovum, infecting future progeny (Lee and Evelyn, 1990). The pathway of infection between this and residence within MP is unknown but the process is so effective that BKD is

prevalent in salmonid populations. Other vectors as water (Mitchum and Sherman, 1981) or feces (Balfrey et al., 1991) from infected animals provide additional routes of infection through phagocytes in the gills and intestines. The success of *R. salmoninarum* as a pathogen likely results from its ability to survive in the cytoplasm of MP and maintain slow or nonexistent replication that is uninhibitory to the fish until conditions favor maximum reinfection.

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Chapter 5

Assessment of Liposome-Encapsulated Erythromycin for Control of Bacterial Kidney Disease in Salmonids

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Abstract

Erythromycin encapsulated in liposomes and injected into rainbow trout (*Oncorhynchus mykiss*) at one or seven days after infection with *Renibacterium salmoninarum* reduced mortality from 83 to $\leq$21%. An equivalent amount of unencapsulated erythromycin (15 mg drug/kg fish) resulted in similar mortalities; but at 92 days post infection, 70-80% of the fish harbored low level infections in contrast to those treated with erythromycin liposomes (EL) (20-40%) as detected by an enzyme-linked immunosorbent assay (ELISA). Gross pathological examination revealed no unusual side effects due to administration of EL and better growth was noted in the uninfected control fish compared with those treated with unencapsulated erythromycin. Mortality was not significantly different between the fish given the control liposomes (CL) or saline; however, the mean day to death was reduced in fish given CL. The more rapid disease onset in these fish was supported by advanced gross pathological changes and elevated p57 levels, the major protein produced by *R. salmoninarum*. The components of the liposomes, dipalmitoyl phosphatidylcholine and cholesterol, may influence disease progress and severity of bacterial kidney disease, an effect which is mitigated by encapsulation of erythromycin in the liposomes.

Introduction

Renibacterium salmoninarum, the causative agent of bacterial kidney disease (BKD) in salmonid fish, survives and replicates within host macrophages (see previous chapter). Intracellular residence offers protection from chemotherapy because many antibiotics that are effective in vitro have limited or no ability to penetrate the membrane of phagocytes (Fountain et al., 1985; Sanchez et al., 1986). Antibiotic treatment (generally, oxytetracycline or erythromycin) appears to alleviate the mortalities caused by BKD, but the bacterium may remain dormant intracellularly in undetectable numbers until individual fish are immunocompromised, i.e. by environmental stress and/or secondary infections, or conditions associated with life cycle changes (Banner et al., 1986; Fryer and Sanders, 1981; Mitchum et al., 1979). Because the disease is vertically transmitted from dam to offspring (Bruno and Munro, 1986; Evelyn et al., 1986), elimination of intracellular bacteria prior to infection of eggs is important.

Erythromycin is the drug of choice for control of BKD in salmonids although elimination of *R. salmoninarum* is incomplete (Elliott et al., 1989). Erythromycin seems to concentrate within phagocytic cells (Brittain, 1987), but studies indicate that it is bacteriostatic and generally does not eliminate all intracellular bacterial cells (Anderson et al., 1986; Sanchez et al., 1986; Vilde et al., 1986). Research shows that erythromycin kills *R.*

salmoninarum in vitro (Gutenberger et al., 1989) and with effective presentation should eliminate the bacterium in fish.

Liposomes, used as therapeutic delivery systems of antibiotics, have successfully combated diseases caused by intracellular organisms (Alving et al., 1978; Bermudez et al., 1987; Fountain et al., 1985). The immune system identifies the microscopic liposomes as foreign and macrophages phagocytose them. Once inside the macrophage, the phospholipid coating of the liposome is lysed by enzymes in the phagolysosome and the antibiotic is released. This method concentrates antibiotic in the macrophage thus avoiding systemic dilution.

The use of liposome-encapsulated antibiotics for treatment of microbial diseases in fish is relatively unexplored, but two studies suggest that the route of administration is important. Austin (1985) compared equivalent oral doses of clindamycin with and without liposome-encapsulation in rainbow trout at ten days post infection with *R. salmoninarum.* Clindamycin alone reduced mortality to 28%, but the clindamycin liposomes provided no protection and mortalities were equivalent (>92%) to the untreated controls. A study by Power et al. (1990), however, found that liposomes administered to rainbow trout by intraperitoneal injection localized in the major sites of macrophage distribution, including the spleen, the anterior and posterior kidney, and to a lesser extent, the liver. These results indicated that liposome-encapsulated antibiotics should target sites critical in the pathogenesis of *R. salmoninarum.*

To determine if a liposome delivery system enhances the efficacy of erythromycin in vivo, the antibiotic was encapsulated in liposomes and injected into rainbow trout at one or seven days post infection with *R. salmoninarum*. This was compared to an equivalent treatment with unencapsulated erythromycin. In addition, liposomes containing saline only were tested to evaluate potential toxicity or side effects from the cholesterol and phospholipid components. In addition to mortality and mean day to death assessment, the soluble protein (antigen F) of *R. salmoninarum*, composed primarily of a 57 kilo-dalton protein (p57), was measured to determine disease progression (Rockey et al., 1991). The gross pathological changes were also noted.

Methods and Materials

Preparation of liposomes. Liposome preparation was based on the procedure of Alving and Swartz (1984). Cholesterol (C8667, Sigma Chemical Co., St. Louis, MO) was recrystallized two times in hot ethanol to remove immunosuppressive oxidative products (Radin and Gramza 1963; Humphries and McConnell 1979). Stock solutions of cholesterol and L-α-phosphatidyl dipalmitoylcholine (DPPC; P6267, Sigma) were prepared at 0.1 M in chloroform (HPLC grade). The cholesterol and DPPC solutions were mixed in a ratio of 0.75:1.0 and rotary evaporated to a film (Buchi HB 40, Brinkmann Instruments, Inc., Westbury, NY) in a round bottom flask at 40°C. The dried lipid films were stored

dessicated under vacuum for 1 to 24 h before addition of the swelling solutions.

The liposomes were prepared the day before injection. Erythromycin phosphate (Argent Chemical Laboratories, Redmond, WA), 50 mg/ml, was dissolved in sterile, triple distilled water and filtered-sterilized. Erythromycin-containing liposomes (EL) were formed by adding the erythromycin swelling solution at a volume ten times that of the original lipid and vortexing until all lipid was in solution (~30 min). Control liposomes (CL) were made using water as the swelling solution. The liposomes were sized by filtration through 1.2 μm acrodisc filters and stored at 4°C overnight. Prior to injection, aliquots of the liposome mixtures were withdrawn for quantification of erythromycin, and the remaining solution centrifuged at 22,000 x *g*, and resuspended in sterile PBS, pH 7.2, at a concentration of 3 mg erythromycin/ml, an amount equivalent to 15 mg erythromycin/kg of fish in an injection volume of 0.1 ml. The CL were treated and diluted identically.

Quantification of erythromycin in liposomes. Aliquots of the CL and EL were centrifuged 10 min at 4°C in a micro-centrifuge (Fisher Scientific, Pittsburgh, Pennsylvania). The EL pellets were dissolved in isopropanol and the erythromycin content determined by spectrophotometric comparison (280 nm wavelength) to a standard curve made from the CL pellets dissolved in isopropanol containing known amounts of erythromycin phosphate. Initial attempts to quantify

erythromycin uptake by liposomes were deterred by the formation of crystals upon dissolution of the EL in isopropanol. Use of water as the basis for the swelling solution, rather than PBS, eliminated this problem and also increased the solubility of erythromycin phosphate. The uptake of erythromycin by the liposomes, as determined by linear regression, was 0.71 $\pm$ 0.04 mg/ml for the one and seven day injections.

In vivo infection and treatment. The D-6 strain of *R. salmoninarum*, originally isolated from coho salmon (*Oncorhynchus kisutch*) in salt water (obtained from C. Banner, Oregon Department of Fish and Wildlife, Corvallis, OR), is virulent in trout and salmon and has been used in numerous studies by our laboratory. The second passage of this strain was grown to log phase in KDM broth (Evelyn, 1977) without serum at 16°C on a shaker. The bacterial culture was centrifuged at 5000 x *g* and the pellet diluted with 0.1% peptone saline to an absorbance of 0.7 at a wavelength of 520 nm (approximately 1 x 10^9 colony forming units/ml). This suspension was further diluted to obtain the desired injection dose which was verified by colony forming units (CFU) assay. Rainbow trout (*Oncorhynchus mykiss* Shasta), 20 g, were injected intraperitoneally (IP) with 50 µl (1.0 x 10^5 CFU) of *R. salmoninarum*. Control fish were injected with an equivalent amount of 0.1% peptone saline. At one or seven days after infection, the fish were injected IP with 0.1 ml of one of four solutions: EL, CL, erythromycin, or PBS (pH 7.2). The erythromycin solution consisted of erythromycin phosphate in

PBS, a dose equivalent (3 mg/ml) to that in the EL. Prior to injection, fish were briefly anesthetized (0.25 ml benzocaine/l water). All injections were done using a 26 gauge needle fitted on a 2.5 ml Combitip reservoir on an Eppendorf Repeator 4780 pipet (Brinkmann Instruments, Westbury, New York). This method delivers a controlled amount of solution with greater precision than the usual injection methods. Fish were held at the Oregon State University Salmon Disease Laboratory in pathogen-free water at 12.5°C.

Mortality and mean day to death determination. Each of the four treatments, given at one or seven days, were represented by 8-10 infected fish in each of three tanks arranged in a balanced block design within one bank of 24 tanks. The uninfected control fish, 8-10 fish/tank, were kept in two adjacent banks (one each for the one and seven day injections) with three, two, and one tank(s) allotted for the CL and EL, unencapsulated erythromycin, and saline treatments, respectively. The tanks (25 l) had a flow rate of 2.2 l/min. Dead fish were collected daily, weighed, and necropsied. For verification of *R. salmoninarum*, kidney tissues from dead fish were cultured on KDM-2 medium (Evelyn, 1977) and the concentrations of p57 measured. At 92 days post infection, ten fish from the EL, erythromycin (one and seven day injections), and the uninfected control groups were removed for pathological examination and determination of p57 concentration.

Determination of disease progression. Four tanks (100 l, with a flow rate of 2.8 l/min) containing 27 fish each were allotted for periodic sampling for pathological examination and concentrations of p57. These fish were infected with *R. salmoninarum* and at one day post infection, injected with EL, CL, erythromycin or saline as described. As a control, 27 uninfected fish were injected with EL only and kept in three 25 l tanks. At 3, 12, 24, 36, and 60 days, 5-6 fish from each treatment group were removed, weighed and examined. The entire kidney was collected, mixed 1:1 (weight:volume) with 1% BSA in TTBS (50 mM tris, 0.75% NaCl, 0.1% tween 20; final pH 7.2) and frozen at -20°C until assay of the p57 levels.

Determination of p57 by ELISA. We used the double monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) developed by Rockey et al. (1991) to quantify concentrations of the p57 protein produced by *R. salmoninarum*. The p57 concentrations in kidney tissue supernatant were calculated from the standard curve using Δ Soft software (Biometallica). A total of 39 non-infected rainbow trout were sampled, to determine the upper limit of p57 values in uninfected fish (Ott, 1977). A value greater than 4.19 ng/ml indicated the presence of BKD.

Feeding trials. To ascertain activity of fish in each treatment at 20, 22, and 27 days, 0.93 (± 0.04) g of Oregon Moist Pellet feed (3/32 in. diameter, Bioproducts, Warrenton, OR), was dropped into each tank and the fish were timed for ≥98 %

consumption. All tanks contained 8-10 fish. The timing for each tank was limited to 5 min because all food was devoured rapidly if health permitted feeding.

Statistical analysis. Analysis of variance followed by comparison of paired means by the Student's t test were performed to determine statistical significance. The multiple range test, LSD, was used to compare treatments (Day and Quinn, 1989).

Results

Mortality and mean day to death. Erythromycin liposomes (EL) and erythromycin were similarly effective in reducing mortality when given at either one ($P>0.05$) or seven ($P>0.05$) days after infection with *R. salmoninarum* (Table 5.1). When the fish were treated with EL or erythromycin at one day post infection, mortality was 13.3% and 16.7%, respectively. Antibiotic delivery at seven days did not significantly alter mortality in either the EL (21.0%) or the erythromycin (13.7%) treatments. Few fish died in the antibiotic treatments and the mean day to death was significantly higher ($P<0.001$) than in the CL or saline groups (Table 5.1).

Mortalities from BKD were substantially higher (78.7-90.0%) for the fish unprotected by antibiotic (Table 5.1). Mortality for fish given CL was not significantly different from fish given saline at either one ($P>0.05$) or seven ($P>0.05$) days. However, the mean

Table 5.1. Efficacy of antibiotic treatments injected at one or seven days after infection with *Renibacterium salmoninarum*. Rainbow trout were injected with erythromycin liposomes and erythromycin at a dose of 15 mg drug/kg fish. Control liposomes and saline served as controls. Each treatment/injection day was represented by three tanks of fish.

Treatment	Injection day	No. dead/ No. exposed	Cumulative dead/exposed	Cumulative percent mortality	Mean day to death[1]	Average mean day to death
Erthromycin liposomes	1	3/10 1/10 0/10	4/30	13.3	51 50 NA	51
	7	4/10 1/10 1/8	6/28	21.0	41 47 37	41
Erythromycin	1	1/10 2/10 2/10	5/30	16.7	47 43 49	46
	7	1/10 2/10 1/9	4/29	13.7	62 44 62	53
Control liposomes	1	10/10 10/10 7/10	27/30	90.0	31 33 35	33
	7	10/10 7/10 8/10	25/30	83.3	35 37 33	35
Saline	1	8/11 8/11 9/10	25/32	78.7	35 37 36	36
	7	6/10 8/9 9/10	23/29	79.7	35 37 37	37

[1]NA=not applicable

day to death significantly decreased ($P \leq 0.03$) for fish given CL (33 days) compared to those given saline (36 days) at one day post infection (Fig. 5.1). An upward trend in the mean day to death was evident from fish injected at seven versus one day and with saline versus CL (Table 5.1). This trend, however, was not supported by statistical differences ($P > 0.05$) between or among groups.

p57 concentrations. Sampling at periodic intervals for levels of p57 provided a quantifiable estimate of disease severity (Fig. 5.2). Through 60 days, the mean p57 levels in fish treated with EL were not significantly different ($P > 0.05$) from uninfected fish. In comparison, the mean p57 concentrations in the erythromycin group were slightly elevated at 24 and 60 days. This increase was contributed by two individuals with p57 values of 9.1 ng/ml (24 days) and 39.0 ng/ml (60 days), the other nine fish having undetectable levels. Contradictorily, one of the fish (60 days) with undetectable concentrations of p57 had notable pathological changes indicative of BKD.

At 92 days after infection, the mean p57 values for the EL (3.3 ng/ml) or erythromycin (5.3 ng/ml) groups were similar or slightly elevated compared with those of the non-infected fish (1.8 ng/ml) but there was an increased number of individuals with p57 values above 4.19 ng/ml, the level determined to be indicative of infection. Both groups treated with antibiotic contained infected individuals, but the number infected in the erythromycin group was more than double that in the EL group.

Figure 5.1 Days to death for rainbow trout infected with *Renibacterium salmoninarum* and given control liposomes (CL) or saline at (A) one or (B) seven days post infection. Administration of CL at one day post infection significantly shortened (P=0.03) the mean day to death in comparison to saline.

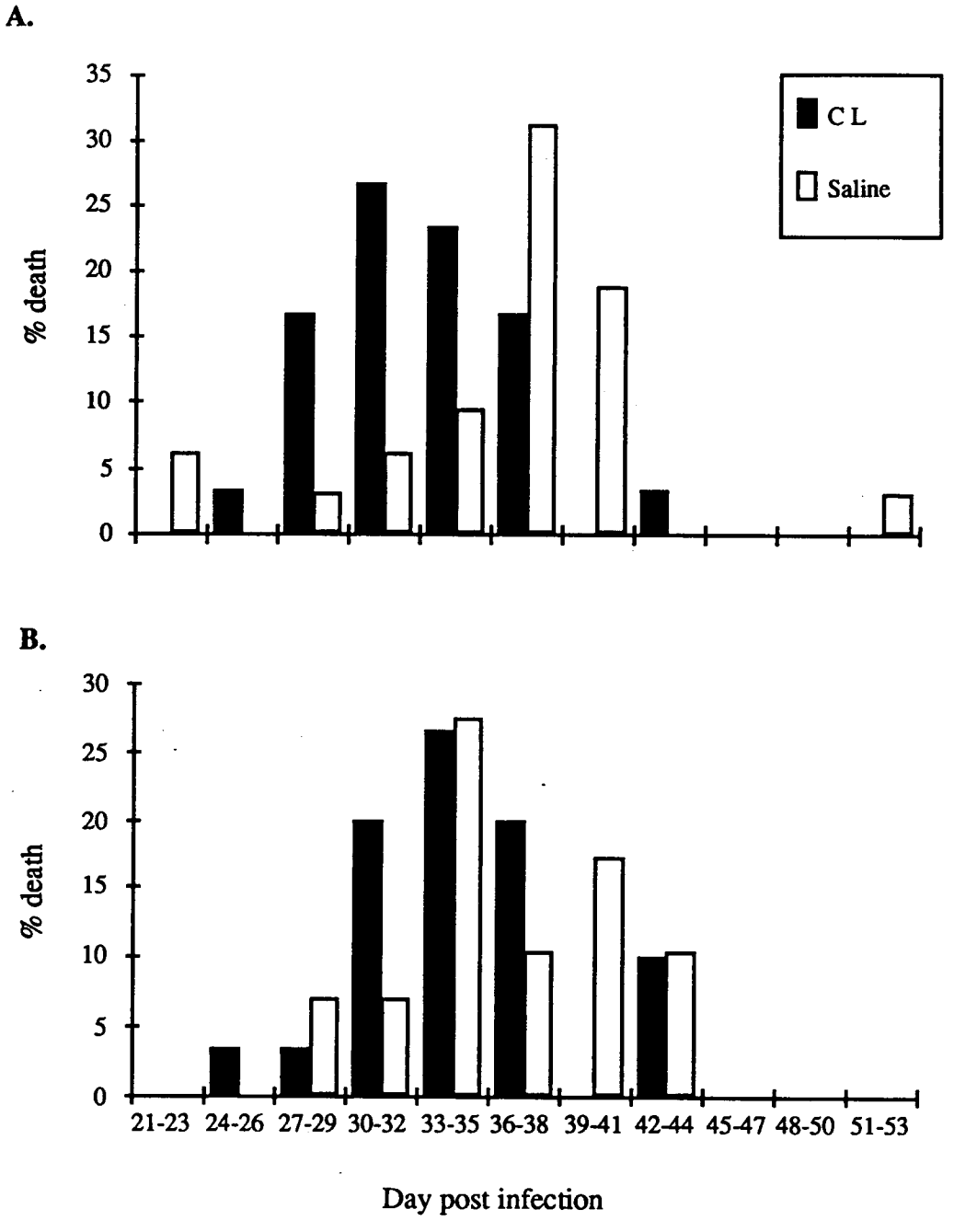
A.
35
30
25
20
15
10
5
0
% death
CL
Saline
B.
30
25
20
15
10
5
0
% death
21-23
24-26
27-29
30-32
33-35
36-38
39-41
42-44
45-47
48-50
51-53
Day post infection

Figure 5.1

Figure 5.2 The mean p57 values for rainbow trout injected with erythromycin liposomes (EL), erythromycin, control liposomes (CL), or saline one day after infection with *Renibacterium salmoninarum*. At 36 days, the last fish surviving in the saline and CL treatments were sampled. Values >4.19 ng/ml (--) indicate infection. Each point represents 3-10 replicates.

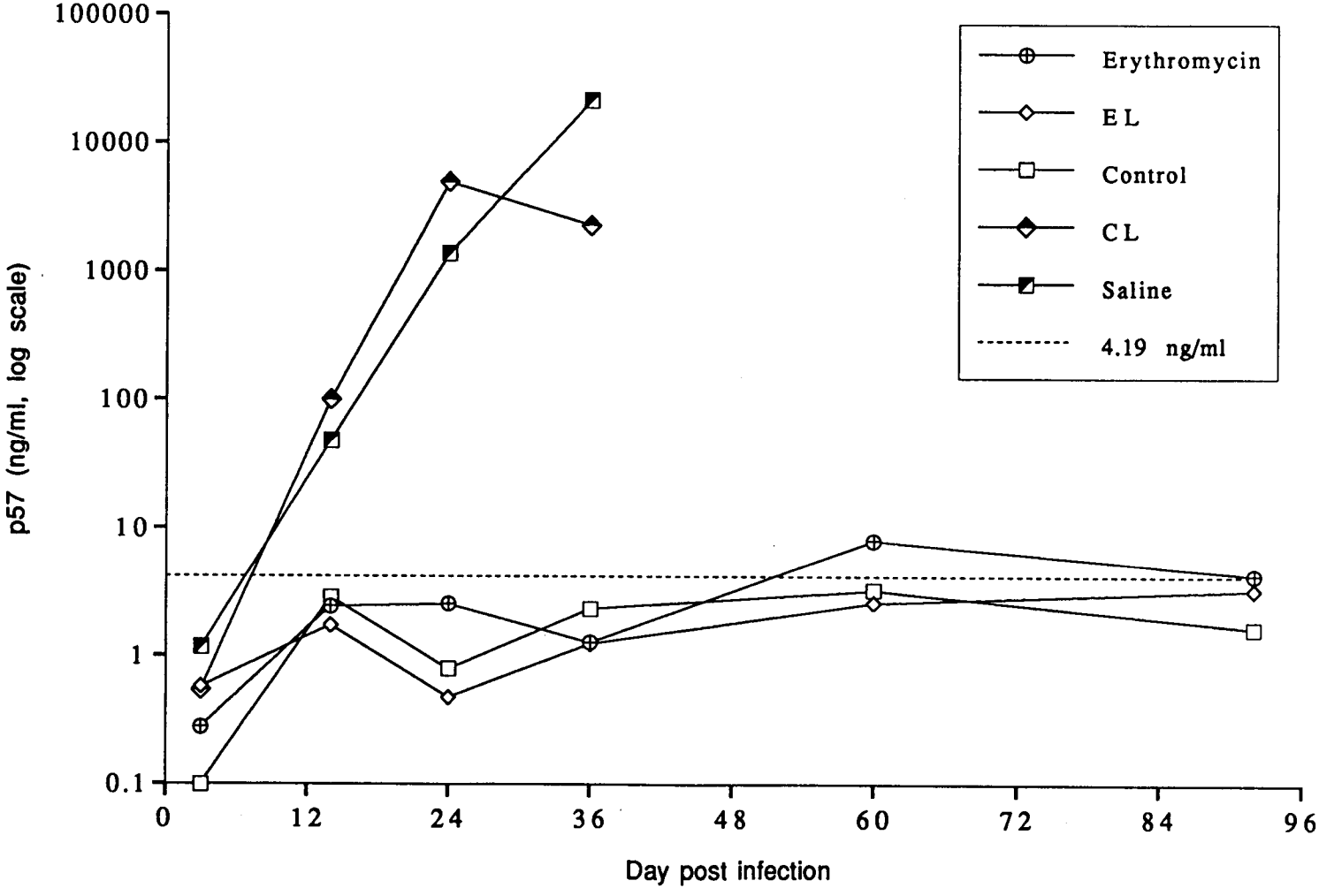
100000
10000
1000
100
10
1
0.1
p57 (ng/ml, log scale)
0
12
24
36
48
60
72
84
96
Day post infection
Erythromycin
EL
Control
CL
Saline
4.19 ng/ml

Figure 5.2

When fish were treated at one day post infection, 70% in the erythromycin group and 40% in the EL group harbored low level infections at 92 days. Treatment at seven days resulted in 80% infection in the fish treated with erythromycin and 20% infection in the EL treatment.

In fish given CL or saline, the concentrations of p57 continued to escalate with time (Figure 5.2). Three days after infection, no p57 was detected in any fish but by 14 days, the mean p57 level (101 ng/ml) in the fish given CL was significantly higher (P=0.002) than for fish in the saline group (48 ng/ml). By 24 days, mean p57 levels in the CL group (4,979 ng/ml) were higher than for the fish given saline (1,381 ng/ml). Many fish died prior to sampling at 36 days thus the mean p57 values represent a combination of moribund and surviving fish. Only one of five fish remaining in the CL group tested positively for p57 (11,509 ng/ml). The other four fish were active, appeared unlikely to develop the disease, and had undetectable levels of p57. The results were similar in the saline group and only two of the four remaining fish had high levels of p57 (28,327 and 57,569 ng/ml).

Fish that died from BKD had high levels of p57 that ranged from 802 to 80,151 ng/ml. Mortalities from the CL, saline, EL and erythromycin groups did not differ significantly (P>0.05) in p57 levels (Table 5.2).

Gross pathology. Gross pathological examination revealed no unusual side effects due to administration of EL. Of 46 live fish

sampled from the EL treatment, one fish at 92 days exhibited a slightly enlarged spleen in a fibrinous cast (Table 5.2). In comparison, three fish from the erythromycin treatment had more explicit signs of infection. At 24 days, one of these fish demonstrated splenomegaly, a pale liver, and ascites. Two fish, at 60 days, displayed a disease onset of greater severity with opaque, thickened swim bladders, swollen, grayish red kidneys, and extreme peritonitis including excess ascites (~3-6 ml) and enlarged spleens enclosed in fibrinous casts. All other fish, whether treated with EL or erythromycin, appeared normal throughout the 92 day study period. The appearance of external petechial hemorrhage on the body or fins at 92 days was deemed more indicative of extended confinement rather than BKD infection because this was also seen in the uninfected controls.

In fish not treated with antibiotic, gross pathological changes were first noted at 14 days (Table 5.2). At this time, the fish in both the saline and CL groups were feeding well and the internal and external appearance was relatively normal apart from the development of ascites and a dark red, grainy spleen that adhered to the peritoneal fat in some fish. By 24 days, the severity of disease onset judged by gross pathology was more apparent in the fish given CL than in those given saline. In all fish examined from the CL group, the kidneys, with or without lesions, had doubled in size, and enlarged spleens and pale livers were conspicuous. Ascites was apparent in most fish and anorexia was noted. Externally, reddened vents, abdominal distention, exophthalmia,

Table 5.2. Mean p57 levels and gross pathological changes for rainbow trout infected with *Renibacterium salmoninarum* and treated with erythromycin liposomes (EL), erythromycin alone (15 mg erythromycin/kg fish, equivalent to EL), control liposomes (CL), and saline. No pathological changes were evident in the uninfected fish and their mean p57 levels were no greater than 2.9 (±1.6) ng/ml throughout the 92 day sampling period.

Treatment	Day post infection[1]	No. fish[2]	Mean p57 (±SD)[3] (ng/ml)	Percent fish with observed pathological changes[4]									
				Gastrointestinal system	Kidney	Spleen	Liver	Internal Hemorrhage	Casts	Ascites	Intraperitoneal clot	External Hemorrhage	External Ulcer
EL	3	5	0.6 (+0.6)	0	0	0	0	0	0	0	0	0	0
	14	5	1.7 (+1.4)	0	0	0	0	0	0	0	0	0	0
	24	5	0.5 (+0.6)	0	0	0	0	0	0	0	0	0	0
	36	5 (4)	1.3 (+1.7)	0	0	0	0	0	0	0	0	0	0
	60	6	2.6 (+2.0)	0	0	0	0	0	0	0	0	0	0
	92	10	3.3 (+1.6)	0	0	0	0	0	0	0	0	50	0
	92*	10	2.8 (+2.1)	0	0	10	0	0	10	0	0	50	0
	Mortalities	9 (7)	16930 (+28526)	100	100	100	67	89	78	44	22	89	89
Erythromycin	3	4	0.3 (+0.6)	0	0	0	0	0	0	0	0	0	0
	14	5	2.4 (+1.3)	0	0	0	0	0	0	0	0	0	0
	24	5	2.6 (+3.8)	0	0	20	20	0	0	20	0	0	0
	36	5 (4)	1.3 (+1.0)	0	0	0	0	0	0	0	0	0	0
	60	6	8.0 (+15)	0	33	33	17	0	33	33	0	0	0
	92	10	4.3 (+2.9)	0	0	0	0	0	0	0	0	50	0
	92*	10	5.3 (+2.1)	0	0	0	0	0	0	0	0	50	0
	Mortalities	8 (5)	9,081 (+7,404)	100	88	100	75	75	63	50	75	88	75
CL	3	3	0.6 (+0.7)	0	0	0	0	0	0	0	0	0	0
	14	5	101 (+20)	0	0	20	0	0	0	20	0	0	0
	24	5	4,979 (+4,276)	100	100	100	80	40	0	60	20	100	0
	36	5	2,302 (+5,147)	20	20	20	20	20	20	20	0	0	0
	Mortalities	58 (6)	23,061 (+4,789)	97	95	81	60	93	55	24	5	98	90
Saline	3	5	0.6 (+0.7)	0	0	0	0	0	0	0	0	0	0
	14	5 (4)	48.0 (+9.4)	0	0	60	0	0	0	40	0	0	0
	24	5	1,721 (+858)	100	100	0	60	0	0	100	0	60	0
	36	4	21,475 (+27,519)	100	50	50	50	0	50	50	50	25	50
	Mortalities	55 (17)	18,531 (+15,105)	96	93	89	67	82	53	40	11	84	91

[1]Live fish, treated at one (or *seven) day post infection, were sampled at each time period. Fish that died from BKD at various times are included as mortalities.

[2]Number of fish examined by necropsy (number of fish measured for p57 levels).

[3]Levels of p57>4.19 ng/ml indicate presence of BKD.

[4]Discoloration and/or hypertrophy of an organ, emptied gastrointestinal system, hemorrhage in one or more tissues (intestine, stomach, pyloric caeca, peritoneal fat), the presence of ascites, fibrinous casts, intraperitoneal clots, external hemorrhage and ulcers at injection site constituted a pathological change. The % observed change was calculated by 100 X (#changes/total #fish) at each time point.

and darkened skin were seen in some fish and petechial hemorrhage on the head and at the base of fins was seen in all fish. The fish in the saline group demonstrated many of the signs noted above but the severity of the disease was reduced, as indicated by the absence of splenomegaly and internal hemorrhage. Most fish died prior to the last sampling and by 36 days, only one fish of five in the CL group and two fish of four in the saline group had the typical pathology of BKD.

In addition to live sampling, all dead fish were examined for gross pathological changes. Regardless of treatment group, the mortalities exhibited analogous pathological changes with one exception (Table 5.2). There was an increased incidence of peritoneal blood clots seen in the erythromycin treatment (75%) compared with the other three treatments (5-22%).

Other mortalities. Although BKD was responsible for all deaths occurring in fish given CL or saline, three fish from the antibiotic treatments (two from the erythromycin treatment and one from the EL treatment) and one uninfected fish died from other causes. These fish all lacked measurable p57 levels and inoculation of kidney tissue on KDM-2 failed to reveal *R. salmoninarum* or other bacteria.

Feeding activity. In three of the the six tanks in the CL group, there was a cessation of feeding activity by 20 days post infection which resembled acute toxic shock and preceded death by four to twenty days. The feeding times for the fish in these tanks (>300 sec) was significantly different ($P<0.001$) from the

other three tanks of fish in the CL group and from the fish given saline. Mortality was 100% in the affected tanks, although this was insignificant ($P>0.05$) compared with the other tanks in this group. Extraneous toxic contaminants were not considered a problem because the same solutions injected for infection or for treatment were given to all fish within a treatment group. During injections, fish were subjected to minimal stress from anethesia and handling, and recovery was rapid in all 49 tanks. Lethargy was not noted until about 13 days post infection. Gross pathological examination provided no clue as to the toxemia.

Weight. The effect of treatment (EL, erythromycin, CL, saline) on the growth of the infected and uninfected fish was insignificant ($P>0.05$) at 92 days. However, differences in weight gain were noticeable in the non-infected fish after 155 days (Table 5.3). Treatment with unencapsulated erythromycin reduced growth by an average of 2 g (6%) compared to treatment with EL and significantly reduced growth by an average of 4 g (11%) compared to treatment with saline or CL ($P>0.04$).

Table 5.3. Weights of the uninfected control fish given erythromycin liposomes (EL), erythromycin (15 mg drug/kg fish, equivalent to EL), control liposomes (CL), or saline at 155 days after initiation of study.

Treatment	No. fish	Weight (±SD)[1] (g)
Erythromycin	20	31.6 (±5.3)[a]
EL	30	33.8 (±4.7)[ab]
CL	30	35.3 (±3.8)[b]
Saline	10	35.8 (±4.4)[b]

[1]Values followed by different letters are significantly different ($P \leq 0.04$).

Discussion

The results from this study suggest that antibiotic delivery by liposomes is an effective means for controlling BKD. Erythromycin liposomes given at one or seven days after infection reduced mortality by BKD from 82% to 14%. The number of low level infections detected at 92 days was reduced by 43-75% compared to unencapsulated erythromycin. This suggests that liposome encapsulation improved intracellular killing of *R. salmoninarum*. The increased incidence of intraperitoneal blood clots (infected fish) and reduced growth (uninfected fish) in animals given unencapsulated erythromycin suggests that liposome encapsulation may also decrease antibiotic side effects.

Although there was a reduction in numbers of low level infections, there was no difference in overall mortality between the EL and erythromycin treatments. This might be attributed to the liposome formulation which may have reduced the full potential of liposome delivery of erythromycin. Although the components of the liposomes (DPPC:cholesterol) without entrapped erythromycin did not result in higher mortality, their use aggravated disease progression and resulted in earlier death. Gilbreath et al. (1989) discovered some phospholipids commonly used to make liposomes inhibit one or more early events in the activation of murine peritoneal macrophages by lymphokines and reduce cytotoxicity against the intracellular parasite, *Leishmania major*. They found that the nature of the phospholipid head groups and the degree of fatty acid unsaturation in the liposome

composition each significantly affect macrophage microbicidal activity. Mukherjee et al. (1988) demonstrated that the microviscosity of murine macrophage membranes is altered by addition of liposomes containing cholesterol:phosphotidylcholine. As the cholesterol to phospholipid increases there is a subsequent decrease in the number of *Leishmania donovani* internalized by macrophages. In addition to induced physiological changes, certain components in liposomes can be toxic. In murine macrophage cell lines, liposomes containing stearylamine at a concentration of 60 μM kill 50% of the macrophages (Kronberg et al., 1990). In our study, fish were injected with a total phospholipid:cholesterol content of 23 mg/fish (0.12% of body weight with 57% contributed by the synthetic saturated phospholipid DPPC and 43% from cholesterol). Based on the effectiveness of the EL in preventing mortality, these components were not toxic; however, the elevation in phospholipid:cholesterol may have directly affected macrophage activity. This would explain the earlier mean day to death seen in fish given liposomes without entrapped erythromycin.

That liposome components can affect membrane composition is particularly relevant in fish in which environmental changes such as temperature shifts generate a corresponding change in cell membrane fluidity. As temperature decreases, there is a significant reduction in the level of saturated fatty acids and an increase in polyunsaturated fatty acids (Hazel, 1979; Lie et al., 1989). It is likely that other environmental changes also result in membrane changes. In our liposome composition, the fatty acid

(palmitic acid) of DPPC was saturated and this may have resulted in membrane changes incompatible with certain enviromental changes. The stressor inducing the abrupt cessation of feeding and high mortality rate in half (three) of the tanks of fish given CL is unknown. Coincidentally, the highest mortalities for the EL group occurred in adjacent tanks.

Studies relating the effects of lipids to disease in fish are few. Sheldon and Blazer (1991) found that feeding catfish a diet high in monounsaturated and saturated fatty acids significantly reduces the ability of macrophages to kill ingested bacteria. This finding in addition to other evidence (Blazer, 1991) suggests that dietary lipids have an influence on fish macrophages, possibly through induced changes in macrophage membranes and/or the activity of soluble mediators and enzymes. The results from another study in which lipids were fed in the form of therapeutic liposomes supports this view. Austin (1985) tested liposomes made of phosphatidylcholine in an effort to improve the uptake of orally administered clindamycin in rainbow trout. Instead of reducing mortality, the liposomes had an apparently debilitating effect, with the recipient fish dying as fast, or faster, than the untreated fish. Ten days after infection with *R. salmoninarum*, fish were fed 100 mg of clindamycin/kg body weight per day which reduced mortality to 28%. In contrast, there was a mortality of 100% in the fish fed an equivalent amount of clindamycin in liposomes. The use of Tweens (compositions of saturated fatty acids), in combination with clindamycin resulted in equally poor

survival. Extrapolation of these results indicates that liposomes containing saturated fatty acids might aggravate progression of BKD.

If lipids do have an effect on macrophages, it is not apparent in healthy fish. An immersion vaccine of liposomes composed of phosphatidylcholine containing formalin-inactivated toxoids and whole cells of *Aeromonas salmonicida* given to rainbow trout ten weeks prior to a natural challenge of furunculosis reduced cumulative mortality from 37% to 11% (Rodgers, 1990). The use of liposomes in the vaccination solution was significantly more effective than the vaccine without liposomes. In addition, growth was better in the fish treated with the liposome vaccine. In our study, there were no adverse effects from liposome treatment (with or without erythromycin) in the uninfected fish and compared with fish treated with erythromycin, there was better growth.

The efficacy of liposomes as an antibiotic delivery system in fish could be improved by adjustments in their components, size, or antibiotic. Power et al. (1990) compared two preparations, multilamellar liposomes (1-5 μm) and unilamellar liposomes (250 nm), and found that there was more efficient uptake of the smaller liposomes by the various tissues. In our study, 1.48% of the available erythromycin phosphate in the swelling solution was entrapped inside large multilamellar liposomes which is low relative to other antibiotic entrappments (5-25%) with liposomes of similar composition and type (Alving and Swartz, 1984). The

use of another erythromycin salt or antibiotic which is more soluble in lipid or aqueous solution should increase antibiotic entrappment and concomittantly reduce the amount of lipid given to infected fish.

Our study, in conjunction with other work, suggests that lipids may play an important role in the activity of macrophages and hence in the control of BKD. The DPPC:cholesterol composition of our liposomes did not increase mortality but did cause earlier mortality suggesting that one or both components adversely affected macrophage killing. Erythromycin encapsulated inside the liposome contradicts the debilitating effect its components have on the ability of the fish to resist BKD. However, another formulation of liposome might improve the efficacy of liposome-encapsulated erythromycin. In our study, a single intraperitoneal injection of liposomes containing erythromycin, given at one or seven days post infection with *R. salmoninarum*, effectively reduced mortality by BKD. Although the reduction in mortality was not significantly different from an equivalent amount of unencapsulated erythromycin, results indicated that the encapsulation of erythromycin in liposomes reduced the number of low level infections.

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Summary and Conclusions

1. The 16S rRNA of *Renibacterium salmoninarum* was sequenced by reverse transcriptase to produce a nearly complete (97%) sequence of 1475 nucleotides.

2. Comparison of a 1224 nucleotide sequence containing both hypervariable and highly conserved regions (84% of the molecule) to those from genera in the three subdivisions of gram-positive bacteria verified the relationship of *R. salmoninarum* to the high G+C (or actinomycetes) subdivision. This relationship was further confirmed by signature sequences which distinguish the gram-positive subdivisions. This led to a reevaluation and publication of a new G+C value (55.5%) which substantiates the inclusion of *R. salmoninarum* in this subdivision, as opposed to the low G+C subdivison.

3. A phylogenetic tree of *R. salmoninarum* and ten actinomycetes revealed that it was most closely related to *Terrabacter tumescens*, a soil bacterium, and more distantly related to the intracellular pathogens *Mycobacterium bovis* and *Nocardia asteroides*. Based on these data and the unique phenotypic characteristics of the bacterium, the classification of *R. salmoninarum* (Sanders and Fryer, 1980) as a separate genus was warranted.

4. *Renibacterium salmoninarum* survived in significant numbers within mononuclear phagocytes (MP). A mechanism contributing

to intracellular survival included the bacterium's ability to lyse the phagosomal membrane and escape into the cytoplasm of the MP. This capability, although slightly diminished and slower, was also retained by the formalin-killed *R. salmoninarum*. Rapid exit into the cytoplasm (<4.5 hours) reduced bacterial cell wall damage suffered from extended residence in the enzymatic and hydrolytic environment of the phagosome. The same cell wall-associated factor that lysed the phagosome was likely responsible for the embedding and penetration of *R. salmoninarum* into cells resembling lymphocytes.

5. A durable bacterial cell wall also provided protection against the killing mechanisms of the MP. Intact live and formalin-killed *R. salmoninarum* were found in phagosomes in the midst of extensive enzymatic activity. Throughout the ten day interval, despite repeated phagocytosis by MP and exposure to antibiotics in the medium, numerous intact bacteria were seen.

6. Dividing bacteria inside MP were seen in the electron micrographs through 96 h; however, attempts to detect intracellular replication by quantitative measures proved unsuccessful. Because antibiotics in the media killed extracellular bacteria, the observations of dividing bacteria at 96 h and ten days suggested that intracellular replication occurred despite a simultaneous depletion in bacterial number attributed to losses of habitable MP.

7. The MP withstood the conditions of in vitro culture better than the other leucocytes and by 96 hours, >70% of the cell population consisted of MP. The MP actively phagocytosed bacteria and debris through 96 hours. The appearance of deformed and irregular bacterial forms in the phagosomes indicated that *R. salmoninarum* could be killed by the MP; however, infection with live *R. salmoninarum* significantly reduced the viability of the MP.

8. The MP phagocytosed melanin released by melanocytes and melanomacrophages during in vitro culture. Although the melanin was often seen in association with enzymatic activity in phagosomes (with or without bacteria), in deteriorating phagosomes, or in the cytoplasm, it neither contributed to nor negated the function or viability of bacteria or MP.

9. Encapsulation of erythromycin into liposomes promoted the killing of intracellular *R. salmoninarum* in vivo and reduced the mortality to $\leq$ 21% when given at 15 mg drug/kg fish at either one or seven days post infection. Ninety-two days later, only 20-40% of the fish treated with erythromycin liposomes (EL) had low level infections compared with 70-80% in the group treated with erythromycin alone. The EL also reduced the toxicity of the drug as evidenced by gross pathological changes in the infected fish and by weight gain in the uninfected control fish.

10. Fish given liposomes without erythromycin died from BKD in numbers equivalent to those given saline only (79-90%). The mean day to death was hastened significantly if the liposomes were injected at one day post infection, suggesting that the liposome components (dipalmitoyl phosphatidylcholine and cholesterol) might interfere in MP function, thus enhancing disease progress and severity.

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