

Thesis  
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**University of Stirling**  
**Institute of Aquaculture**  
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**U.K.**

**Immunopathological studies on *Renibacterium salmoninarum*  
the causative agent of bacterial kidney disease (BKD)**

**Thesis presented for the degree of  
Doctor of Philosophy**

**by**

**Carlos Salvador Farias Rojas M.V.**

**1995** 

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*To my wife Patricia and my children Denise and Felipe, the most treasured people in my life.*

## **DECLARATION**

I hereby declare that this thesis has been composed entirely by myself and has not been submitted in any previous application for a degree.

The work of which it is a record has been carried out by myself. The nature and extent of any work carried out by, or in conjunction with others, has been specifically acknowledged by reference.

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## ABBREVIATIONS

6PG	6-phosphogluconic acid
AGE	agarose gel electrophoresis
ALD	aldose
APS	ammonium persulphate
ATCC	American type culture collection
BA	blood agar
BKD	bacterial kidney disease
BSA	bovine serum albumin
CA	cytophaga agar
CFU	colony forming unit
CMI	cell mediated immunity
CNBr	cyanogen bromide
CW	cell wall
DMEM	Dulbecco's modification of Eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxynucleic acid
dNTP	deoxyribonucleoside triphosphate
DPCT	drop plate culture technique
ECP	extracellular product
ECP <sup>+</sup>	induced extracellular product
ECP <sup>-</sup>	non induced extracellular product
EDTA	ethylenediaminetetra-acetate
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EST- $\alpha$	alpha esterase
EST- $\beta$	beta esterase
ET	electrophoretic type
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
G6P	glucose-6-phosphate
HAT	hypoxanthine, aminopterin and thymidine
HI	humoral immunity
HRP	horse radish peroxidase
I.U.	international units
IFAT	indirect fluorescent antibody technique
IFN- $\gamma$	gamma interferon
Ig	immunoglobulins
IHC	immunohistochemistry
IL-1	interleukin-1
IL-2	interleukin-2
IP	intraperitoneally
IR	immunoreactivity
Kbp	kilobase pair
kDa	kilodalton
KDM-2	kidney disease medium 2
KDM-CH	kidney disease medium charcoal
Lf	lactoferrin

Tf	transferrin
LB/amp	Luria Berthini /ampicillin medium
LMWp	low molecular weight protein
MA	marine agar
MAb	monoclonal antibody
MAF	macrophage activation factor
MCA	marine cytophaga agar
MeOH	methanol
MH-C	Mueller Hilton cysteine
MHC	Major Histocompatibility Complex
MLEE	multilocus enzyme electrophoresis
mM	milli molar
MPI	mannose phosphate isomerase
MTT	3(4.5-di-methylthiazoyl-2-y)2.5 diphenyltetrazolium bromide
MW	molecular weight
MWCO	molecular weight cut off
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
p57	57 kDa renibacterial protein
PAb	polyclonal antibodies
PAGE	polyacrylamide gel electrophoresis
PAP	peroxidase anti peroxidase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylenglycol
pen/strep	potassium benzyl-penicillin / streptomycin sulphate
PG	peptidoglycan
PMN	polymorphonuclear leukocyte
PMS	phenazine methosulphate
PMSF	phenyl methyl sulphonyl fluoride
PPM	parts per million
PS	polysaccharide
R.s	<i>Renibacterium salmoninarum</i>
rpm	revolution per minute
RPS	relative percentage survival
SAS	saturated ammonium sulphate
SC	subcutaneous
SDS	sodium dodecyl sulphate
SKDM	selective kidney disease medium
SM	soultom medium
TBS	Tris- buffered saline
TCA	trichloro acetic acid
TE	Tris-EDTA buffer
TEMED	N,N,N,N,-tetromethyl-ethylendiamine
TNF	tumour necrosis factor
TNT	Tris-NaCl-Tween-20 solution
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
TSA	tryptic soya agar
TSB	tryptic soya broth
WB	Western blot

## ABSTRACT

*Renibacterium salmoninarum* isolates collected from the United States, Chile, Japan and Scotland were characterised using API ZYM, multilocus enzyme electrophoresis (MLEE), polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs). The API ZYM profiles of bacteria cultured in kidney disease medium 2 (KDM-2) without foetal calf serum (FCS) were identical in all isolates tested with only slight differences in the intensity of the reaction. However, bacteria cultured in KDM-charcoal showed a notable reduction in the production of butyrate esterase, caprylate esterase and  $\alpha$ -glucosidase, while bacteria grown in Muller Hinton Cysteine (MH-C) failed to produce acid phosphatase. The MLEE analysis showed positive reactions for four out of the seven enzymes tested (glucose 6-phosphate, 6-phosphogluconic acid, mannose phosphate isomerase and aldose). Each of these enzymes showed the same electrophoretic type in all the isolates tested.

Polyclonal antibodies anti-cell wall and anti-57 kDa protein (p57) plus seven monoclonal antibodies were produced. Three MAbs recognised peptidoglycan epitopes (14C2, 9F5, and 12B7) and three recognised p57 (5A11, 1C7/1E1 and 1C7/3D1). MAb 11D11 recognised both p57 and peptidoglycan. All MAbs were able to recognise the collection of *R. salmoninarum* isolates using ELISA, immunohistochemistry or Western blot. The crossreactivity of the PAbs and MAbs to 31 different bacterial pathogens was determined. No crossreaction was observed using MAbs C7/3D1 and 1C7/1E1. MAb 11D11 and 12B7 showed crossreaction only with *Micrococcus luteus* at very low level. PAbs showed crossreaction with several Gram positive species, particularly *M. luteus* and *Arthrobacter sp.*

The ELISA analysis of the 18 renibacterial isolates using a library of PAbs and MAbs showed no significant differences between isolates except isolate B88151 which showed a significant reduction in p57. In addition, the production of renibacterial antigens was, however, shown to be dependent on the age, isolate and culture media used.

The humoral immune response of rainbow trout to *R. salmoninarum* was investigated by injecting fish with a variety of purified antigens. The cell wall induced a strong immune response, especially when it was administered with Freund's complete adjuvant (FCA). Immunisation of trout with iron restricted extracellular products (ECP), normal ECP, live or heat killed bacterial cells also induced specific antibodies. Passive immunisation using some of these antibodies, followed by experimental challenge of fish, showed no evidence of protection, however there was a delay in the onset of mortality in some of the groups. By the time that 100 % mortality was reached in most of the experimental groups (day 34), only 57% mortality was observed in the group injected with MAb 1C7/1E1 and 60 % by the one injected with PAb anti cell wall.

The antibody probes were also utilised to screen a *R. salmoninarum* gene bank and identify a gene coding for an unknown cell wall antigen. The gene encoding for the major soluble antigen of *R. salmoninarum* (p57) was isolated and amplified from genomic renibacterial DNA using polymerase chain reaction (PCR). The gene was then inserted into the expression vector pBTtrp2 and the p57 protein synthesised by activating the tryptophan promoter.

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## GENERAL INTRODUCTION

Bacterial Kidney Disease (BKD) is a serious infectious disease of salmonid fish. It has been detected in fresh and sea water farms and also in cultured and feral species of the family Salmonidae (Earp, Ellis and Ordal, 1953; Rucker, Earp and Ordal, 1953; Pippy, 1969; Evelyn, Hoskings and Bell, 1973; Wood, 1974; Mitchum, Sherman and Baxter, 1979; Banner, Long, Fryer and Rohovec, 1986; Souter, Dwilow and Knight, 1987).

The disease, initially recorded in Scotland in 1930 (Mackie, Arkwright, Pryce-tannatt, Mottram, Johnson and Menzies, 1933; Smith, 1964), is now recognised in almost all countries with salmon industry (Fryer and Sanders, 1981; Klontz, 1983). BKD has become a particularly serious problem in the Pacific Northwest, and the Great Lakes region of the United States (Fryer and Lannan, 1993), Canada (Evelyn 1993) and Chile (Lindbergh, 1993). The disease causes up to 80 % losses in stocks of Pacific salmon (*Onchorhynchus sp*) and 40 % losses among infected Atlantic salmon (*Salmo salar*), as reported by Bruno (1986a).

BKD is caused by *Renibacterium salmoninarum* (R.s), a fastidious, slow growing, strongly Gram positive diplobacillus which produces a chronic, systemic infection characterised by granulomatous lesions in the kidney and other organs (Fryer and Lannan, 1993). The disease usually results in low grade but persistent mortality or sporadic epizootics either of which cause significant economic loss to farmers. Due to the chronic nature of the infection, fish seldom succumb before six to eighteen months of age, therefore, considerable financial investment is incurred by the time mortalities arise (Evelyn, 1988).

*R. salmoninarum* has been the subject of extensive research in the past few years aimed at prevention and control of BKD through chemotherapy (Wolf and Dunbar, 1959; Austin, 1985), diet modification (Wedemeyer and Ross, 1973; Bell, Higgs and Traxler, 1984), vaccination (Paterson, Desautels and Weber, 1981; Evelyn, Ketcheson, Prosperi-Porta, 1984a; McCarthy, Croy and Amend, 1984), genetic manipulation and broodstock selection (Suzumoto, Schreck and McIntyre, 1977), treatment of eggs and spawning adult fish (Amend and Pietsch, 1972; Ross and Smith, 1972; Bullock, Stuckey and Mulcahy, 1978, Evelyn *et al.*, 1984b). Important advances have allowed rapid and sensitive detection of *R. salmoninarum* to be developed using a variety of methods. However, in spite of this, BKD continues to be a serious problem for both wild and cultured salmonid fish around the world.

The serological and biochemical homogeneity of *R. salmoninarum* isolates has been suggested for quite some time (Earp, 1950; Ordal and Earp, 1956; Embley, Goodfellow, Minnikin and Austin, 1983; Goodfellow, Embley and Austin, 1985; Bruno and Munro 1986a). However, using monoclonal antibodies, some differences between isolates have been reported (Arakawa, Sanders and Fryer, 1987), although the antigens responsible for these differences have not been identified.

Early experimental vaccination indicated that, in all salmonid species a significant but slow to develop, non protective agglutinating antibody response is generated. This is probably due to inadequate immunising preparations, perhaps because they contain the wrong antigen, insufficient of those necessary to confer protection, or perhaps the important antigens are too weakly immunogenic or responses to them suppressed by other antigens (Munro and Bruno, 1988). More recent studies suggest that protection against *R. salmoninarum* infection could be induced, particularly in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (Paterson *et al.*, 1981; McCarthy, 1984; Campbell, Thiry and Richards, 1995).

The use of molecular biology techniques has been recently applied to the study of *R. salmoninarum* and some favourable results have already been obtained (Evenden, Gilpin and Munn, 1990; Etchegaray, Martinez, Krauskopf and Leon, 1991; Grayson, Evenden, Gilpin and Munn, 1995).

This thesis sets out to investigate the following aspects:

- To prepare a library of *R. salmoninarum* isolates and characterise these by serological, electrophoretic, and enzymatic techniques.
- To develop polyclonal and monoclonal antibodies against different extracellular and cell surface antigens.
- To analyse a number of bacterial and subunit preparations with reference to their immunogenic properties in rainbow trout and the induction of potential protective immunity against *R. salmoninarum*
- To identify, clone and express selected genes encoding for renibacterial antigens using genetic engineering.

Part of the work described in this dissertation has been performed at and in collaboration with the Department of Microbiology, Moredun Institute of Animal research, Edinburgh, Scotland U.K.; The Department of Microbiology of the University College Galway, Ireland; and PHAROS S.A. Liège, Belgium. This project was financially supported by the Government of Chile through the Ministry of National Planification and partially sponsored by PHAROS S.A. Liège, Belgium.

# CHAPTER ONE

## LITERATURE REVIEW

### 1.1 HISTORICAL BACKGROUND

Bacterial kidney disease (BKD) was first described in 1930 in Atlantic salmon (*Salmo salar*) from the rivers Dee and Spey in Scotland under the name of Dee Disease (Mackie *et al.*, 1933; Smith, 1964). In 1935, the disease was reported in Massachusetts, United States of America where it occurred in a hatchery rearing brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) (Belding and Merrill, 1935). The disease spread to the Pacific coast of America by 1936 (Earp *et al.*, 1953). It was referred to as "white boil" disease and affected brook, rainbow trout and brown trout reared in state and commercial hatcheries in the state of California. In the USA BKD problems were exacerbated prior to 1960 by the feeding of unpasteurized salmon carcasses and viscera to juvenile fish (Wood and Wallis, 1955). The first evidence of the presence of BKD in Chinook salmon (*O tshawytscha*), Coho salmon (*O kisutch*) and Sockeye salmon (*O nerka*) was reported by Rucker *et al.*, (1953) and Earp *et al.*, (1953). In Alaska the disease was not reported until 1975 (Didier, 1981).

BKD was introduced to Chile via imported eggs from the Northern hemisphere. It was first detected in 1970 in two rainbow trout hatcheries and since then the disease has been frequently found in salmon farms (Lindberg, 1993).

Between 1930 and 1961, a period during which no significant fish farming was carried out in Scotland, 149 cases of BKD were recorded in wild Atlantic salmon, principally involving the Dee river system (Smith, 1964). The first recorded outbreak in farmed fishes occurred in 1976 when the causative

bacterium was detected in two hatcheries rearing rainbow trout. This resulted, in 1978, in the addition of BKD to the list of notifiable fish diseases in Great Britain (Bruno, 1986a).

To date, the disease has been detected in 13 species of salmonid fish, and is present in virtually all parts of the world where wild and cultured salmonids exist (Fryer and Sanders, 1981; Klontz, 1983).

## **1.2 THE ETIOLOGICAL AGENT OF BKD**

### **1.2.1 Taxonomy.**

Based on cellular morphology, Ordal and Earp (1956) first suggested that the kidney disease bacterium should be placed in the genus *Corynebacterium* as *C. salmonis* and the disease was called Corynebacterial kidney disease. In addition, the bacterium was linked with *Rickettsia* (Snieszko and Griffin, 1955), *Brevibacterium* (Smith, 1964), *Listeria* (Bullock, Stuckey and Wolf, 1975), and *Lactobacillus* (Vladik, Vitovec and Carvinka, 1974).

Austin and Rogers (1980) reported biochemical differences in the kidney disease bacterium and proposed dividing the isolates into 2 groups, one of which they equated with *Corynebacterium pyogenes*, currently named *Actinomyces pyogenes* (Cowan, 1992), and the other as a novel taxon. The numerical phenotypic study of Goodfellow, Embley and Austin, (1985) confirmed the homogeneity of the taxon, and demonstrated its dissimilarity to *Lactobacillus* and *Listeria*.

Sanders and Fryer (1980) reported that the bacterium contains a peptidoglycan, cell wall sugar and G-C content different from those described for the genus *Corynebacterium*. These authors realised that the

pathogen belonged in a new, as yet undescribed genus and, therefore, placed the bacterium within the *Coryneform* group as a new genus and proposed *Renibacterium*. Thus, the pathogen became classified as *R. salmoninarum* (R.s) with the type strain termed Lea-1-72 (ATCC 33209).

## 1.2.2 Cell morphology and structure

*Renibacterium salmoninarum* (R.s) has been described as a small (0.3 - 1.5  $\mu\text{m}$  by 0.1 - 1.0  $\mu\text{m}$ ) strongly Gram positive, non sporulating coccobacillus. It is neither acid fast, nor motile. It often occurs in pairs, and short chains are sometimes observed (Sanders and Fryer, 1980). Evidence of pleomorphism, metachromatic granules and a coryneform appearance have been reported (Ordal and Earp, 1956; Smith, 1964). Transmission electron microscopy of negatively stained cells, obtained from 28 days old cultures on KDM-2, revealed the presence of pleomorphism and intracellular vacuoles and granules (Austin and Rayment, 1985).

As in almost all prokaryotic microorganisms, with the exception of Mollicutes (Mycoplasmas, Spiroplasma), the cell envelope of R.s is composed of two distinct components: an inner cytoplasmic membrane and a strong outer cell wall.

Very little information about the intracellular and cytoplasmic membrane components of R.s is available, but as in many other Gram positive bacteria, the cytoplasmic membrane controls the substrate and electron transport process of the cell and is the site of biosynthesis of lipoteichoic acid, lipomannan, and other molecules. The intracellular content includes the genetic material (DNA, RNA), nucleoproteins, enzymes, and probably also lipoteichoic acid, and lipomannan

Renibacterial cell wall is composed mainly by a unique polysaccharide which is covalently attached to the peptidoglycan (Kusser and Fiedler, 1983; Fiedler and Draxl, 1986). The bacterium also possesses short, flexible and peritrichous fimbriae less than 2 nm in diameter (Dubreuil, Jacques, Graham and Lallier, 1990). On further analysis it was observed that these fimbriae shared many characteristics with the 57 kDa protein first described by Getchell, Rohovec and Fryer, (1985) and it seems likely that this fimbrial adhesin and p57 are the same surface component.

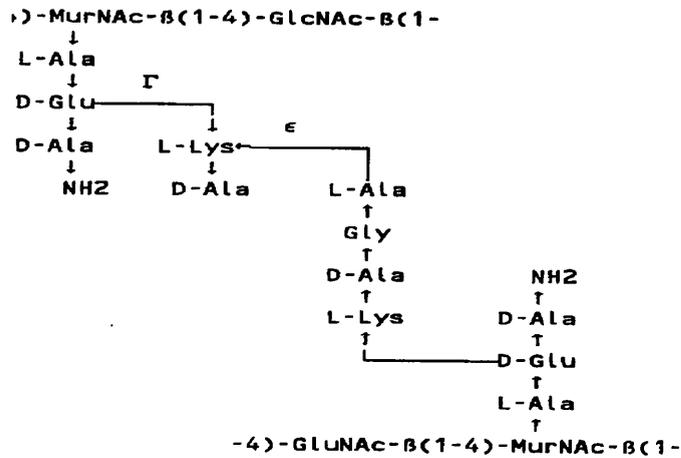
In addition, a capsular material of 50-60 nm thickness has been recently reported on the surface of the pathogen (Dubreuil, Lallier and Jacques, 1990). The exact nature of this material was not investigated but as in other encapsulated Gram positive bacteria, capsular material tends to disappear in old cultures and is generally protein or sugar in nature.

The guanine plus cytosine (G+C) content of the bacterium ranged from 52.47 to 53.55, with an average of  $53 \pm 0.46$  moles % (Sanders and Fryer, 1980). More recently, Banner, Rohovec and Fryer, (1991) reported a modification of the average G+C to 55.5 mol %.

The fatty acid analysis of 21 isolates of *R. salmoninarum* (Embley *et al*, 1983) showed that they are composed almost exclusively (92%) of methyl branched fatty acids. Over 81% of the total fatty acids were composed of the lower melting point anteiso acids, which may contribute to membrane fluidity at low temperature. No mycolic acid, phosphatidylinositol or related dimannosides, or diamminobutyric acid have been reported. Straight chain fatty acids generally accounted for 1% of the total fatty acid, and unsaturated fatty acids were not detected at all. All strains examined contained diphosphatidylglycerol, two major and six or seven minor glycolipids and two unidentified minor phospholipids. In addition, a large amount of glycolipid on the surface of the pathogen has been reported by Collins, (1982).

Chemical analysis of *R. salmoninarum* cell wall performed by Kusser and Fiedler, (1983) showed that two components have been found to be characteristic of this pathogen: a unique peptidoglycan and an unusual polysaccharide. The phosphate content of the cell wall was determined to be 0.1 mmol/mg. Further analysis demonstrated that the polysaccharide amounts to more than 60% of the dry weight of the cell wall and is covalently linked to the peptidoglycan (Fiedler and Draxl, 1986). No mycolic acid (Fryer and Sander, 1981) or teichoic acids (Fiedler and Draxl, 1986) has been detected, contrary to the observations of Fryer and Sanders (1981), who suggested the presence of low amounts of the latter.

Renibacterial peptidoglycan (PG) contains D-alanine, D-glutamic acid and glycine. The diamino acid corresponded to L-lysine (Sanders and Fryer, 1980). The PG content in the type strain (ATCC 33209) is about 230 mmol/mg of cell wall. As a consequence of the low amount of peptidoglycan in the cell wall and the high degree of substitution of N-acetyl muramyl residues, Fiedler and Draxl (1986) suggested that the surface of a renibacterium cell is formed mainly by its unique polysaccharide. The same authors reported that the composition of the peptidoglycan and structure of the cell wall derived from cells grown in either KDM-2 or SKDM were found to be identical. It contains lysine in the third position of the peptide subunit, a glycyl alanine interpeptide bridge between lysine and the D- alanine of adjacent peptide subunits and a D- alanine- amine substitution at the  $\alpha$ -carboxyl group of D-glutamic acid in position 2 of the peptide side chain. A diagram showing the structure of the *R. salmoninarum* peptidoglycan is presented in Figure 1.1.



**Figure 1.1** Proposed primary structure of the peptidoglycan of *R. salmoninarum* isolate ATCC 33209 (Kusser and Fiedler, 1983).

The lysine containing PG of *R.s* is classified according to Schleifer and Kandler (1972) as a type of A3 $\alpha$  variation. The biochemical composition of the renibacterium peptidoglycan in a number of different isolates showed a marked similarity (Kusser and Draxl, 1983).

The accessory cell wall polysaccharide of *R.s* includes similar molecular weight types each containing an approximate MW of 10 kDa. Renibacterial polysaccharide is composed mainly of galactose (1.3 mmol/mg of cell wall), comprising 25% of the dry weight of the cell wall. In addition, N-acetylglucosamine, N-acetylfucosamine (2-acetamide-2,6-dideoxy-galactose), and rhamnose (6-deoxy-galactose) are also present in an amount of 10% to 20% for each component as compared with galactose. Galactose appeared to be in a furanoid configuration (Fiedler and Draxl, 1986). These findings are in contrast to the data of Sanders and Fryer (1980), who found glucose, mannose, arabinose and rhamnose as the major sugars in the renibacterial cell walls.

Recently, the polysaccharide has been analysed with respect to monosaccharide and glycosyl linkage composition. (Sørum and Robertsen, 1994). Alditol acetate analysis of acid hydrolysed polysaccharide showed the presence of galactose, rhamnose, glucose, mannose, N acetyl-glucosamine, and N acetyl-fucosamine in an approximate ratio of 5:1:0.5:0.5:1.5:1. Methylation analysis showed 1,3-galactofuranosyl, 1,3,6-galactopiranosyl; terminal rhamnosyl; and terminal galactofuranosyl residues in a ratio of 3:1:0.5:0.5

The polysaccharide appeared to be similar in the 13 different renibacterial isolates tested with respect to both its quantity in the cell and in the chemical composition. Based on these findings it was suggested that this is a unique cell wall polysaccharide (Fiedler and Draxl, 1986).

Immunisation experiments using trypsinized R.s cells (Sørum and Robertsen, 1994) and trypsinized cell walls (Fiedler and Draxl, 1986) showed the formation of antibodies to polysaccharides in rabbits. No information about its immunogenicity in fish has been reported to date.

Some proteins have also been associated with the renibacterial cell wall or ECP. The 57 Kda protein is the best known of all (Getchell *et al.*, 1985), however some others have been detected by culturing the bacterium in special conditions or by enzymatic techniques, these include a low molecular weight protein (Thiry, personal communication), 70 Kda protein (Kusser and Fiedler, 1983; Dubreuil *et al.*, 1990), proteases of MW higher than 100 Kda (Rockey, Wiens, Cook and Kaattari, 1991) and haemolysin (Evdenden, Gilpin and Munn, 1993).

P57 is the predominant protein antigen found on the bacterial cell surface and is the major component of the extracellular protein (ECP) isolated from bacterial culture supernatant (Getchell *et al.*, 1985; Wiens and Kaattari, 1989, 1991). High concentrations of a 57 kDa protein (1 mg ml<sup>-1</sup> of serum), can be found in

experimentally and naturally infected fish tissue and sera (Turaga, Wiens and Kaattari, 1987a; Wiens and Kaatari, 1989).

Recently, Bandin *et al.*,(1992) have reported the presence of a vascular permeability factor in the ECP of ten isolates of *R.s.* They inoculated rabbits with ECP and observed the development of haemorrhagic and/or oedematous zones at the injection site with a diameter ranging from 10 to 30 mm. However, the ECP samples did not display toxic effects in fish at the same dose as inoculated into rabbits (180 - 400 µg protein per 0.1 ml).

### **1.2.3 Habitat.**

*R. salmoninarum* is an obligate pathogen of farmed and wild salmonid fish found intra and extracellularly. There has been no evidence to suggest that this bacterium is a component of the normal aquatic microflora. On the contrary, the organism has so far only been found routinely associated with asymptomatic and clinically diseased fish (Austin and Rayment, 1985). The precise source of infection is unclear, but may include clinically or asymptomatic diseased fish (Wood and Wallis, 1955; Mitchum *et al.*, 1979; Paterson, Gallant, Desautels and Marshall, 1979; Fryer and Sanders, 1981; Austin and Rayment 1985), although *R.s* has the potential to survive outside of fish for a limited period (Austin and Rayment, 1985). The exact ecological status of *R.s* is unclear. To date, many studies have been limited by the relative lack of sensitivity and selectivity available to detect the pathogen in the environment.

#### 1.2.4 Cultural characteristics.

The first successful attempts to culture *R.s* were performed using minced chick embryo tissue embedded in 1% agar and Dorset's medium (Earp *et al.*, 1953), Cysteine blood agar medium (Ordal and Earp, 1956), and Mueller Hinton agar supplemented with L-cysteine (Bullock, Stuckey and Chen, 1974). The substitution of blood in this medium by serum (Evelyn, 1977) has been widely used and is referred to as kidney disease medium 2 (KDM-2).

More recently a semi-defined medium was devised by Embley, Goodfellow and Austin, (1982) which was recommended only for studies on the growth requirement of the bacterium, due to its low recovery of colony forming units (CFUs) compared with MH-C or KDM-2. The formulation of a selective medium (SKDM) by Austin, Embley and Goodfellow (1983) allowed the effective isolation of the pathogen from diluted samples and permitted the recovery of the *R. salmoninarum* from seeded river water and from kidney and faeces of experimentally infected fish.

SKDM enhanced the selective isolation of pathogen from infected animals because of the addition of antibiotics such as D-cycloserine, polymyxin B sulphate and oxolinic acid. These compounds were found to be antibacterial for most of the common contaminating organisms, while *R.s* was insensitive.

The replacement of the serum in KDM-2 with activated charcoal (Daly and Stevenson, 1985) generated an effective and less expensive culture medium referred to as KDM-Charcoal. Of all culture media reported so far to culture *R.s*, those currently in use today are KDM-2, KDM-Charcoal, MH-C and less frequently SKDM.

The effectiveness of these common media for the primary isolation of the pathogen over a 14 weeks incubation period was compared by Gudmundsdottir, Helgason and Benediktsdottir,(1991). Of all positive samples, 91% were positive on SKDM, 60% on SKDM-C and 35% on KDM-2.

On cysteine serum agar, renibacterial colonies are circular and convex, white to creamy yellow, and of varying sizes (Smith, 1964). On KDM-2, the colonies are cream, non pigmented, shiny, smooth, round, raised, entire, 2 mm diameter colonies after incubation at 15 °C for 20 days. Old cultures (more than 12 weeks) may become granular or crystalline in appearance (Austin and Austin 1993). On Mueller Hinton agar supplemented with L- cysteine, virulent strains are sticky, smooth, slightly, raised, creamy yellow and shiny (Bruno, 1988).

The optimal growth temperature for the bacterium is 15 °C. They grow very slowly at 5 °C and 22 °C, and not at all at 37 °C. (Smith, 1964).

Primary isolation of R.s requires the use of peptone saline solution (0.1% w/v peptone, 0.85% w/v NaCl) as a diluent to remove any inhibitory factors against the pathogen which may be present in kidney tissue (Evelyn, Ketcheson and Proserpi-Porta, 1981).

The median incubation time for the primary isolation of the bacterium on S-KDM is 5 weeks for samples with confluent growth, 6 weeks for samples with 10 - 500 CFUs and 8 weeks for samples with one to nine CFUs. (Benediktsdottir, Helgason and Gudmundsdottir, 1991). These authors also reported that the growth pattern of the bacterium at primary isolation was characterised by a prolonged lag phase followed by a relatively rapid growth in one week from microscopic to macroscopic size of the colonies.

The primary isolation of the bacteria from infected fish in KDM-2 required 3-4 weeks (Evelyn, 1977). The bacterium requires cysteine to grow (Ordal and Earp, 1956) which may be used to synthesise the 57 kDa protein, as suggested by Bandin *et al.*, (1992).

The major problem working with R.s is its fastidious and slow growing nature in artificial laboratory media. This fact allows other contaminating bacteria and fungi to overgrow the culture quickly. The development of SKDM has been of particular help in overcoming this problem.

Evelyn (1977), recommended the drop plate culture technique (DPCT) over the traditional spread plate method to work with R.s. The DPCT consists in the dilution of the bacterial sample, one drop (50 µl) of each dilution is then plate it out in the selected culture media. This technique reduced considerably the chances of overgrowth of the cultures with contaminating microorganisms but the method is very time consuming and practically impossible to perform with large numbers of samples.

An improved culturing technique developed by Evelyn, Prosperi-Porta and Hetcheson, (1989) accelerates the growth of the pathogen in KDM-2 considerably. The technique is based on a spent culture of R.s which is used as a source of "feeder" micro-organisms.

### **1.2.5.- Biochemical characteristics**

Ordal and Earp (1956) demonstrated catalase and proteolytic activity of the bacterium and realised that there was a growth requirement for cysteine. Embley *et al.*, (1983) and Goodfellow *et al.*, (1985) fully described the biochemical characteristics of the bacterium.

Bruno and Munro (1986a) compared the biochemical properties of 26 isolates of R.s from natural outbreaks from U.K., Canada, and USA, including the American type strain ATCC 33209. They found properties not previously recorded for this bacterium including haemolytic and proteolytic activity, DNase, the presence of glycogen in cells, and negative reactions in tests for nitrates, tween-80 hydrolysis, amylase, bile solubility, arginine hydrolysis and phosphatase.

The enzymatic analysis of R.s by API ZYM showed the following profile: alkaline phosphatase, caprylate esterase, leucine arylamidase, trypsin, acid phosphatase, phosphoamidase,  $\beta$ -glucosidase, and  $\alpha$ -galactosidase (Austin and Austin,1987). Other authors have reported differences in some enzymatic activities, probably due to the geographical distribution of the pathogen (Bruno and Munro, 1986; Sakai, Soliman, Yoshida and Kobayashi, 1993).

### **1.3.- EPIZOOTIOLOGY**

#### **1.3.1. Host and geographical distribution**

BKD has been reported to occur naturally in 13 species of the family Salmoninidae including genus *Oncorhynchus*, *Salmo* and *Salvelinus*. It is encountered among fish confined to rearing facilities, but wild fish are also affected (Ellis, Novolty and Harvell, 1978; Banner *et al.*, 1986).

The etiological agent, *R. salmoninarum* (R.s), has been detected also in feral populations (Pippy, 1969; Evelyn *et al.*, 1973; Mitchum *et al.*, 1979; Paterson *et al.*, 1979). It has been reported in Canada (Evelyn *et al.*, 1973), France (De Kinkelin,1974), Germany (Pfeil-Putzien, Hoffmann and Popp, 1985), Iceland (Evelyn,1977), Italy (Ghittino,Andrueto and Vigliani, 1977), Japan (Kimura and Awakura,1977; Awakura,1978), Spain (Martinez-Millan,1977), Yugoslavia (Fijan,1977), Scotland (Smith,1964; Bruno,

1986a), Ireland (Fryer and Sanders, 1981; Hoffman, Popp and van de Graff, 1984) and Chile (Sanders and Barros, 1986; Lindbergh, 1993).

The organism has also been isolated from non-salmonid fish. It was recovered from Pacific herring (*Clupea harengus pallasii* L) present in cages in which coho salmon with clinical BKD were being held (Munro and Bruno, 1988). It has been also found in the Danube salmon (*Hucho*) (Pfeil-Putzien, *et al.*, 1985) and the grayling (*Thymallus*) (Kettler, Pfeil-Putzien and Hoffmann, 1986).

The disease has been experimentally reproduced in *Pinephales promelas* (*Rafinesque*) the common shiner, (*Notropis cornutus*; Hicks, Daly and Ostland, 1986), sablefish, (*Anoploma fimbria*; Bell, Hoffmann and Brown, 1990) and Pacific herring (*Clupea harengus pallasii*; Traxler and Bell, 1988)

Resistant species of fish to the bacterium include the Pacific lamprey, *Lamprata tridentata* (Bell and Traxler, 1986) and carp, *Cyprinus carpio* (Sakai, Ogasawara, Atsuta and Kobayashi, 1989).

R.s antigens have been detected in greenling (*Hexagrammos otakii*), Japanese sculpin (*Cottus japonicus*), and in scallops (*Patinpecten yessoensis*) collected in Japanese waters from the vicinity of netpens containing R.s infected coho salmon (Sakai and Kobayashi, 1992). The possible role of vectors such as molluscs (Sakai and Kobayashi, 1992) or other non-salmonid fish hosts (Evelyn, 1988; Munro and Bruno, 1988) has been suggested.

### **1.3.2. Transmission**

Although the principal means of transmission is considered to be vertical (Allison, 1958; Evelyn *et al.*, 1984b), horizontal transmission within production facilities, as well as from farmed to feral fish stocks

have been adduced (Mitchum and Cherman, 1981). Horizontal transmission has been reported in both fresh water and sea water, as well as between different species of salmonids (Bell, 1961). The disease has been experimentally transmitted via ingestion of contaminated flesh and viscera (Wood and Wallis, 1955), via the skin and eye (Hendricks and Leek, 1975; Hoffman *et al.*, 1984), cohabitation and via intraperitoneal inoculation. Furthermore, the pathogen has been detected in the faeces of infected fish (Mitchum *et al.*, 1979; Mitchum and Sherman, 1981).

Much of the work concerning transmission has been performed on vertical transmission which appears to be the principal way in which the pathogen is transmitted in the wild. Egg infection studies suggest that only a few organisms are needed for infection (Brown, Albright and Evelyn, 1990). The bacterium has been detected in 11.6-15.1% of eggs from a coho salmon which was infected with BKD. The infection was sometimes so severe that the coelomic fluids were cloudy because of the high numbers of the organism (Evelyn *et al.*, 1984b). These authors suggested that R.s was present in the yolk of the eggs. On hatching, progeny shed bacteria which are capable of persisting outside of the host for sufficient duration of time to invade and infect other susceptible individuals to establish an enzootic state.

The pathogen is able to be transmitted to progeny of eggs even after surface disinfection with povidone iodine (Bullock *et al.*, 1978; Evelyn *et al.*, 1986a,b,c), to which R.s has been shown to be susceptible (Amend and Pietseh, 1972). The stage at which R.s is naturally incorporated into the eggs is unknown, but Bruno and Munro (1986b) observed the bacterium in maturing oogonia of experimentally infected rainbow trout. Evelyn *et al.* (1986c) infected eggs by immersion in high concentrations of R.s suggesting that transmission could also take place through contact of the eggs with infected coelomic fluid. The male fish is not believed to pose a significant risk of vertical transmission because it has not been possible to infect eggs with heavily contaminated milt or to increase the egg infection rate by fertilising eggs while they are immersed in heavy suspension of renibacterial cells (Evelyn *et al.*, 1986c).

### 1.3.3. Pathogenesis

Bacterial kidney disease of salmonid fish is a very complex disease which appears to exploit a variety of pathogenic mechanisms (Kaatari *et al.*, 1989). Its manifestations depend on the species of fish affected, the genetic constitution of the host, virulence of the pathogen, and a series of environmental factors such as water temperature, salinity, water hardness, and diet.

Within the genus *Oncorhynchus*, Sanders, Pilcher and Fryer, (1978) and Bell *et al.*, (1984) consider pink salmon (*O. gorbushka*) the most susceptible followed by sockeye (*O. nerka*), chinook (*O. tshawytscha*) and perhaps the chum (*O. keta*) with coho (*O. kisutch*) the least susceptible. With regard to rainbow trout (*O. mykiss*) and Atlantic salmon (*Salmo salar*), Bruno (1988) reported these species to have a moderate resistance.

Suzumoto *et al.*,(1977) have demonstrated that the genetic makeup of the juvenile coho salmon may be responsible for their resistance to BKD. They demonstrated that among the three transferrin genotypes of coho salmon (e.g., AA, AC, CC), the AA genotype was the most susceptible while the CC genotype was the most resistant to experimental infection.

The virulence of the pathogen appears to be related to its autoagglutinating properties, hydrophobicity of the bacterial cell surface components, and the presence of a 57 Kda soluble protein (Bruno, 1988,1990). The same authors reported that highly virulent isolates exhibit cultural characteristics different to those of low virulence. Virulent isolates grow as sticky and autoagglutinating cultures with the production of sediment in a relatively clear medium, while low virulence isolates grow as a uniform suspension, and are non agglutinating. The presence of fimbrial protein was found only in virulent isolates.

Natural outbreaks of BKD are strongly seasonally related, occurring from late autumn to early spring. As the temperature increases from spring to summer the prevalence of diseased fish decreases (Bruno, 1986a,b,c). Transmission of BKD was shown to be increased during the autumn and the winter when the water temperature declined (Belding and Merrill, 1935). Deaths occur 30-35 days post exposure at temperatures greater than 11°C and 60-90 days post-exposure at 7.2-10°C (Wood, 1974). Sanders *et al.*, (1978) also observed that the infection progressed more rapidly at higher temperature (15-20.5 °C) but noted that the greatest mortality occurs at or below 12°C.

Bacterial kidney disease has been diagnosed in fish following movement from fresh to sea water (Earp *et al.*, 1953; Bell 1961). Fryer and Sanders, (1981) found increased BKD related mortality (17.2% vs 4%) among coho salmon transferred to saltwater holding tanks over that experienced by a comparable group held in fresh water. Similar results were obtained by Sanders, Long, Arakawa, Bartholomew and Rohovec, (1992) who observed increased mortality (45% vs 6%) and increased incidence of detectable R.s (46% vs 9%) among chinook salmon held in salt water when compared with a control group of the same fish held in fresh water.

The disease may be of paramount importance to the ability of fish to become established at sea and survive in the oceanic environment (Fryer and Sanders, 1981).

The nutritional status of fish may be an important factor in the manifestation of BKD. Infected fish have been shown to have lower levels of vitamin A, zinc, and iron than non-infected fish (Paterson, Lall and Desautels, 1981). In nutritional experiments which followed, these authors showed reduced levels of BKD in fish which had been fed with a high level of trace element i.e iron, copper, manganese, cobalt and iodine, or lowered calcium ( Paterson, Lall, Airdrie, Greenham and Poy, 1985).

Experimentally infecting fish by feeding infected fish has yielded varying results. 100% transmission of BKD was accomplished by feeding infected flesh and viscera of adult Chinook salmon to young Chinook salmon (Wood and Wallis, 1955). Smith, (1964) however, found that fish could not be infected, if the infected fish tissues were stored at -17 to -20 °C for more than four months.

Parasites which cause skin lesions and skin penetration of parasites have been suggested as being vectors in the transmission of BKD (Bullock, Stuckey and Wolf, 1975).

The 57 kDa protein and its breakdown products, also designated as fimbrae, p57, haemagglutinin, and lethal toxin, have been found closely related to the virulence of the pathogen. Low virulence isolates lack fimbrae, while highly virulent ones exhibit a great amount of it (Bruno, 1990).

Although the initial focus of the infection appears to be the kidney (Wood and Yasutake, 1956) with subsequent haematological dysfunction, the disease eventually becomes systemic, with lesions occurring in many organs and tissues (Turaga, Wiens and Kaattari, 1987a).

Host cells resembling macrophages or polymorphonuclear leukocytes with phagocytosed bacterial cells often appeared in lesions. Electron microscopy of these cells showed the morphological integrity of the phagocytosed bacterial cells and evidence of their active division, indicating intracellular survival (Young and Chapman, 1978).

## 1.4 PATHOLOGY

The predilection sites for the bacterium are the anterior and posterior kidney. Due to the inherent functional reserves of renal tissue, coupled with nitrogenous waste and monovalent ion excretion predominantly via the gills, clinical manifestation of BKD occurs only in the terminal stages of infection and reflects osmoregulatory, coupled with acid-base imbalances. Moribund fish commonly appear lethargic, anaemic and inappetent. The infection often results in a significant decrease in the haematocrit, red cell diameter, haemoglobin and an increase in the number of monocytes, thrombocytes and neutrophils. A significant increase in bilirubin, blood urea nitrogen, and potassium were also found in infected fish, along with a decrease in total serum protein, cholesterol and sodium (Bruno, 1986c).

Post mortem findings of fish infected with BKD are variable depending on the species of salmonid affected (Kaatari *et al.*, 1989; Sakai, Atsuta and Kobayashi, 1991a,b; Raverty, 1992). Gross external lesions are variable, ranging from no disease signs to a distended abdomen (ascites), exophthalmia, cutaneous ecchymosis, petechiae, especially around the bases of the fins and the presence of blood filled blisters on the flanks and the presence of ulcer abscesses. (Belding and Merrill, 1935; Hedricks and Leek, 1975; Fryer and Sanders, 1981; Klontz, 1983; Ferguson, 1992).

Bruno (1986c) and Raverty (1992) fully described the gross and histological lesions of an experimentally developed BKD in rainbow trout and salmonid species. Internal gross lesions of fish appear to be species-specific (Raverty, 1992). In Pacific salmon, lesions include diffuse, subacute to chronically active fibroperitonitis, nephritis, splenitis, and pericarditis; whereas in Atlantic salmon, fish featured multifocal chronic, pyogranulomata, predominantly in the reticuloendothelial tissue with cicatrization and resolution of lesions (Wood and Yasutake, 1956; Bruno, 1986c).

Delayed type hypersensitivity reaction (DTH III) have been suggested as a possible pathogenic mechanism of BKD, based on the strong granulomatous response with scant bacterial cells. Coagulopathies, probably due to mechanical endothelial injury have also been reported to occur in experimentally induced BKD (Raverty, 1992). Death of infected fish is usually due to the obliteration of normal kidney and liver structure by the dissemination of large granulomatous lesions. Impaired renal function and heart failure was also considered to be a contributing factor to mortality due to the invasion of myocardium by phagocytic cells containing the bacterium (Bruno, 1986c).

## 1.5 Diagnosis

Usually the diagnosis of BKD has been performed by means of the macro and microscopic examination of the tissue and supported by the Gram's stain. Gross and histopathological lesions are characteristic but not pathognomonic of the disease. They are present only in the latest stages of the infection and generally they are absent in asymptotically diseased fish and carriers.

Culturing technique have improved diagnosis but its effectiveness as a diagnostic procedure is marred by the slow growth of the organism which may take up to 8 weeks.

A variety of serological tests have also been developed. They include immunodiffusion (Chen *et al.*, 1974; coagglutination (Kimura and Yoshimizu, 1981a,b), direct or indirect fluorescent antibody techniques (Wedemeyer and Ross, 1973; Mitchum *et al.*, 1979; Bullock and Stunckey, 1975; Austin, Frist and Rayment, 1985; Yoshimizu *et al.*, 1987), ELISA (Dixon, 1987b; Turaga *et al.*, 1987a; Pascho and Mulcahy, 1987; Kaattari *et al.*, 1989; Hsu *et al.*, 1991; Rockey *et al.*, 1991), immunohistochemistry (Hoffmann *et al.*, 1989; Farias and Cubillos, 1991; Jansson *et al.*, 1991), Western blot (Sakai *et al.*, 1987; Griffiths and Lynch, 1991).

Serological methods using polyclonal antibodies often involve crossreactions with other bacterial species. This problem was initially overcome by preabsorbing the antisera (Dixon, 1987a) and more recently through utilising monoclonal antibodies directed against specific renibacterial epitopes (Hsu *et al.*, 1991; Rockey *et al.* 1991).

The most recent approach for detecting the pathogen involves the use of gene probes such as DNA, RNA or oligonucleotide sequences specific for R.s. Some initial successes with these type of techniques has been published (Brown *et al.*, 1994; Leon *et al.*, 1993).

## **1.6 Treatment and control**

BKD is one of the most difficult bacterial fish diseases to control. The reason for this may be that the bacterium appears to exploit a variety of pathogenic mechanisms to infect and survive within the fish. Its ability to survive and even multiply within phagocytic and blood monocytes has been reported (Young and Chapmann, 1978) and has often been cited as a major factor limiting the usefulness of many of the therapeutic measures that have been tested (Wolf and Dunbar, 1959; Getchell *et al.*, 1985; Sakai *et al.*, 1986). This is the reason why the control of BKD by chemotherapy provides only a poor and temporary relief (Fryer and Sanders, 1981).

Control of BKD by immunisation has not been successful. Rainbow trout, Atlantic and Pacific salmon are able to develop antibodies against certain renibacterial extracellular antigens but only rainbow trout and Atlantic salmon appear to develop a limited degree of protective immunity (Paterson *et al.*, 1981,1985; McCarthy *et al.*, 1984; Campbell *et al.*, 1994).

BKD has been considered as one of the most difficult bacterial fish diseases to treat (Bullock *et al.*, 1975; Fryer and Sanders, 1981). Treatment by chemotherapy is of limited effectiveness because of the intracellular nature of the pathogen (Young and Chapman, 1978) and the failure of many antibiotics to reach the actual loci of the infection (Evelyn, 1993). Antimicrobial therapy has proven effective in reducing disease levels but unsuccessful in eliminating the pathogen from host populations (Wolf and Dunbar, 1959; Austin, 1985).

Wolf and Dunbar (1959) compared 34 compounds and concluded that erythromycin, dosed at 100 mg/kg body weight of fish per day for 21 days, gave the most promising results. This concentration of erythromycin was confirmed by Austin *et al.*, (1985) although it was suggested that treatment need only be continued for 10 days. The former authors, showed that sodium penicillin and the macrolide antibiotics, specifically erythromycin phosphate, erythromycin thiocyanate, clindamycin and spiramycin, were the antibiotics of choice for therapeutic purposes, while erythromycin phosphate, erythromycin thiocyanate, rifampicin, clindamycin and kitasamicyn were the antibiotics of potential value for prophylaxis.

Disinfection of egg surfaces has also been utilised to control BKD. Iodophores, at 25-100 mg/ml for 5 minutes have proved beneficial in reducing transmission of the disease (Amend and Pietsch, 1972; Ross and Smith, 1972; Bullock *et al.*, 1978), although this does not eliminate the pathogen from inside eggs (Evelyn *et al.*, 1984b). Disinfection of the eggs with 100 ppm organic iodine for 15 minutes as reported by Ross and Smith, (1972) was not useful for avoiding carrier fish. Groman and Klontz (1983) and later Evelyn *et al.*, (1986c) concluded that erythromycin did not enter the yolk but remained in the perivitelline fluid and diffused out after the eggs were placed in antibiotic free running water.

As with treatment with iodophores, water hardening of eggs for 30 minutes in 1-2 mg l<sup>-1</sup> of erythromycin is ineffective in eliminating the bacterial infection. Bullock and Leek (1986) and Evelyn *et al.*, (1986c)

demonstrated that although the antibiotic is taken up by the egg during water hardening, the intra-ovum antibiotic concentration drops too rapidly to provide a bactericidal effect.

The results from experimental vaccination indicate the production of a significant but slow to develop non protective agglutinating antibody response (Munro and Bruno, 1988). The studies of Hastings and Ellis, (1988) indicate that fish may be much more restricted in their abilities to produce antibodies with a wide variety of specificities, as opposed to that observed in mammals. Moreover, there may be intrinsic differences between Pacific salmon, Atlantic salmon and rainbow trout in their capacity to develop a protective immune response to the pathogen, as suggested by Paterson *et al.*, 1981; Evelyn, 1984; McCarthy *et al.*, (1984) respectively.

Although only few accounts of attempts at immunisation for BKD have been published, there are data suggesting protection may be most readily achieved in Atlantic salmon and rainbow trout rather than Pacific salmon (Kaattari *et al.*, 1989).

In the U.K., measures Employing the Power of the Disease of Fish Act, 1937 and 1983, states that when infection is found, a Designated Area Order (DAO) is made under the act which is used to regulate all movements of live fish and ova from infected sites (Bruno,1988). As there are so many waters in use for fish farming, Department policy is to eliminate infection, or when farmers cannot afford to do this, to restrict its spread as much as possible. Breeding from infected stocks is prevented, and to prevent the spread of disease, the movement of infected fish is restricted (Bruno 1988).

At the moment control of BKD is best achieved by avoidance. Evelyn, (1993) suggested a series of preventive measures to avoid the transmission and reduce the incidence of the disease which are summarised below:

Vertical transmission could be prevented by using seedstock (eggs and smolts) derived from broodstock that have been screened and found free of the pathogen. The sample of choice for screening is coelomic fluid because it is known to be a source of infection for eggs and because its *Renibacterium* load appears to reflect the infection status of ovarian tissue. Female broodstock should be injected with erythromycin (20mg/Kg fish) prior to spawning. Penicillin G, Oxytetracycline, cephadrine and rifampicin (Brown *et al.*, 1990) also have shown to be useful for this purpose. Eggs from treated broodstock should be surface disinfected with an iodophore to eliminate any surface-borne renibacterial cells. Those eggs should be grown in ground water or in surface water that has been treated to kill fish pathogens as suggested by Elliot *et al.*, 1989) or is devoid of salmonids. Considerations should be also be given to treating fry with orally administered erythromycin shortly after first feeding.

In attempting to avoid horizontal infections, the farm should be located well away from major salmon rivers or from other salmonid farms. Also it is highly advisable that only one age group of fish is held on any given farm at any given time. This procedure prevents one age group of fish (usually the older fish) from infecting another age group (usually the younger fish), and it provides an opportunity to disinfect the farm during its fallow period.

Ozone disinfection and ultraviolet (UV) irradiation have been used successfully for the destruction of several fish pathogenic bacteria (Conrad *et al.*, 1975; Bullock and Stuckey, 1977; Colberg and Ling, 1978; Austin, 1983; Sako and Sorimachi, 1985). However, their use for controlling *R.s* has not been investigated.

## CHAPTER 2

### GENERAL MATERIALS AND METHODS

The methodology outlined in this chapter consists of a general description of the techniques used throughout this study. Variations in the experimental parameters and design will be discussed in the appropriate chapters. Preparation of standard buffers and stock solutions are given in Appendix 1. Standard techniques and miscellaneous are presented in Appendix 2.

#### 2.1 PREPARATION OF CULTURE MEDIA

##### 2.1.1 Kidney disease medium-2 (KDM-2)

KDM-2 was made up according the method described by Evelyn *et.al.*,(1977) with modifications. Special peptone (Oxoid, 5 g), yeast extract (Oxoid, 0.25 g), L-Cysteine Hydrochloride (Sigma Chemical Company, 0.5 g) and agar N°1 (Oxoid, 7.5 g) were dissolved in 450 ml of nanopure water, and the pH adjusted to 6.5. The medium was autoclaved at 121°C and 15 psi for 20 minutes. After cooling the medium to 45°C in a water bath, 50 ml of heat inactivated (56°C for 30 minutes) foetal calf serum (FCS Sigma Chemical Company) was filter sterilised through 0.22 mm pore size filter (Whatman) and added to the medium. KDM-2 was then dispensed in petri dishes to give 20 ml of medium each.

KDM-2 broth was made up as described previously but without the addition of agar. For the isolation of R.s directly from naturally or artificially infected fish, cyclohexamide (Sigma Chemical Company) was added at 0.05 g per litre prior to autoclaving.

### **2.1.2 Selective kidney disease medium (SKDM)**

SKDM was made up following the method described by Austin *et al.*,(1983). Tryptone T (Oxoid, 5 g), yeast extract (Oxoid, 0.25 g), L-Cysteine Hydrochloride (Sigma Chemical Company, 0.5 g) and agar N°1 (Oxoid, 7.5 g), and cycloheximide (Sigma Chemical Company, 0.025 g), were dissolved in 450 ml of nanopure water and the pH adjusted to 6.8 with 1 M sodium hydroxide (NaOH). The medium was then autoclaved at 121°C and 15 psi for 20 minutes. After cooling to 45°C in a water bath, heat inactivated foetal calf serum (5 % v/v), polymyxin B sulphate (Sigma Chemical Company, 0.0025 % w/v), oxolinic acid (Sigma Chemical Company, 0.00025 % w/v) and D-cycloserine (Sigma Chemical Company, 0.00124 % w/v) were filtered sterilised through 0.22 mm pore size filter (Whatman) and added to the basal medium. The medium was then dispensed under sterile conditions in petri dishes to give 20 ml each. SKDM broth was made up as described previously but without the addition of agar.

### **2.1.3 Kidney disease medium charcoal (KDM-C)**

KDM-C was made up according the method described by Daly and Stevenson,(1985). Special peptone (5 g), yeast extract (0.25 g), L-Cysteine Hydrochloride (0.5 g) agar N°1 (7.5 g) and activated charcoal (Sigma Chemical Company, 0.5 g) were dissolved in 485 ml of nanopure water and the pH adjusted to 6.8 with 1 M NaOH. The medium was then autoclaved at 121°C and 15 psi for 20 minutes. After cooling the medium to 45°C in a water bath it was dispensed in petri dishes. KDM-C broth was made up as described previously but without the addition of agar. In order to obtain a clear medium, the charcoal was placed in dialysis tubing loosely tied before autoclaving. The medium was allowed to stand at 22°C for 24 h in an orbital rotary incubator and then aliquoted under sterile conditions.

#### 2.1.4 Mueller Hinton Cysteine (MH-C)

MH-C was made up by dissolving Mueller Hinton Agar (Oxoid, 38 g) and L-Cysteine (1 g) with 1 litre of nanopure water. The pH was adjusted to 6.8 with 1 M NaOH and autoclaved at 121°C for 20 minutes. The medium was cooled at 45 °C and aliquoted in petri dishes.

#### 2.1.5 Tryptone soya agar (TSA)

TSA was prepared by dissolving 40 g of TSA (Oxoid) in one litre of distilled water, heated to dissolve, pH adjusted to 7.2 to 7.4 and autoclaved at 121 °C for 20 minutes. After cooling to 45°, the medium was dispensed in petri dishes. Supplemented TSA was prepared for *Haemophilus piscium* (TSA, 1% NaCl, 2 mg ml<sup>-1</sup> cocarboxylase) and *Pasteurella piscicida* (TSA, 1.5 % NaCl). *Nocardia asteroides* was cultured in tryptone soya broth in order to facilitate harvesting.

#### 2.1.6 Marine agar (MA)

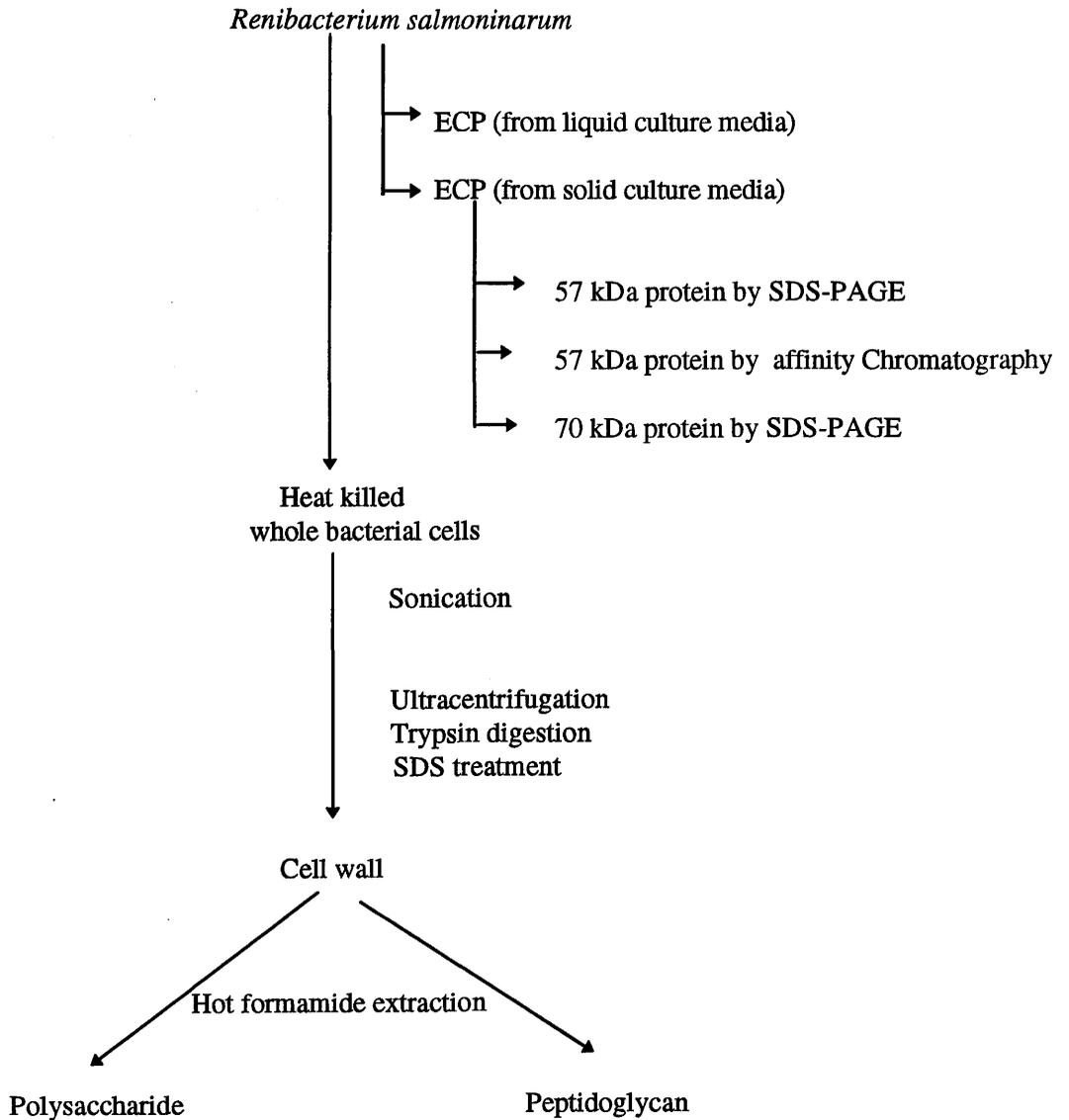
MA was prepared by mixing sodium acetate (0.08 g), beef extract (0.08 g), agar (4.4 g), sea water (300 ml) and distilled water (100 ml). The culture was autoclaved (121°C for 20 minutes) and then aliquoted in sterile universal tubes.

### **2.1.7 Cytophaga agar (CA) and marine cytophaga agar (MCA)**

CA was prepared by mixing tryptone (0.5 g), beef extract (0.2 g), yeast extract (0.5 g), sodium acetate (0.2 g), and agar (9 g) in distilled water (1 litre). The pH was adjusted to 7.2 to 7.4 and autoclaved at 121 °C for 15 minutes. For soft agar for the maintenance of active cultures, the content of agar was reduced to 4 g l<sup>-1</sup>. The culture was dispensed in 3 to 4 ml amounts in sterile universal tubes. Marine cytophaga medium was prepared as described previously but including 70 % filtered sea water.

## **2.2 PREPARATION OF RENIBACTERIAL ANTIGENS**

The following methodology was used to obtain different bacterial fractions. Heat killed whole cells were used for ELISA screening in the production of monoclonal and polyclonal antibodies, for the immunological analysis of 18 R.s isolates and as a starting material for the extraction of renibacterial cell wall, polysaccharide and peptidoglycan. Extracellular products were obtained from liquid and solid culture media following published protocols. The general methodology used in this section is summarised in Figure 2.1.



**Figure 2.1 Summary of the extraction of renibacterial subunits**

### **2.2.1 Preparation of heat killed whole bacterial cells**

Freshly harvested bacterial cells were suspended in 5 ml of sterile PBS (50 mM phosphate, 0.15 M NaCl, pH 7.5). Bacteria were heat inactivated at 60°C for 60 minutes in a water bath and the cell concentration adjusted to  $1 \times 10^{10}$  cell  $\text{ml}^{-1}$  ( $\text{OD}_{610}$  approx. 10). This was achieved by adjusting the  $\text{OD}_{610}$  of a 1/10 dilution to 1.0 using sterile PBS. Thiomersal was added to a final concentration of 0.01%. The cell suspension was aliquoted and stored at 4°C for up to one week.

### **2.2.2. Preparation of sonicated renibacterial cells.**

Renibacterial whole cells (1 g wet weight in 10 ml sterile PBS) prepared freshly as described in section 2.2.1, were disrupted by sonication using a sonicator at maximum power and amplitude set at position 3. Samples were placed on ice and sonicated for 20 cycles of 1 min each followed by 1 minute intervals between sonication. An aliquot of 10  $\mu\text{l}$  was taken before and after the treatment and stained by Gram to detect unbroken cells. Thiomersal was added to a final concentration of 0.01%. Samples were aliquoted and stored at -70°C until use.

### **2.2.3 Preparation of renibacterial cell wall**

Renibacterial cell wall was prepared following the procedure described by Kusser and Fiedler, (1983) and Fiedler and Draxl, (1986). Heat inactivated renibacterial cells (see section 2.2.1) were suspended in sterile distilled water and disrupted by sonication as described in section 2.2.2. They were then centrifuged at 45,000 $\times g$  in a ultracentrifuge for 20 minutes at 4°C. The supernatant was discarded and the upper white layer of cell wall was carefully removed from the small pellet of unbroken cells. The cell walls were washed twice with sterile deionised water by centrifugation and resuspension as described previously. The pellet was

resuspended in filter sterilised 20 mM Tris buffer pH 8.0 containing 100 IU of trypsin ml<sup>-1</sup> (Sigma Chemical Company) and incubated overnight at 37°C with gentle agitation and with the addition of two drops of toluene (50 µl) to prevent microbial contamination. Cell walls were then recovered and washed once by centrifugation and resuspension as above. The final pellet was resuspended in 4% boiling SDS for 15 minutes and then allowed to cool overnight at room temperature. The cell walls were recovered and washed by centrifugation (45,000xg) four times in sterile deionised water. They were lyophilised and stored at 4°C until use. The total amount of cell wall recovered from 3 g of wet bacterial cells was 3.86 mg. The whole procedure was repeated twice more to obtain enough material for polysaccharide and peptidoglycan extraction.

#### **2.2.4 Extraction of polysaccharide and peptidoglycan**

Renibacterial polysaccharide and peptidoglycan were obtained from protein free cell wall as described by Kusser and Fiedler, (1983) and Fiedler and Draxl, (1986).

Freeze dried cell wall (6 mg) prepared as described in section 2.2.3 were boiled for 30 minutes with 10 ml of formamide (Sigma Chemical Co.). After cooling, 10 ml of acidified ethanol (5 ml of 2 N HCl and 95 ml of absolute ethanol) was added and allowed to stand at room temperature for 1 hour. The peptidoglycan pellet was collected by centrifugation at 45,000xg for 20 minutes at room temperature. The supernatant was filter sterilised through 0.22 µm pore size filter and then precipitated in 5 volumes of cold acetone (kept at -20°C) and left overnight at 4°C. Polysaccharide was recovered by gently centrifugation at 600 rpm (MS Mistral) at 4°C for 10 minutes in a glass test tube with cap. The residue was washed three times in fresh cold acetone by centrifugation and resuspension as above. The final pellet was resuspended in 1 ml of deionised water and the total sugar content was measured by the phenol/sulphuric acid method (1.29 mg ml<sup>-1</sup>) before lyophilization. The pellet of peptidoglycan was washed three times in fresh acidified ethanol. The formamide extraction and the

peptidoglycan precipitation was repeated twice more. The pellet from the last centrifugation was suspended in deionised water (1 ml) and assayed for sugar content by the phenol/sulphuric acid method. Peptidoglycan was then aliquoted, and freeze dried. The total concentration of peptidoglycan was estimated to be 2.82 mg ml<sup>-1</sup>. Samples were stored at 4°C.

### **2.2.5. Extraction of renibacterial ECP from liquid media**

Renibacterial ECP was obtained from liquid culture media following the procedure described by Turaga *et al.*, (1987b). Liquid culture media (KDM-2 without FCS, 3 litres) was inoculated with 10% of spent culture (isolate ATCC 33209) and incubated at 15°C to an OD<sub>610</sub> approximately 1.0 (1x10<sup>9</sup> cell ml<sup>-1</sup>). The cells were removed by centrifugation at 6000 g (High speed centrifuge) for 30 minutes at 4°C. The supernatant was collected, filtered through 0.22 µm pore size filter (Millipore) and concentrated using polyethyleneglycol (PEG-8000, Sigma Chemical Company). The filtered supernatant was placed inside dialysis tubing (MWCO 12 kDa), previously boiled for 10 minutes in 0.01 M EDTA and washed in deionised water. The tubing was then completely covered in PEG powder and left at 4°C for 10-16 hrs until the volume has been reduced 10 fold. The retentate was further concentrated using 50% saturated ammonium sulphate (SAS, Sigma Chemical Company)) precipitation. The mixture was stirred for 3-4 hrs at 4°C and the precipitate was collected by centrifugation at 3000 rpm (MS Mistral) for 30 minutes at 4°C. This precipitate was dissolved in a volume of PBS (0.02 M phosphate, 0.15 M NaCl, pH 7.2), equivalent to 10% the original retentate volume. The solution was then reprecipitated by SAS as described above and dialysed extensively against PBS at 4°C. The protein concentration was estimated to be 1.93 mg<sup>-1</sup>. The concentrated ECP was characterised by SDS-PAGE, lyophilised and stored at 4°C.

### **2.2.6. Extraction of renibacterial ECP from solid media**

Renibacterial ECP was obtained from solid culture media following the procedure described by Dubreuil *et al.*,(1990). This procedure was originally developed for the purification of bacterial fimbriae but it was used here to obtain extracellular products larger than 30 kDa.

Solid culture media (MH-C, 3 litres) was inoculated with 100 µl plate<sup>-1</sup> of spent culture (isolate AVU 9202 at 1x10<sup>9</sup> cell ml<sup>-1</sup>) and incubated at 15°C for 14 days. The lawn of cells was harvested and suspended in sterile PBS to approximately 3 g wet weight in 50 ml PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma Chemical Company). The suspension was stirred vigorously for 20 minutes at room temperature. Whole cells were then removed by centrifugation at 12,000 g for 15 minutes (ultracentrifuge). The supernatant was filter sterilised (0,22 µmm pore-size filter, Millipore) and then ultrafiltered by centrifugation at 3000 rpm (MS Mistral) using Amicon units (MWCO 30 Kda) at 4°C for 15 minutes to concentrate and eliminate salts and other substances lower than 30 kDa. The retentate was diluted to 10 ml with sterile PBS/PMSF and ultrafiltered again. The total protein concentration of the retentate was 3.21 mg ml<sup>-1</sup>. The prepared ECP was confirmed by SDS-PAGE analysis, lyophilised and stored at 4°C.

### **2.2.7 Purification of 57 kDa protein by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).**

57 kDa protein was purified from concentrated ECP (prepared as described in section 2.2.6) by preparative SDS-PAGE under reduced conditions (Harlow and Lane, 1988). Electrophoresis was carried out in the discontinuous buffer system of Laemli (1970) described in section 2.3.4. Sample wells were formed by inserting a preparative well-forming comb into the stacking gel. After polymerisation (60 minutes), 200 µl of

sample (containing 200 mg ECP protein in sample buffer 1:1) were loaded in the preparative well with a micro syringe. The samples had previously been heated to 98°C for 4 minutes in sample buffer. Low molecular weight markers (BioRad Labs Ltd, 10 µl) were added to the smallest well. The gel was electrophoresed against gel buffer at 200 V until the dye front (visualised by the addition of bromophenol blue (0.2% w/v) to sample buffer) was at the bottom of the gel (Approximately 45 minutes). The gel was washed four times in deionised water and then stained for 10 minutes with 0.05% of Coomassie brilliant blue R-250 prepared in deionised water. The gel was washed again several times in deionised water for 2 hours with agitation. The 57 kDa proteins bands from four gels were excised using a scalpel and finely chopped. The fragmented gels were homogenised by passing them through two 2.2 ml syringes in 1 ml of sterile PBS containing 0.01% thiomersal. The process was repeated at least five times, passing the gel fragments back and forth between the two syringes. A 21-Gauge needle was placed onto the outlet of the syringes, and the process repeated. The samples were stored at 4°C overnight and the protein concentration determined (approx. 160 µg in 500 µl)

### **2.2.8 Purification of 70 Kda antigen by SDS-PAGE**

The 70 kDa antigen was purified from fresh R.s isolate AVU 9202 cultured in MH-C agar for 15 days at 15°C. The lawn of cells were harvested and suspended in PBS to approximately  $1 \times 10^9$  cell ml<sup>-1</sup>. The cell suspension (15 ml) were centrifuged at 12000 g (Microcentrifuge), the supernatant was discarded and the cell pellet suspended in 800 µl sample buffer. Samples were boiled for 10 minutes and then loaded into four preparative SDS-PAGE gels. The gels were prepared and run as described in section 2.3.4. The 70 kDa antigens were excised from the gel and homogenised in PBS as described in section 2.2.7.

### 2.2.9 Purification of 57 kDa antigen by affinity chromatography

CNBr activated sepharose 4B (Sigma Chemical Company, 1 g) was mixed with 10 ml of 1 mM HCl in a universal tube and gently shaken for 10 minutes at room temperature. The liquid was removed slowly by placing the gel in a sintered filter G3 connected to a water vacuum pump but without allowing the gel to become dry. The gel was washed twice more with fresh 1 mM HCl as previously described.

Rabbit anti p57 IgG (2 ml) containing  $8.2 \text{ mg ml}^{-1}$  protein was dialysed in coupling buffer (0.1 M  $\text{NaHCO}_3$ , 0.5 M NaCl, 0.5% Tween 80, pH 8.3) and mixed gently with the washed gel in a universal tube for 10 minutes at room temperature and then in a rotary incubator at room temperature for 2 hrs. The liquid was removed using a sintered filter and the gel washed once with 20 ml of blocking buffer (0.2 M glycine pH 8.0). After removing the liquid, the IgG coupled gel was transferred to a fresh universal tube and 20 ml of fresh blocking buffer were added. The liquid was removed again in a sintered filter and the gel was washed with 50 ml of coupling buffer, then with 50 ml of acetate buffer (0.1 M pH 4.0, 0.5 M NaCl, 0.5% Tween 80) and finally with 50 ml of coupling buffer. The IgG coupled gel was stored at 4°C in PBS containing azide (0.02 % v/v) for up to one month.

Before assembling the column, all buffers and the gel were allowed to warm at room temperature for 30 minutes. The gel was washed with 20 ml of freshly prepared running buffer (0.02 M phosphate, 0.15 M, 0.02% azide, 1 mM PMSF) and each 5 ml fraction collected and the  $\text{OD}_{280}$  measured. Lyophilised ECP (prepared as described in section 2.2.6) was dissolved in 1 ml coupling buffer to  $1.45 \text{ mg ml}^{-1}$ . ECP protein solution (0.5 ml) was added to the column and incubated for 15 to 20 minutes at room temperature. The column was then washed with 150 ml of running buffer. The antigen (p57) was eluted by adding elution buffer (0.1 M glycine/NaOH pH 11), fractions of 5 ml were collected and the protein concentration measured at  $\text{OD}_{280}$ . The collected fractions were neutralised with 0.6 ml of Tris buffer (0.1 M, pH 7.2). The fractions containing the major peaks were pooled, concentrated by Amicon 30 kDa MWCO filter units and dialysed against PBS. The

total protein concentration was  $586 \mu\text{g ml}^{-1}$ . The samples were checked by SDS-PAGE and stored at  $-70 \text{ }^{\circ}\text{C}$  until use.

#### **2.2.10 Measurement of the protein concentration**

Total protein concentration was determined in duplicate using the BioRad protein assay. A standard curve was prepared from a stock solution of BSA in PBS ( $1 \text{ mg ml}^{-1}$ ). Aliquots of 0.8 ml of test sample (diluted 1/10, 1/100, 1/1000 in PBS) or 0.8 ml of standard (100, 80, 40, 20, 15, 10, 5, and  $0 \mu\text{g ml}^{-1}$ ) in PBS were placed in tubes. Dye concentrated reagent (0.2 ml) was added, and the tubes were vortexed prior to incubating at room temperature for 5 minutes. Supernatants were read using a spectrophotometer at 595 nm (UV/Visible 4050 Ultraspec II spectrophotometer, LKB Instruments, S. Croyden, Surrey, England), blanking against PBS/dye solution

#### **2.2.11 Measurement of sugar concentration**

The total carbohydrate content of the samples was determined using the phenol sulphuric acid assay. All the determinations were performed in duplicate. A standard curve was prepared from a stock solution of galactose (Sigma Chemical Company,  $1 \text{ mg ml}^{-1}$ ) in deionised water. Aliquots of 0.8 ml of test sample (diluted 1/10, 1/100, 1/1000 in deionised water) or 0.8 ml of standard (100, 80, 40, 20, 15, 10, 5, and  $0 \mu\text{g ml}^{-1}$ ) in deionised water were placed in very clean and sterile glass tubes. 200  $\mu\text{l}$  of aqueous phenol solution (5% v/v in water) was added to each tube followed by 1 ml of concentrated sulphuric acid ( $\text{H}_2\text{SO}_4$ ). Samples were left at room temperature for 10 minutes and then shaken vigorously. The  $\text{OD}_{490}$  was measured after 30, blanking against deionised water /phenol/ $\text{H}_2\text{SO}_4$ .

## 2.3 SCREENING TECHNIQUES

### 2.3.1 ELISA detection of antibodies against particulate antigens

Microtitre plates (Immunolon 4, Dynatech Ltd.) were coated with 100  $\mu\text{l}$  well<sup>-1</sup> of coating buffer (0.1 M carbonate/bicarbonate pH 9.6) containing 0.05 mg ml<sup>-1</sup> poly-L-lysine (PLL). Plates were incubated for 60 min at room temperature and washed three times with Tris-Tween buffer. On the last wash, plates were allowed to soak for five minutes. 100  $\mu\text{l}$  of diluted heat killed whole bacteria were dispensed into each well (10<sup>7</sup> cells well<sup>-1</sup>). Cell wall and peptidoglycan were diluted directly into coating buffer, without PLL, to 100  $\mu\text{g}$  ml<sup>-1</sup> and 100  $\mu\text{l}$  added to each well. Plates were incubated overnight at 4°C and then washed in low salt wash buffer followed by the addition of 250  $\mu\text{l}$  well<sup>-1</sup> of blocking solution (1% BSA in PBS containing 0.01% thiomersal). Plates were incubated at room temperature for 2 hours or covered with adhesive cellophane sheet and stored at 4 °C for up to one week.

Plates were warmed at room temperature and washed with three washes of low salt wash buffer. Undiluted hybridoma cells supernatant or polyclonal antibodies (diluted 1/20,000 in low salt wash buffer) were added to each well (100  $\mu\text{l}$  ml<sup>-1</sup>). Preimmune sera (rabbit, mouse or goat, diluted at 1:20,000) and MAbs anti-*Aeromonas salmonicida* subsp *salmonicida*, MAb anti-*Mycobacterium fortuitum* or MAb anti-PKX were used as negative control. Polyclonal antibody PVIR (1:20,000) was used as positive control. Plates were incubated at room temperature for two hours, washed with five washes of high salt buffer (PBS pH 7.3, 0.02M phosphate, 0.5 M NaCl containing 0.01% (v/v) Tween-20 and 0.01 % (w/v) Thiomersal). Conjugate (donkey anti rabbit IgG-HRP, donkey anti mouse IgG-HRP or donkey anti sheep/goat IgG-HRP) diluted 1/1000 in conjugate buffer (1% BSA in low salt wash buffer) were added to each well (100  $\mu\text{l}$  well<sup>-1</sup>) and incubated 60 minutes at room temperature. After five washes with high salt wash buffer, 100  $\mu\text{l}$  of chromogen (42 mM tetramethyl dihydrochloride in 2 M acetic acid) were mixed with 10 ml of substrate buffer (0.1 M citric acid,

0.1 M sodium acetate, 0.33% hydrogen peroxide) and added to the wells ( $100 \mu\text{l ml}^{-1}$ ). Plates were incubated at room temperature for 10 minutes. The reaction was stopped with  $100 \mu\text{l well}^{-1}$  of stop solution (2 M sulphuric acid). Absorption at 450 nm was measured using an ELISA reader (Dynatec MR5000). Positive wells were yellow in colour while negative wells remained clear. The end point of the titre, was taken as mean values of duplicate wells with an absorbance 3 times that of the background.

### **2.3.2 ELISA detection of antibodies against soluble antigens**

ECP, p57, and low molecular protein (LMWp) were diluted to  $80 \mu\text{g ml}^{-1}$  in freshly prepared coating buffer. The protein solutions were plated out into each well ( $100 \mu\text{l ml}^{-1}$ ) and incubated overnight at room temperature. Plates were washed 3 times in low salt wash buffer allowing to stand for 5 minutes in the last wash and then  $250 \mu\text{l}$  of blocking agent. Plates were further processed as described in section 2.3.1.

### **2.2.3 ELISA detection of anti-polysaccharide antibody**

ELISA plates were sensitised with  $0.1 \text{ mg ml}^{-1}$  of polymyxin-B-sulphate (Sigma Chemical Company) in carbonate/bicarbonate buffer. The plates were covered with adhesive cellophane sheets, incubated overnight at room temperature, and washed three times with low salt wash buffer. In the last wash the plates were allowed to soak for five minutes and then postcoated with 1% BSA (w/v) in PBS containing 0.01% Thiomersal ( $250 \mu\text{l well}^{-1}$ ). After incubating the plates for at least two hours at room temperature the wells were washed four times in low salt wash buffer as described previously. Renibacterial polysaccharide ( $100 \mu\text{l}$ ) containing  $240 \mu\text{g ml}^{-1}$  in low salt wash buffer was added to each well and incubated two hours at room temperature. The plates were washed again in low salt wash buffer as above and processed as the standard ELISA described in section 2.3.1.

### 2.3.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out in the discontinuous buffer system of Laemmli (1970) with a 4% stacking gel and 12% separating gel by using a Mini Protean II dual slab cell system (BioRad Labs Ltd.). The gel holder was assembled following the directions provided by the manufacturer.

Stock solution of acrylamide/bisacrylamide (30% total, 2.67% crosslinker) was made up as follows: 29.2 grams of acrylamide (Sigma Chemical Company), 0.8 grams of N,N'-bis-methylene-acrylamide (BioRad Labs Ltd.) were dissolved up to 100 ml of deionised water and filtered through a Whatman N°3 qualitative paper filter and stored at 4°C in the dark (covered in foil) for a maximum of one month. The stock solution of 10% Sodium dodecylsulphate (SDS, Sigma Chemical Company) was prepared by dissolving 10 grams of SDS in 90 ml of deionised water. The solution was stored at room temperature indefinitely.

Separating gel was prepared to yield a final concentration of 12% acrylamide/bisacrylamide, 0.1% SDS and 0.375 M Tris-HCl. Deionised water (3.35 ml) was mixed with 2.5 ml of 1.5 M Tris-HCl buffer pH 8.8, 100 µl of 10% stock solution of SDS and 4 ml of acrylamide/bisacrylamide stock solution. The solution was de-gassed *in vacuo* for 15 minutes at room temperature and then 50 µl of a freshly made 10% ammonium persulphate (APS, Sigma Chemical Company), and 5 µl of N,N'-tetra-methylenediamine (TEMED, Sigma Chemical Company) were added to give a final concentration of 0.05% (w/v) of APS and 0.05% (v/v) of TEMED. The gel was quickly poured between the plates, taking care not to introduce bubbles, and 200 ml of butanol was layered onto the gel, before allowing it to polymerise overnight at 4°C or 1 hour at room temperature.

Sample wells were prepared using stacking gel prepared to yield a final concentration of 4% of acrylamide/bisacrylamide, 0.1% SDS and 0.125 M Tris-HCl. Deionised water (6.1 ml) was mixed with 2.5 ml of 0.5 M Tris-HCl pH 6.8, 100 µl SDS stock solution and 1.3 ml of acrylamide/ bisacrylamide stock solution. The solution was de-gassed *in vacuo* for 15 minutes and then 50 µl freshly prepared 10 % solution of

APS and 10  $\mu$ l of TEMED was added to give a final concentration of 0.05% (w/v) and 0.1% (v/v) respectively. The separating gel was washed with distilled water to remove the butanol before layering on the stacking gel. The stacking gel was then poured in and allowed to polymerise for 45 minutes with a 10 well comb placed on top. Just before electrophoresis, the samples were thawed and boiled for 5 minutes. Samples (10  $\mu$ l) were loaded in each well and run at 200 volts for 45 minutes. Gels were stained 30 min with Coomassie blue and destained overnight in destaining solution (methanol 40 %, acetic acid 10 %).

### 2.3.5 Western blot

Samples were submitted to SDS-PAGE (see section 2.3.4) and then transblotted onto nitrocellulose membranes according to the method of Wiens *et al.*, (1990). The gel was gently removed from the electrophoresis plates into transfer buffer for 10 minutes. This and the transblotting material (filter paper and nitrocellulose membranes), were equilibrated in transblot buffer. Three filter papers were placed on the cathode side of the transblotter (BioRad). The gel, and then the nitrocellulose membrane were positioned on the assembly, with a further three filter papers and the anode on top of this. The gel was blotted onto nitrocellulose membrane by applying 20 volts for 20 minutes.

Non-specific binding sites, present on the nitrocellulose membrane, were blocked with 1% (v/v) BSA in Tris buffered saline (TBS) with 0.1 % (v/v) Tween-20 for 2 hours with agitation at room temperature. The membrane was washed with three 5 minute washes of TBS and placed in a mini protean II multiscreen (BioRad). Each line was incubated with undiluted hybridoma cell supernatant or polyclonal antibodies (diluted 1/200) and left overnight at 4 °C. The blot was washed as described previously and incubated with conjugate (donkey anti rabbit IgG-HRP, donkey anti mouse IgG-HRP or donkey anti sheep/goat IgG-HRP) diluted 1/100 for 60 minutes at room temperature. The plate was washed again, but including an additional rinse of 1 minute

using TBS with no Tween-20. Colour was developed with 2 ml of TMB solution (BioRad) until bands materialised. The reaction was stopped by soaking the membranes in distilled water for 10 minutes.

### **2.3.6 Immuno dot blot for whole bacteria**

Nitrocellulose membranes (2 cm x 5 cm) were washed with saline solution (0.85% w/v NaCl) for 2-3 minutes and allowed to dry over a filter paper in oven at 37°C for 30 minutes. Samples (25 ml) of heat killed bacteria were blotted on the membrane and allowed to dry over a filter paper for 15 minutes at 37°C. The membranes were blocked, incubated with antibodies and developed as described in section 2.3.5.

### **2.3.7 Indirect immunoperoxidase detection of *R. salmoninarum***

Paraffin embedded tissues, prepared as described in appendix 1, were cut at 5 µm and mounted in poly-L-lysine coated slides. The tissue sections were dewaxed in xylene (2 x 5 minutes) and hydrated in 100 % ethanol (5 minutes), 70 % ethanol (3 minutes) and rinsed in distilled water. Tissue sections were surrounded by a waxy circle using a PAP pen (BDH Ltd.) to allow the minimum use of reagent in the test. The endogenous peroxidase activity was blocked by immersing the slides in 10% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol. After washing for 15 minutes with three washes of Tris saline buffer (TBS, 0.05 M Tris, 0.15 M NaCl, pH 7.6) the non-specific binding sites were blocked with normal goat serum diluted 1/10 in TBS for 10 minutes at room temperature. The blocking solution was then poured off by tapping the edges on paper towel. Hybridoma supernatant (undiluted) or antisera (1/1000 in TBS) were added onto the tissue section and incubated at room temperature overnight in a moist chamber. Slides were washed with three washes of TBS (3 x 5 minutes) and then incubated with conjugate (donkey anti rabbit IgG-HRP, donkey anti mouse IgG-HRP or donkey anti sheep/goat IgG-HRP) diluted 1/1000 in TBS for 1 hour at room temperature. After washing with TBS as above, they were developed by incubating the

slides with 3'3' diaminobenzidine tetrahydrochloride (DAB) in the presence of hydrogen peroxide (100  $\mu$ l of 1 %  $H_2O_2$  , 0.5 ml of 1.5 mg  $ml^{-1}$  DAB and 5 ml of TBS). The reaction was stopped by immersing the slides in tap water. Slides were counterstained with haematoxylin for 3-4 minutes, dehydrated in 70 % ethanol (3 minutes), 100 % ethanol (10 minutes) and rinsed in xylene (2 x 5 minutes). Slides were mounted using pertex/xylene (5:1).

## CHAPTER 3

### ISOLATION, PATHOLOGY AND ELECTRON MICROSCOPY OF *R. salmoninarum* ISOLATES FROM RAINBOW TROUT NATURALLY INFECTED WITH BKD.

#### 3.1 Introduction

The intracellular nature of *R.s* was first suggested by Young and Chapman, (1978) who found that host cells resembling macrophages or polymorphonuclear leukocytes with phagocytosed bacterial cells appeared in lesions. Electron microscopy of these cells showed the morphological integrity of the phagocytosed bacterial cells and evidence for their active division, indicating intracellular survival. Recently, this finding was also observed by Gutenberger, (1993).

*R. salmoninarum* cultured for prolonged periods have been reported to lose important antigens, particularly p57 (Bruno , 1990). The same findings has been reported for many other bacterial pathogens which are unable to produce important antigens or virulence factors when they are cultured under artificial conditions. Therefore, the library of new isolates of *R. salmoninarum* and their subsequent biochemical and antigenic analysis is an essential step in the search for antigens for vaccine development.

The main goal of this study is to collect “fresh” isolates of *R. salmoninarum* which will be then comparatively analysed, in the following chapters of this dissertation, with other isolates collected from four different countries, particularly with reference to the expression of novel antigens, and their antigenic

and biochemical characteristics. Gross pathology, histopathology, bacteriological and immunological techniques were used in the present study mainly to investigate the characteristics of the pathogen in tissue rather than to provide an exhaustive analysis of the immunopathology of the disease since excellent reviews about this matter already exist (Bruno, 1986, Austin and Austin, 1987, Fryer and Lannan, 1992, Raverty, 1992, Evelyn 1993). *R. salmoninarum* isolates were characterised using standard bacteriological techniques and ELISA. Kidney tissue samples from infected fish were also submitted to EM to study the intracellular nature of this bacterium.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Postmortem Procedures**

Diseased rainbow trout (30 to 1200 g) were obtained from two local commercial trout farms in Scotland (see Plate 3.1). Moribund fish were anaesthetised with benzocaine (50 mg l<sup>-1</sup>), bled out by caudal puncture using two ml syringes fitted with 21G needles, and then killed by transection of the spinal cord behind the head.

Dissection was performed using sterile standard instruments. An incision was made in the midline 5-10 mm cranial to the cloaca and extended to the level of the pectoral fins, avoiding damage to the viscera. A skin flap was reflected cranially and dorsally to expose the abdominal organs. Gross pathological changes were noted. The alimentary tract, liver, spleen and swim bladder were carefully dissected out. Bacteriological samples were collected as described in section 3.2.2. An operculum was excised to expose the gill rakers and part of one raker was removed. The heart was exposed and removed. The head was cut from the body and the skull bisected by a dorsoventral, longitudinal, midline incision, exposing and transecting the brain. The eyes were also removed.

Samples for histological and immunohistochemical analysis were placed in 10% neutral buffered formalin, for at least 24 hours and embedded in paraffin following the method described in Appendix 1. Tissues for ultra-structural examination were cut into very small pieces (1 mm<sup>3</sup>), using razor blades then immersed in Karnovsky fixative at 4°C overnight and processed for EM using the standard protocol described in Appendix 1.

### **3.2.2 Isolation and characterization of *R. salmoninarum***

Infected kidney tissues (obtained from section 3.2.1) were homogenised with peptone water (0.1 % peptone, 0.85 % NaCl) and plated out in KDM-2 agar supplemented with cycloheximide (see section 2.1.1). Plates were incubated at 15 °C for up to 10 weeks in plastic bags. Suspected colonies were submitted to Gram's stain, catalase, cytochrome oxidase activity (see Appendix 1) and indirect ELISA using affinity purified polyclonal antibodies to *R. salmoninarum* (Kinkergaard Perry Labs, USA) and following the method described in section 2.3.1.

## **3.3 RESULTS**

### **3.3.1 Gross and immunohistopathological examination**

An average of 20 moribund and unhealthy fish (50-1000 g body weight) were sampled during each trip to the farm. The clinical signs of diseased fish included abnormal swimming behaviour where the fish remained in the corner of the cage and moved very slowly (Figure 3.2). Externally, some of the fish showed exophthalmos, ascites (Figure 3.3 and 3.4 respectively) and anemic gills. Internally a clear liquid

was observed in the abdominal cavity and a white creamy membrane was sometimes detected in the surface of the kidney of bigger fish. The kidney tissue was often swollen and soft in small fish, and swollen with a granulomatous appearance in larger ones.

Larger fish sometimes showed a thin white membrane covering the heart, petechial and ecchymotic haemorrhage, especially in the base of the pectoral fins, fat tissue, liver and the wall of the abdominal cavity (Figure 3.5).

Histopathological examination of the kidney tissue showed a severe interstitial hyperplasia of macrophage like cells (Figure 3.6). Immunohistochemical (indirect peroxidase) examination of the same tissue revealed massive numbers of renibacterial cells in the cytoplasm of the macrophages (Figure 3.7). Moderate to severe hydropic and hyaline degeneration was sometimes observed. The melanin granules were widely dispersed throughout the kidney tissue but not inside the melanomacrophages as occurs in healthy fish. The heart showed moderate to severe infiltration of inflammatory cells in the pericardial area (Figure 3.8), with some bacterial colonies between the muscular fibres (Figure 3.9). Immunohistochemical analysis of these tissue identified the bacteria as *Renibacterium salmoninarum* (Figure 3.10).

### **3.3.2 Isolation and characterization of *R. salmoninarum* isolates.**

Suspected *R. salmoninarum* colonies were obtained after 3 to 6 weeks of incubation in KDM-2 containing cycloheximide. Gram's stain revealed the presence of strongly gram positive bacteria arranged in pairs or as single cells. Many of them were found in clumps and immersed in a proteinaceous (eosinophilic) material, which probably corresponded to secreted extracellular proteins. Bacteria were found to be catalase positive and cytochrome oxidase negative. The bacterium was definitively identified as

*R. salmoninarum* by indirect ELISA using affinity purified polyclonal antibodies to *R. salmoninarum* (Kinkergaard Perry, USA.). The bacterium was recovered only in 12 out of 80 kidney samples tested.

In total four different isolates were obtained. Three of these (AVU 9201, 9202 and 9401) were collected from the same trout farm in the Northwest Scotland but during different years (1992, 1994) and one isolate (B94166) from another commercial farm also in Scotland (1994).

### **3.3.3 Electron microscopy of the intracellular nature of *R. salmoninarum***

The EM analysis was performed on those kidney samples where the presence of *R. salmoninarum* was detected by bacteriological or immunological techniques. *R. salmoninarum* was found only in two of the 10 samples analysed. The bacterium was observed only inside cells (probably macrophages). They were apparently undigested and located both inside phagosome vesicles and also free in the cytoplasm (Figure s 3.11, 3.12, and 3.13). A thick capsule-like material was detected when the bacteria were found inside host cells. The nature of this material was not determined (Figure 3.13).



Figure 3.1 Commercial rainbow farm where periodic outbreaks of BKD were detected

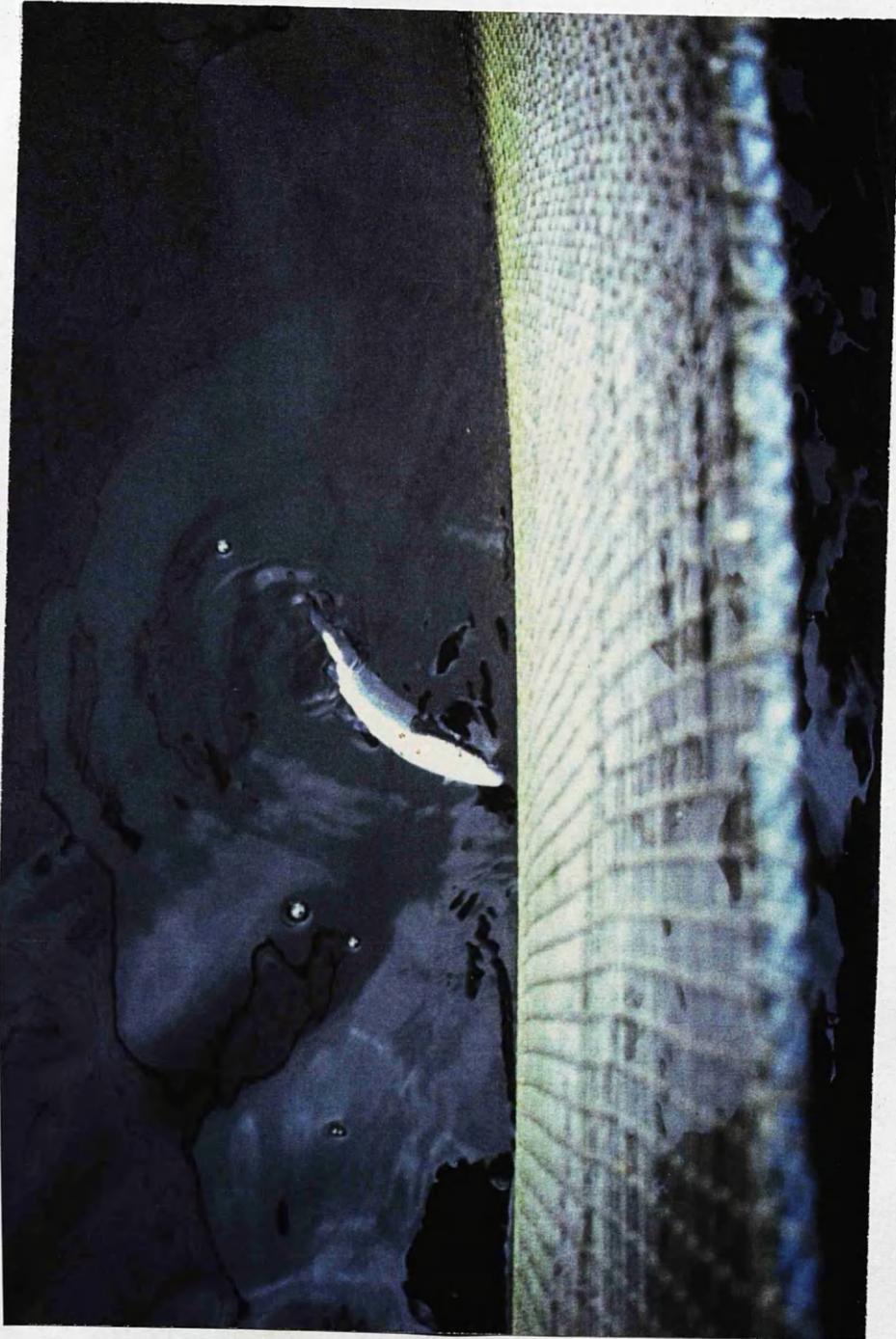


Figure 3.2 Abnormal swimming behaviour of rainbow trout naturally infected with *R. salmoninarum*.



Figure 3.3 Exophthalmus in rainbow trout naturally infected with *R. salmoninarum*.



Figure 3.4 Ascites in rainbow trout naturally infected with *R. salmoninarum*



Figure 3.5 Haemorrhage of internal organs in a rainbow trout naturally infected with *R. salmoninarum*.

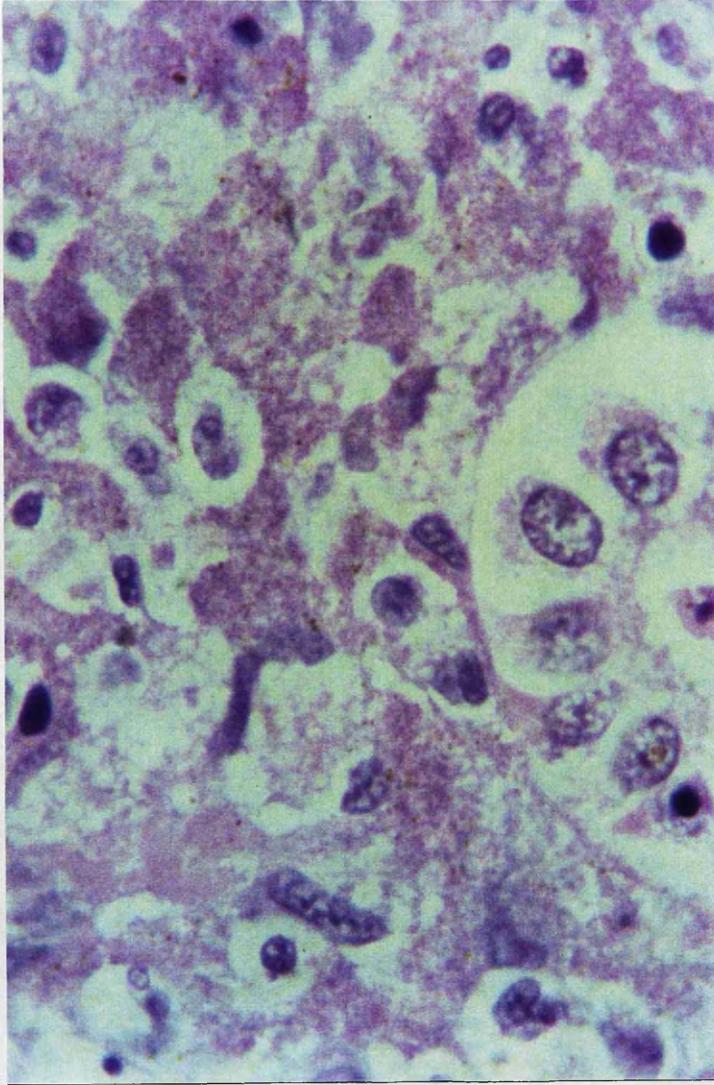


Figure 3.6 Kidney tissue of rainbow trout naturally infected with *R. salmoninarum* isolate AVU 9202 (H/E)

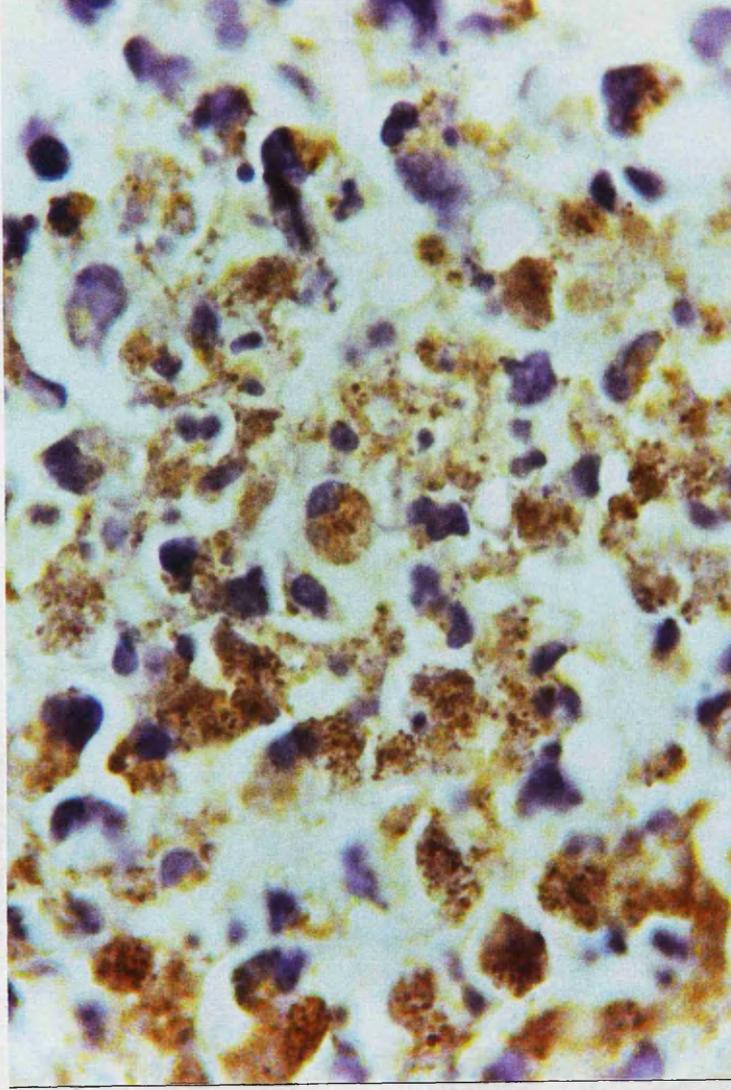


Figure 3.7 Kidney tissue of rainbow trout naturally infected with *R. salmoninarum* isolate AVU 9202. Immunohistochemistry using MAb against cell surface antigens (peptidoglycan)

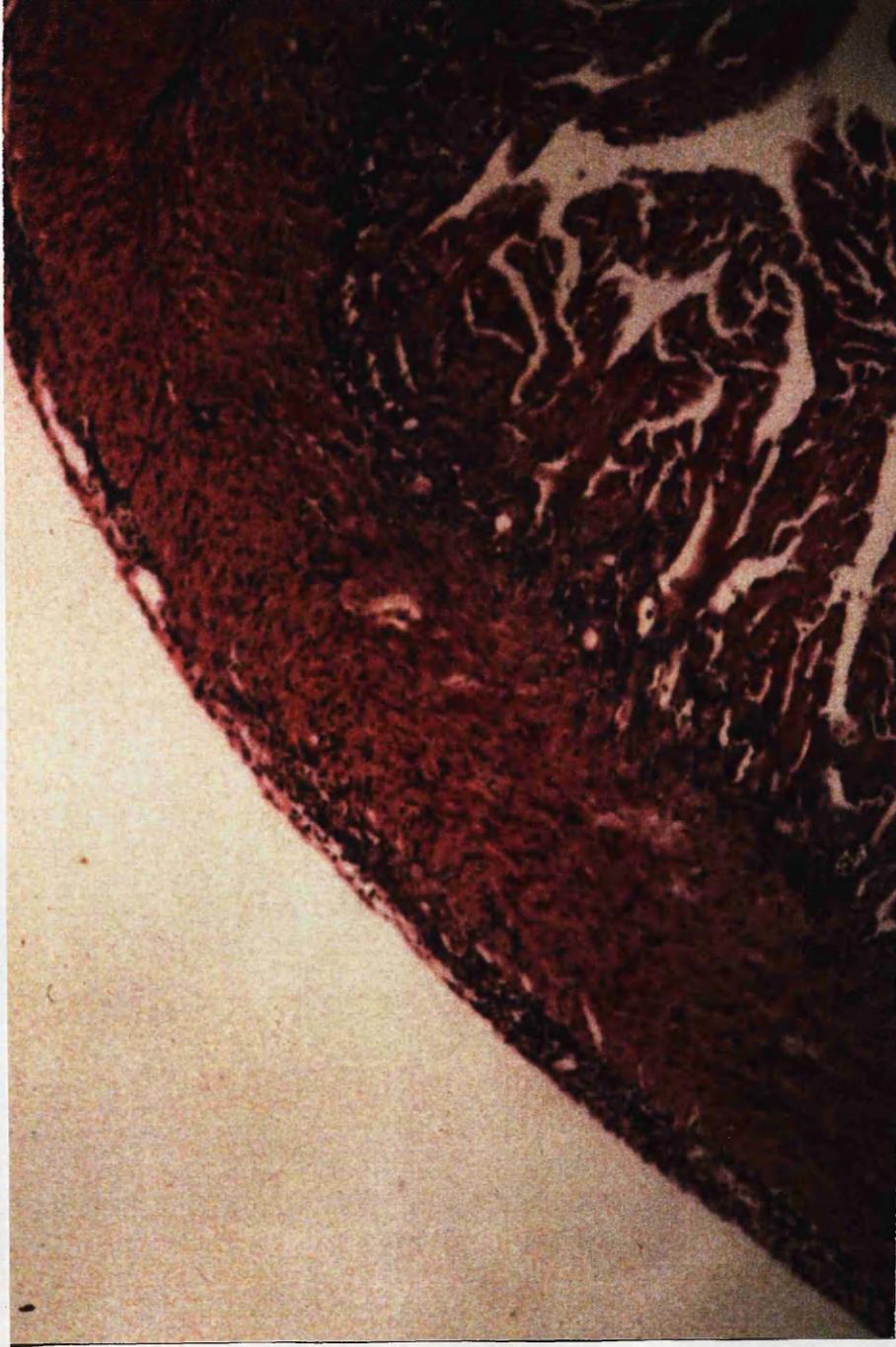


Figure 3.8 Pericarditis caused by *R. salmoninarum* in naturally infected rainbow trout (H/E). Note the infiltration of the pericardium by inflammatory cells

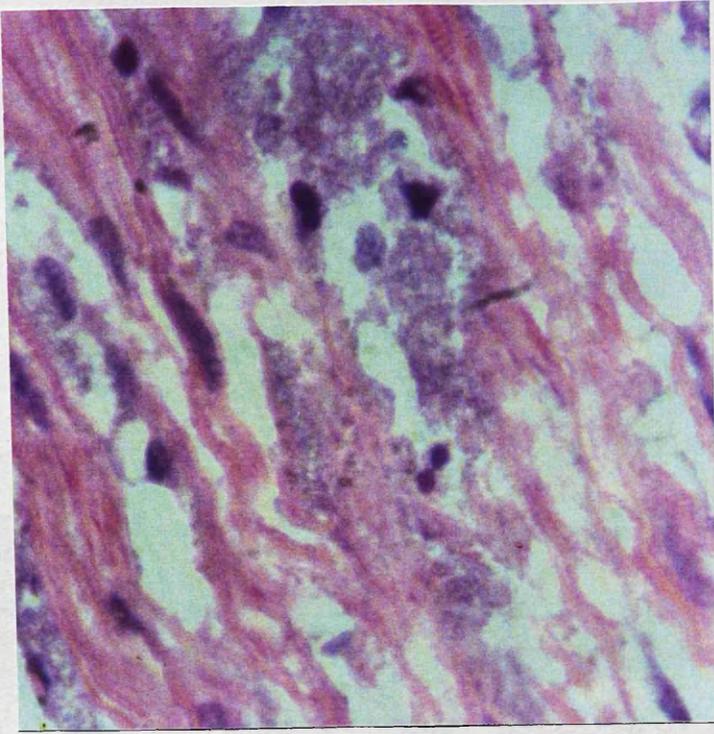


Figure 3.9 Heart tissue of rainbow trout naturally infected with *R. salmoninarum* (H/E). Note the presence of colonies of the pathogen between the muscular fibres.

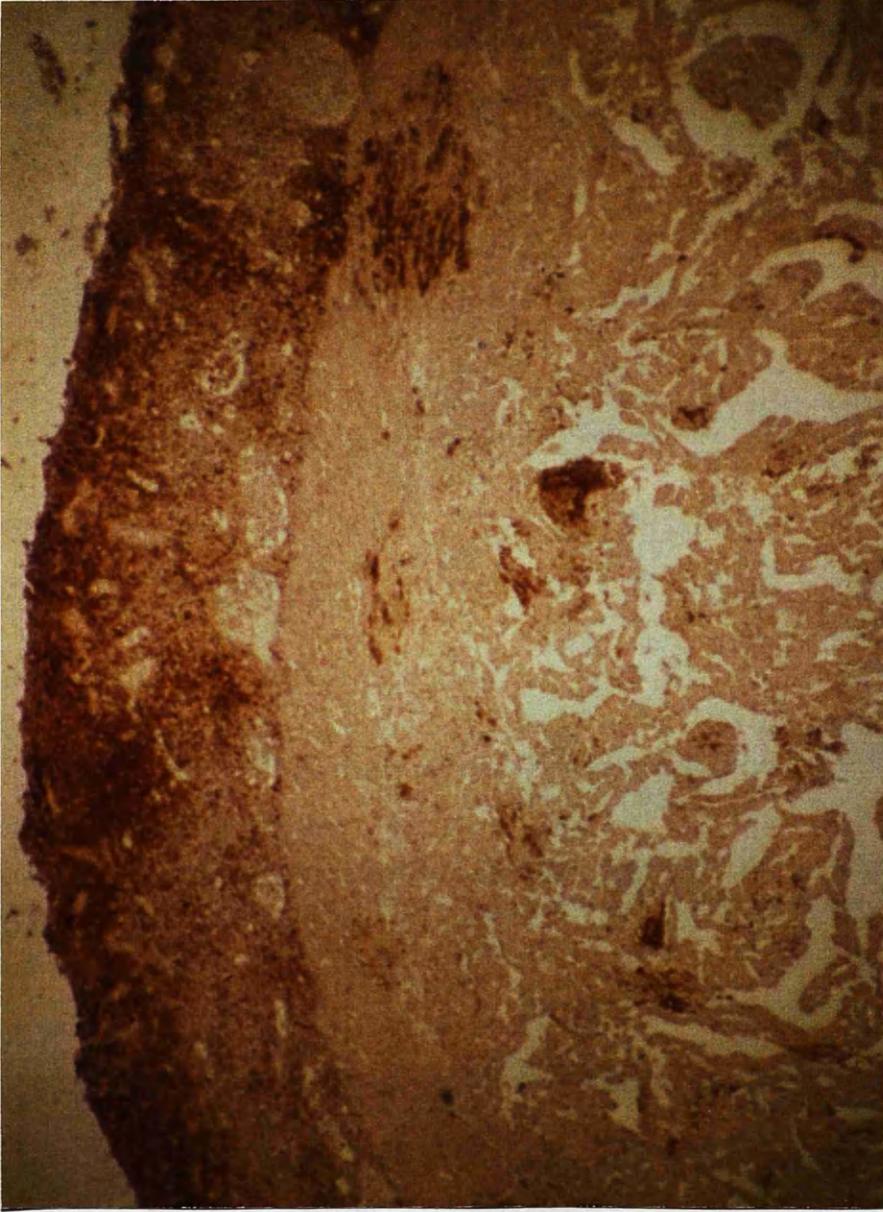
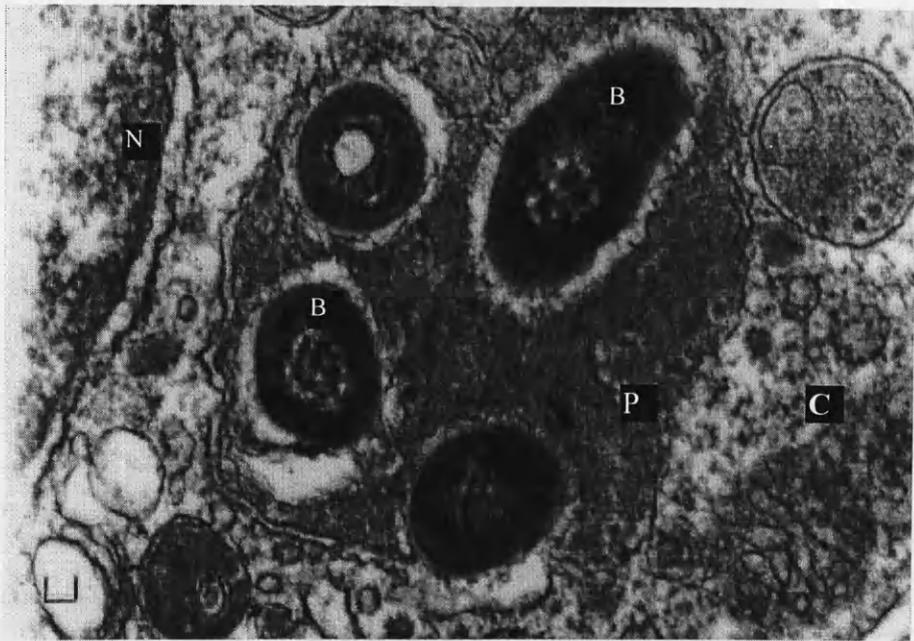


Figure 3.10 Immunohistochemical detection of *R. salmoninarum* in the heart of a naturally infected rainbow trout



**Plate 3.11** *Renibacterium salmoninarum*(B) isolate AVU9202 inside a phagosome (P) like structure in the cytoplasm (C) of a macrophage cell. Rainbow trout were naturally infected. Nucleo (N). Magnification 57600x. Bar marker = 100 nm.

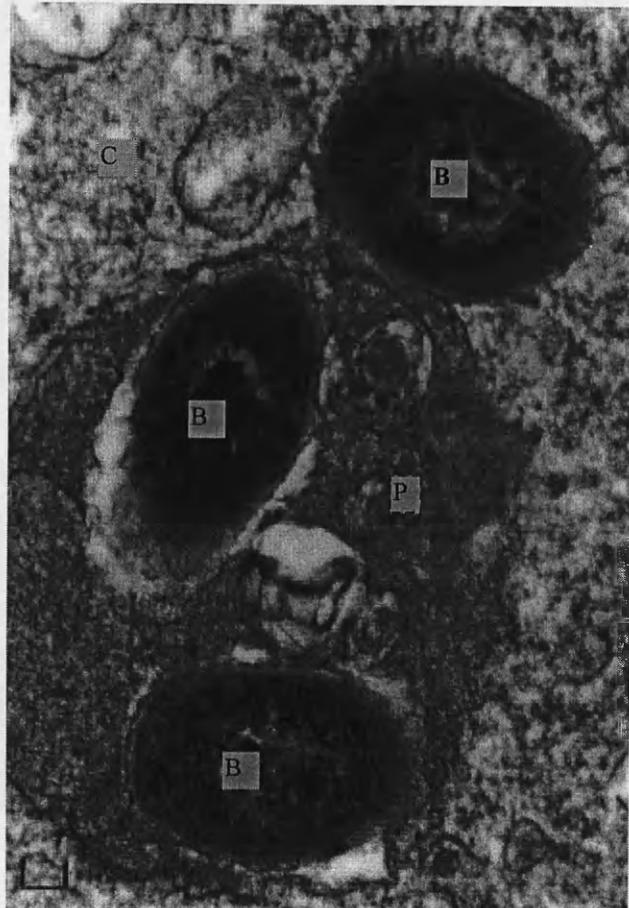


Plate 3.12 *Renibacterium salmoninarum* isolate AVU 9202 in the cytoplasm of a macrophage cell from naturally infected rainbow trout. The bacteria (B) appears to be undigested both in the cytoplasm (C) and inside the phagosome (P). Magnification 76120x. Bar marker = 100 nm.



**Plate 3.13** *Renibacterium salmoninarum* isolate AVU 9202 free in the cytoplasm of macrophage cells from naturally infected rainbow trout. Note the presence of capsule like structure covering the bacterium (arrow). Magnification 94400x, bar marker = 100 nm.

### 3.4 Discussion

The signs and lesions found in the clinical and postmortem examination of diseased fish were in agreement with those reported elsewhere for BKD (Belding and Merrill, 1935; Hedricks and Leek, 1975; Fryer and Sanders, 1981; Klontz, 1983; Ferguson, 1992). However, the farm has been periodically affected by enteric red mouth disease and a number of parasite infections which may have contributed to the signs and pathology observed. The intensity of the lesions was higher in bigger fish where peritonitis and pericarditis were observed. Smaller fish very rarely showed signs of disease. Active bacterial kidney disease was confirmed in some fish by using histopathology, and immunohistochemistry.

Historically the diagnosis of BKD was performed by means of the macro and microscopic examination of the tissue and supported by the Gram's stain. However, gross and histopathological lesions are characteristic but not pathognomonic of the disease. In addition, they are present only in the latest stages of the infection and generally they are absent in asymptomatic diseased fish and carriers. The Gram stain alone is not recommended as a diagnostic procedure because its reliability is impaired by the presence of melanin granules and other morphologically similar bacteria (Chen *et al*, 1974). Using this technique, at least  $10^8$  cell  $g^{-1}$  of tissue should be present in the tissue or smear in order to obtain a positive result (Sakai *et al*, 1989b).

The discovery of glycogen as a component of the bacterium by Bruno and Munro (1982) allows the use of the Lillie's Allochrome stain. However, interference could result from the presence of other morphologically similar, glycogen containing bacteria (Austin and Austin, 1988). Lillie's allochrome was also used for the detection of the bacterial cells which resulted in better clarity when compared to the Gram's stain.

The destruction of tissue observed in severe cases of BKD seems to be related to the release of hydrolytic and catabolic enzymes by ruptured host cells, especially phagocytes, although the liberation of toxic or lytic agents by the bacterium itself or autolysis of dead bacteria may also contribute (Shieh, 1988b, Bandin *et al*,1992). These, together with the release of substances by the cells of the cell mediated immune (CMI) response may also exacerbate the reaction. The route of entry of *R. salmoninarum* into cells is unknown. However the studies of Rose and Levine (1992) demonstrated that complement could be involved. They suggested that opsonisation with rainbow trout complement protein(s) is required for the phagocytosis of the bacteria by rainbow trout head kidney macrophages. In addition, there is information suggesting that the hydrophobic and hemagglutinating cell surface properties of the bacterium may be important (Bruno, 1988). Moreover, the main extracellular and cell surface proteins of the this pathogen (p57) possess certain characteristics resembling the adhesin of other bacterial pathogens (Daly and Stevenson, 1987).

The EM analysis of naturally infected fish with *R. salmoninarum* showed the bacterium only intracellularly, suggesting a carrier state. The pathogen was found apparently undigested both inside phagosomes and also free in the cytoplasm. These finding are in agreement with the observations of Young and Chapman, (1978) and Gutenberger, (1993) who suggested that *R. salmoninarum* possesses a membrane damaging factor which allows the bacterium to escape from the phagosome to the cytoplasm where it apparently can multiply. In addition, survival of the pathogen inside cells has been recently associated with the apparent resistance to reactive oxygen intermediates ( Kaatari *et al*, 1987; Bandin *et al.*, 1993). Other avoidance mechanisms such as catalase and iron reductase activity and phospholipase have been suggested (Bruno and Munro, 1986a; Evenden *et al.*, 1990). However, further investigation is needed in order to better understand the mechanisms for the entry and survival of *R. salmoninarum* inside cells.

## CHAPTER 4

### COMPARATIVE ANALYSIS OF *Renibacterium salmoninarum* ISOLATES BY API ZYM AND MULTILOCUS ENZYME ELECTROPHORESIS (MLEE)

#### 4.1 Introduction

*R. salmoninarum* has been isolated from almost every country where salmonids exist. Some of them, in particular North American and European isolates, have been biochemically, antigenically and enzymatically studied. No major biochemical differences between isolates have been reported (Ordal and Earp, 1956; Embley *et al.*, 1983; Goodfellow *et al.*, 1985; Bruno and Munro 1986a). However, the use of monoclonal antibodies (Arakawa *et al.*, 1987) and API ZYM (Bruno and Munro, 1986a; Austin and Austin, 1987; Sakai *et al.*, 1993) has demonstrated that differences between isolates do exist.

The API ZYM Identification System has been used as a biochemical test for the identification of many other pathogenic bacteria, *Pseudomonas cepacia* (Poh and Loh, 1988), and *Aeromonas hydrophila* (Waltman *et al.*, 1982). Sakai *et al.*, (1993), analysed the use of API ZYM for the diagnosis of eleven common fish pathogenic bacteria. They demonstrated the presence of several biotypes by *Streptococcus sp.*, *Vibrio anguillarum*, and *A. hydrophila*. Other bacterial species tested (e.g. *Aeromonas salmonicida*, *Enterococcus seriolicida*, *R. salmoninarum*, *Pasteurella piscicida*, *Edwardsiella tarda*, *Pseudomonas fluorescens*, *Flexibacter columnaris*, and *F. maritimus*) showed uniform profiles.

Austin and Austin,(1987) reported the following API ZYM profile in 48 *R. salmoninarum* isolates tested: Alkaline phosphatase, caprylate esterase, leucine arylamidase, trypsin, acid phosphatase, phosphoamidase,  $\beta$ -glucosidase, and  $\alpha$ -mannosidase. Similar results have been reported by other authors, with the exception of  $\alpha$ -glucosidase, which was not found in the study of Bruno and Munro, (1986) and the presence of  $\alpha$ -galactosidase (Bruno and Munro, 1986) and butyrate esterase activity (Sakai *et al.*, 1993), in addition to those cited by Austin and Austin, (1987).

The presence of butyrate esterase has been reported so far only in Japanese isolates (Sakai *et al.*, 1993). The authors suggested that the difference in this enzymatic activity may reflect a geographic difference in types of bacterial enzymes present.

Multilocus enzyme electrophoretic (MLEE) analysis has been shown to be a highly sensitive tool to discriminate between isolates of the same bacterial species. By this technique, each isolate is assigned an electrophoretic type (ET), depending on the electrophoretic mobility of known enzymes in a starch gel. As the rate of migration of the protein during electrophoresis is determined by its amino acid sequence, mobility variants (electromorphs or allozymes) of an enzyme can be directly equated with alleles at the corresponding structural gene locus (Selander *et al.*, 1986).

The MLEE has recently been used successfully to estimate the genetic diversity in natural populations of a variety of bacterial species including gram positive and negative organisms. With this technique it is possible to differentiate and identify strains which are morphologically, biochemically and serologically identical (Milkman, 1975; Cougant *et al.*, 1981, 1984, 1985; Ochman and Selander, 1984; Selander *et al.*,1986; Achtman *et al.*, 1986; Musser *et al.*, 1986).

The present work describes the comparative analysis of 18 *R. salmoninarum* isolates using API ZYM and the multilocus enzyme electrophoresis technique (MLEE). The determination of the optimum culture media for growing the bacterium and for their use in API ZYM is also described. This study includes the first MLEE analysis of *R. salmoninarum*. In addition, this is apparently the first time that Japanese and Chilean isolates have been compared with their North American and European counterparts.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Collection of a *R. salmoninarum* library and determination of the optimum culture medium for their culture.**

A library of 18 *R. salmoninarum* isolates, obtained from the United States (2), Chile (1), Japan (2) and the United Kingdom (13) was prepared. Table 4.1 shows the name and origin of all isolates used in this study. All the isolates were cultured in KDM-2 agar unless stated otherwise. They were tested by Gram's stain (Lillie, 1928), catalase and cytochrome oxidase activity using the methods described by Cowan (1992) (see Appendix I and II). The confirmatory identification was performed by indirect ELISA using affinity purified polyclonal antibody (Kinkergaard and Perry Labs, USA) as described in section 2.3.1. Each isolate was inoculated in TSA to detect contamination with other bacteria. If no growth was obtained after a 48 hrs incubation period at 22 °C, the culture were considered pure and suitable for further analysis.

The optimum liquid culture media for the growth of *R. salmoninarum* was investigated by inoculating the type isolate (ATCC 33209) in 100 ml of liquid KDM-Charcoal, KDM-2, SKDM and KDM-2 without FCS (see section 2.1). Cultures were inoculated with 1 ml of spent culture medium (KDM-Charcoal

broth) at OD<sub>610</sub> approximately 1x 10<sup>9</sup> cell ml<sup>-1</sup> and incubated at 15°C for up to 21 days. All the analyses were performed in triplicate. Aliquots of 1 ml were taken every three days and diluted 1:10 in sterile saline solution. The optical density at 610 nm was measured. A graph was constructed plotting the recorded optical density versus incubation time (see Figure 4.1).

**Table 4.1** *R. salmoninarum* isolates used in the present study

Name	Source	Origin
2235	NCIMB	American type strain Lea-1-74 ATCC 33209 from yearling Chinook salmon. Oregon. USA.
2196	NCIMB	ATCC 33739 (81-10B-E-BK) from Brook trout SA.
1111	NCIMB	Scotland
1112	NCIMB	Scotland
1113	NCIMB	Scotland
1114	NCIMB	From <i>Salmo salar</i> . River Dee Aberdeenshire. Scotland
1115	NCIMB	Scotland
1116	NCIMB	From rainbow trout. Scotland
B92256	IOA	From rainbow trout. Scotland
88151	IOA	From rainbow trout. Scotland
B92235	IOA	From rainbow trout. Scotland
B94166	IOA	From rainbow trout. Scotland
AVU9201	IOA	From rainbow trout. Scotland
AVU9202	IOA	From rainbow trout. Scotland
AVU9401	IOA	From rainbow trout. Scotland
UACH9001	UACH	From Coho salmon. Chile.
BOG-9206	MU	Japan
BI-9301	MU	Japan

NCIMB : National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK

ATCC : American Type Culture collection, United States.

IOA : Institute of Aquaculture, University of Stirling, Scotland

UACH : Universidad Austral de Chile

MU : Miyasaki University, Japan

The relationship between bacterial concentration ( $\text{cell ml}^{-1}$ ) and optical density (610 nm) was established and the results are given in Table and Figure 4.2.. Bacteria (isolate ATCC 33209) were cultured in KDM-Charcoal, harvested and heat killed as described in section 2.2.1. The bacterial suspension was diluted in sterile saline containing 0.01% (w/v) Thiomersal (Sigma Chemical Company) to  $\text{OD}_{610\text{nm}}$  0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 and the number of bacteria cells per ml of each dilution was determined under phase contrast microscopy (Olympus BH-2, Japan) at 40x. The counting was repeated three times and the average was taken.

#### **4.2.2 Enzymatic analysis using API ZYM**

The enzymatic analysis of *R. salmoninarum* isolates was performed using the API ZYM Identification System (La Balme les Grottes, 38390 Montalieu, France) following the method provided by the manufacturers and the modifications described by Bruno and Munro, (1986). The gallery was inoculated with  $100 \mu\text{l well}^{-1}$  of freshly harvested bacterial suspension at  $\text{OD}_{610}$  1.0 (approximately  $1 \times 10^9 \text{ cells ml}^{-1}$ ) and incubated in a humid chamber for 28 h at  $22^\circ\text{C}$ . One drop of reagent ZYM A (250 g Tris, 110 ml of 37% HCl, and 100 g SDS made up to a final volume of 1 litre of distilled water) was added to each well followed by one drop of reagent ZYM B (3.5 g of Fast Blue BB per litre of 2-methoxyethanol). The colour was allowed to develop for five minutes with the gallery placed under a powerful light source for approximately 10 seconds with the bulb placed 4 inches above the gallery. This procedure eliminated any yellow colour which could have appeared in the wells due to any excess of fast blue which had not reacted. After light exposure the negative reactions became colourless. The results were read from the gallery using the table provided by the manufacturers.

The API ZYM analysis described in the present study was performed in three steps. In step 1, the effect of four common solid culture media (eg. KDM-2 without FCS, SKDM, KDM-charcoal, MH-C) in the enzymatic profile of the type isolate (ATCC 33209) was studied. In step 2, the effect of broth and agar formulation of the optimum culture media (determined in step 1) was analysed. In step three, the whole *R. salmoninarum* library was analysed using the optimum culture medium determined in steps 1 and 2.

#### **4.2.3 Multilocus enzyme electrophoresis (MLEE)**

MLEE was used to determine and compare the electrophoretic type (ET) of seven isolates of *R. salmoninarum* obtained from USA (ATCC33209, ATCC 33739), Japan (BOG 9206, BI 9301), Chile (UACH 9001) and Scotland (AVU 9202, NCIMB 1112). All the MLEE techniques were performed at the Microbiology department of the Moredun Institute, Edinburgh, Scotland. U.K. in collaboration with Dr. William Donnachie. *Listeria monocytogenes*, a mammalian pathogenic bacterium was used as positive control in all the reactions as the MLEE technique for this bacterium is well standardised in this laboratory. *L. monocytogenes* isolates 262 and 924, cultured on blood agar were obtained from the same laboratory.

At least five plates of culture medium were used per isolate. All the analyses were carried out in duplicate. Bacteria were lysed, electrophoresed on 11 % starch gel and stained for glucose-6-phosphate (G6P), 6-phosphogluconic acid (6PG), mannose phosphate isomerase (MPI), aldose (ALD), leucylglycineglycine (LGG),  $\alpha$ -esterase (EST- $\alpha$ ), and  $\beta$ -esterase (EST- $\beta$ ) as described by Selander *et al.*, (1986); Harris and Hopkins, (1976).

### **4.2.3.1 Bacterial lysate preparation**

The lawn of growth was collected from KDM-2 agar plates (incubated for 18 days at 15 °C) and resuspended into 750 µl of lysate buffer (10 mM Tris, 1 mM EDTA, 0.5 mM NADP, 0.005% DNase, pH 6.8). The cell suspension was then placed into two 2 ml capped tubes (Sarstedt) containing 1.5 ml (approximately 2,5 g) of refrigerated, washed zirconia silica beads 0.1 mm diameter (Biospect Products Inc., Barthesville OK 74003. USA). Cells were disrupted in a mini bead beater (Biospect Products Inc., Barthesville OK 74003. USA) for 2.5 minutes and placed on ice. The supernatant was transferred to a fresh tube and centrifuged at 13,200 g for 20 minutes. The supernatant was recovered and aliquoted into 60 µl amounts. Samples were stored at -70 °C until use.

### **4.2.3.2 Preparation of starch gels**

Starch gels were prepared by mixing 48 g of hydrolysed starch (Connaught Horwell Laboratories Ltd., supplied by Scientific Lab Supplies, Nottingham) and 420 ml of appropriate buffer (A or F buffer, depending on the enzyme of interest ( see sections 4.2.3.4 to 4.2.3.9) in a 2 litres Pyrex side-arm flask. They were mixed thoroughly to avoid clumps and placed in boiling water until the viscosity increased and the opacity decreased. This was continued until the viscosity decreased again and then left in the boiling water bath for 30 minutes.

Starch was then boiled under vacuum for about 1 minute until large bubbles appeared and the mixture was clear. The starch was slightly cooled after boiling and then poured into a 20x20 cm perspex mould, 1 cm thick, previously sealed on a glass plate with vacuum grease. All bubbles were carefully removed with a Pasteur pipette and left to set for 1 hour at room temperature. Gel was wrapped in cling film and kept at 4 °C overnight, if it was not used immediately.

### 4.2.3.3 Electrophoretic separation

The gel was cut using a scalpel, all the way across,  $\frac{1}{4}$  of the distance from one end, to form a slot into which the samples could be loaded. Samples (60  $\mu$ l) were loaded onto two IEF sample application pieces (Pharmacia code 80-1129-46) and placed on the gel slot. Only one sample was loaded per cm with a 4 mm gap between each one. Markers were also loaded at both sides of the gel (Amoranth 0.05% w/v in lysate buffer).

When all the samples were loaded the gel was pushed together at the slot by placing a piece of squeeze mop wicks. The surface of the gel was blotted dry. Two squeeze mop wicks were soaked in the refrigerated electrode buffer and squeezed out gently. The electrode buffer was then poured into the buffer tank and the wicks inserted. The pattern was placed and then the loaded gel (still in the casting mould) put on top. The wicks were folded over so that the cathode wick lay flat on the starch gel nearly reaching the sample slot, and the anode wick lay flat on the starch gel covering  $\frac{1}{4}$  of the gel at the other end. A tray of ice was placed on top of the gel between, but not touching the squeeze mop wicks. This provided sufficient cooling but sometimes needed topping up during the runs as the ice melted.

The gels were run at 80 volts for 1 hour and then 200 voltage with one amp limit set until the dye front had moved 9 cm from the origin, or at 30 volts and 1 amp overnight.

After running, the apparatus was dismantled and the gel cut to size so that it fitted the staining box but none of the loci were lost. Gels were submitted to specific staining procedures depending on the enzyme of interest.

#### **4.2.3.4 Detection of glucose 6 phosphate dehydrogenase (G-6-P)**

Starch gels (12 % w/v) were made up using buffer A (0.661 M Tris, 0.15 M citric acid, pH 8.0) diluted 1:29 following the instructions described in section 2.4.2. Undiluted buffer A was used as electrode buffer.

G-6-P was revealed by staining the gel after electrophoresis with 51 ml of enzyme buffer I (0.2 M Tris HCl, pH 8.0, 0.1 M MgCl<sub>2</sub>) containing glucose 6 phosphate (Sigma Chemical Company, 100 mg), MTT (Sigma Chemical Company, 1 ml stock solution), PMS (Sigma Chemical Company, 0.5 ml stock solution), and NADP (Boehringer, 1 ml). See Appendix 1 for the preparation of stock solutions and buffers.

The staining solution was poured into the gel container and incubated for 30 minutes at 37°C in the dark with constant agitation. Gels were rinsed twice in tap water and then destained with stop solution (acetic acid 10%, methanol 45% in distilled water) for up to 10 minutes. After washing again in tap water as described previously the gels were photographed with a Polaroid camera using Polaroid SS Pos/Neg film.

#### **4.2.3.5 Detection of 6-phosphogluconic acid dehydrogenase (6-P-G)**

6-P-G was revealed by staining the gel, prepared and run in buffer A, with 30 ml of enzyme buffer I containing 6-phosphogluconic acid, Trisodium salt (Sigma Chemical Company, 10 mg), MTT (1 ml, stock solution), PMS (0.5 ml, stock solution), and NADP (0.5 ml). The gel was incubated in the staining solution for 15 minutes at 37°C and then destained, rinsed and photographed as described in section 4.2.3.4.

#### **4.2.3.6 Detection of mannose phosphate isomerase (MPI)**

MPI was revealed by staining the gel, prepared and run in buffer A, with 26 ml of enzyme buffer I containing mannose-6-phosphate (Sigma Chemical Company, 10 mg), glucose-6-phosphate dehydrogenase (Sigma Chemical Company, 20 U), phosphoglucose isomerase (Sigma Chemical Company, 50 U), NAD (20 mg), NADP (10 mg), MTT (1 ml), and PMS (0.5 ml). The gel was overlaid with 25 ml of melted 2% agarose and incubated for 1 hour at 4°C in the dark. As the reaction could not be stopped, it was photographed immediately.

#### **4.2.3.7 Detection of leucylglycineglycine peptidase (LGG)**

LGG was revealed by staining the gel, prepared and run in buffer A, with 52 ml of enzyme buffer II (0.25 M Tris HCl, pH 8.0, 0.25 M MnCl<sub>2</sub>) containing leucylglycineglycine (Sigma Chemical Company, 20 mg), peroxidase (Sigma Chemical Company, 15 mg), snake venom, (Sigma Chemical Company, 10 mg) and 3-amino-9-ethylcarbazole (Sigma Chemical Company, 0.2 ml). The gel was overlaid with 25 ml of melted 2% agarose and incubated for 1.5 hour at 4°C in the dark. As the reaction could not be stopped, it was photographed immediately.

#### **4.2.3.8 Detection of alanine dehydrogenase (ALD)**

ALD was revealed by staining the gel, prepared and run in buffer A, with 50 ml of enzyme buffer III (0.05 M sodium phosphate, pH 7.0) containing DL-Alanine (Sigma Chemical Company, 50 mg), NAD (2 ml), MTT (1 ml), and PMS (0.5 ml). The gel was then incubated, rinsed and photographed as described in section 4.2.3.4.

#### **4.2.3.9 Detection of $\alpha$ and $\beta$ naphthyl propionyl esterase (EST- $\alpha$ and EST- $\beta$ )**

Starch gels was made up using buffer F (0.1 M Tris, 0.1 M maleic acid 0.1 M EDTA, MgCl<sub>2</sub>, pH 8.2). Agarose was prepared as described in section 4.2.3.2 but using diluted Buffer F (46.7 ml of buffer plus 373.3 ml distilled water). Undiluted buffer F was used as electrode buffer.

EST- $\alpha$  and EST  $\beta$  were revealed by staining the gel with 40 ml of buffer III containing  $\alpha$  -naphthyl propionate (Sigma Chemical Company, 15 mg), and Fast blue RR salt (Sigma Chemical Company, 25 mg). The gel was incubated, rinsed and photographed as described in section 2.4.4. Both enzymes were developed by the same staining solution. EST- $\beta$  travelled behind EST- $\alpha$ , approximately half way down the gel.

### **4.3 RESULTS**

#### **4.3.1 Determination of the optimal culture medium for *R. salmoninarum* and the relationship between optical density and bacterial concentration.**

The highest bacterial concentration (OD<sub>610</sub> measured after 21 days incubation period) was obtained when the bacterium was cultured in KDM-2 (1.08±0.045) followed by KDM-charcoal (0.85±0.072), SKDM (0.65±0.037), and KDM-2 without FCS (0.44±0.055). The growth pattern of *R. salmoninarum* in all cultures was characterised by a 3-6 days lag phase followed by a constant but slow growth until day 21. The optimum culture media was found to be KDM-2, followed by KDM-Charcoal. The poorest growth

was obtained using KDM-2 without FCS where the lag phase lasted almost until day 15 (see Figure 4.1). The relationship between the absorbance and bacterial concentration is shown in Table 4.2. A standard curve showing the same relationship was made and is presented in Figure 4.2

**Table 4.2. Relationship between optical density (OD<sub>610</sub> nm) and bacterial concentration (cell ml<sup>-1</sup>)**

OD <sub>610</sub> nm	Bacteria cell ml <sup>-1</sup>
0.1	8.0 x 10 <sup>7</sup>
0.2	1.2 x 10 <sup>8</sup>
0.3	2.0 x 10 <sup>8</sup>
0.4	2.9 x 10 <sup>8</sup>
0.5	3.9 x 10 <sup>8</sup>
0.6	4.5 x 10 <sup>8</sup>
0.7	6.6 x 10 <sup>8</sup>
0.8	8.3 x 10 <sup>8</sup>
0.9	9.1 x 10 <sup>8</sup>

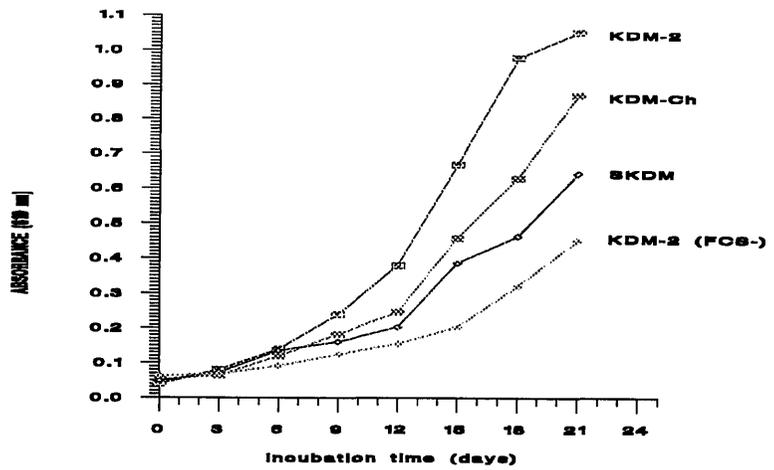


Figure 4.1 Standard curve of *R. salmoninarum* (ATCC 33209) cultured in four different culture media

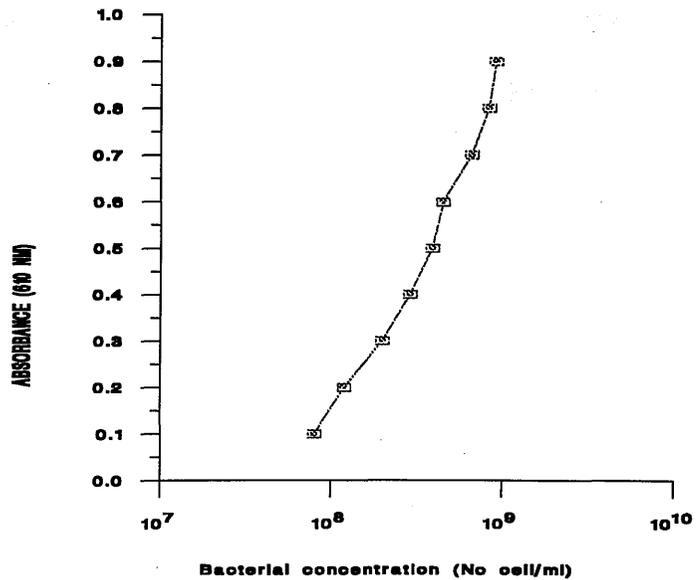


Figure 4.2 Standard curve of *R. salmoninarum* (ATCC 33209) showing the relationship between absorbance (OD<sub>610</sub> nm) and the bacterial concentration (cell ml<sup>-1</sup>).

### 4.3.2 API ZYM analysis of *R. salmoninarum* (ATCC 33209) cultured on four different solid culture media

The API ZYM profiles of *R. salmoninarum* cultured in KDM-2, SKDM and KDM-charcoal were found to be similar and only minor differences with reference to the intensity of the reaction ( $\text{nM ml}^{-1}$ ) were detected. However, bacteria grown on MH-C failed to produce a signal for acid phosphatase activity which was detected in high concentration ( $30\text{--}40 \text{ nM ml}^{-1}$ ) in the other three culture media used (see Table 4.3).

Bacteria obtained from KDM-charcoal, produced a decreased signal for some enzymatic activities, including butyrate esterase, caprylate esterase, and  $\alpha$ -glucosidase. Alkaline phosphatase was detected at a very low level ( $<5 \text{ nM ml}^{-1}$ ) in all the media. Cells obtained from KDM-2 and SKDM showed a very similar profile and even the concentration of the enzymes detected was almost the same. No reaction was obtained for myristate lipase, valine arylamidase, cysteine arylamidase, chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase,  $\beta$ -glucosidase, N-acetyl- $\beta$ -glucosaminidase, and  $\alpha$ -fucosidase. The results suggest that KDM-2 and SKDM are the optimum solid culture media for API ZYM characterisation of *R. salmoninarum*.

Table 4.3 API ZYM ANALYSIS OF *Renibacterium salmoninarum* ATCC 33209 CULTURED ON FOUR SOLID CULTURE MEDIA\*

ENZYME	CULTURE MEDIA			
	KDM-2	SKDM	KDM-Ch	MH-C
Control	--	--	--	--
Alkaline phosphatase	<5	<5	<5	<5
Butyrate esterase (C4)	10	20	5	10
Caprylate esterase (C8)	20	20	5	30
Myristate lipase (C14)	--	--	--	--
Leucine arylamidase	30	30	30	30
Valine arylamidase	--	--	--	--
Cystine arylamidase	--	--	--	--
Trypsin	5	5	5	5
Chymotrypsin	--	--	--	--
Acid phosphatase	30	40	30	--
Phosphoamidase	10	5	5	5
$\alpha$ -galactosidase	--	--	--	--
$\beta$ -galactosidase	--	--	--	--
$\beta$ -glucuronidase	--	--	--	--
$\alpha$ -glucosidase	40	30	10	20
$\beta$ -glucosidase	--	--	--	--
$\beta$ -glucosaminidase	--	--	--	--
$\alpha$ -mannosidase	10	5	20	10
$\alpha$ -fucosidase	--	--	--	--

\* Figure s represents nanomoles (nM) ml-1

### 4.3.3 API ZYM analysis of *R. salmoninarum* cultured in KDM-2 broth and agar

In the previous sections it was found that KDM-2 and SKDM agar are the optimum solid culture media for API ZYM analysis. However, it was also found that SKDM slowed down the growth of the bacterium, probably due to the presence of a mixture of antibiotics. In this section the API ZYM profile of KDM-2 broth and agar was studied. The results are presented in Table 4.4.

Bacteria grown in liquid culture media produced an increased signal for alkaline phosphatase and butyrate esterase in comparison with the same isolate grown in solid media. A false positive reaction was obtained when non inoculated culture media were analysed. This finding was observed for alkaline phosphatase, butyrate esterase and caprylate esterase both in liquid (5-10 nM ml<sup>-1</sup>) and solid media (<5 nM ml<sup>-1</sup>). Trypsin also showed a low level of background (5 nM ml<sup>-1</sup>) but only when bacteria were grown in liquid media. The present results suggest that KDM-2 agar is superior to its broth counterpart for API ZYM studies.

**Table 4.4** API ZYM PROFILE OF *Renibacterium salmoninarum* ATCC 33209 CULTURED in KDM-2 BROTH AND AGAR.\*

ENZYME	KDM-2 BROTH		KDM-2 AGAR	
	Inoculated	Non inoculated	Inoculated	Non inoculated
Control	--	--	--	--
Alkaline phosphatase	20	10	6	6
Butyrate esterase (C3)	20	5	10	6
Caprylate esterase (C8)	40	5	20	6
Myristate lipase (C14)	--	--	--	--
Leucine arylamidase	30	--	30	--
Valine arylamidase	--	--	--	--
Cystine arylamidase	--	--	--	--
Trypsin	5	5	5	--
Chymotrypsin	--	--	--	--
Acid phosphatase	40	--	30	--
Phosphoamidase	5	--	10	--
$\alpha$ -galactosidase	--	--	--	--
$\beta$ -galactosidase	--	--	--	--
$\beta$ -glucuronidase	--	--	--	--
$\alpha$ -glucosidase	30	--	40	--
$\beta$ -glucosidase	--	--	--	--
$\beta$ -glucosaminidase	--	--	--	--
$\alpha$ -mannosidase	5	--	10	--
$\alpha$ -fucosidase	--	--	--	--

\* Figure s represents nanomoles (nM) ml-1

#### 4.3.4 API ZYM analysis of 18 *R. salmoninarum* isolates.

All the isolates tested showed identical API ZYM profiles. However, variations in the concentration of some enzymatic activities ( $\text{nM ml}^{-1}$ ) between isolates were detected (See Table 4.5). The following enzymatic activities were detected: alkaline phosphatase ( $<5\text{-}10 \text{ nM ml}^{-1}$ ), butyrate esterase ( $5\text{-}10 \text{ nM ml}^{-1}$ ), caprylate esterase ( $20\text{-}40 \text{ nM ml}^{-1}$ ), leucine arylamidase ( $10\text{-}30 \text{ nM ml}^{-1}$ ), trypsin ( $<5 \text{ nM ml}^{-1}$ ), acid phosphatase ( $30\text{-}40 \text{ nM ml}^{-1}$ ), phosphoamidase ( $10 \text{ nM ml}^{-1}$ ),  $\alpha$ -glucosidase ( $20\text{-}40 \text{ nM ml}^{-1}$ ) and  $\alpha$ -mannosidase ( $5\text{-}15 \text{ nM ml}^{-1}$ ). Control samples, taken from non inoculated media, showed a low level of false positive reaction for alkaline phosphatase ( $<5 \text{ nM ml}^{-1}$ ), butyrate esterase ( $<5 \text{ nM ml}^{-1}$ ), caprylate esterase ( $<5 \text{ nM ml}^{-1}$ ) and phosphoamidase ( $<5 \text{ nM ml}^{-1}$ ). The most variable enzymatic activity was caprylate esterase ( $20\text{-}40 \text{ nM ml}^{-1}$ ) and the most stable was trypsin ( $5 \text{ nM ml}^{-1}$ ).

No reaction was obtained for myristate lipase, valine arylamidase, cystine arylamidase, chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase,  $\beta$ -glucosidase, N-acetyl- $\beta$ -glucosaminidase, and  $\alpha$ -fucosidase as expected. Figure 4.3 shows the API ZYM profile of selected *R. salmoninarum* isolates obtained from Chile, Japan, USA and UK.

Table 4.5 ENZYMIC ANALYSIS OF *R. salmoninarum* ISOLATES\*

ENZYME	CONTROL	9202	2196	88151	UACH	BOG	BI
Control	--	--	--	--	--	--	--
Alkaline phosphatase	<5	10	10	10	5	10	5
Butyrate esterase (C4)	5	10	5	10	10	10	10
Caprylate esterase (C8)	<5	30	20	20	30	20	30
Myristate lipase (C14)	--	--	--	--	--	--	--
Leucine arylamidase	--	20	10	20	20	30	20
Valine arylamidase	--	--	--	--	--	--	--
Cystine arylamidase	--	--	--	--	--	--	--
Trypsin	--	5	5	5	5	5	5
Chymotrypsin	--	--	--	--	--	--	--
Acid phosphatase	--	40	40	30	40	40	40
Phosphoamidase	--	10	10	10	10	10	10
$\alpha$ -galactosidase	--	--	--	--	--	--	--
$\beta$ -galactosidase	--	--	--	--	--	--	--
$\beta$ -glucuronidase	--	--	--	--	--	--	--
$\alpha$ -glucosidase	--	40	30	30	40	30	30
$\beta$ -glucosidase	--	--	--	--	--	--	--
$\beta$ -glucosaminidase	--	--	--	--	--	--	--
$\alpha$ -mannosidase	--	10	10	5	15	10	10
$\alpha$ -fucosidase	--	--	--	--	--	--	--

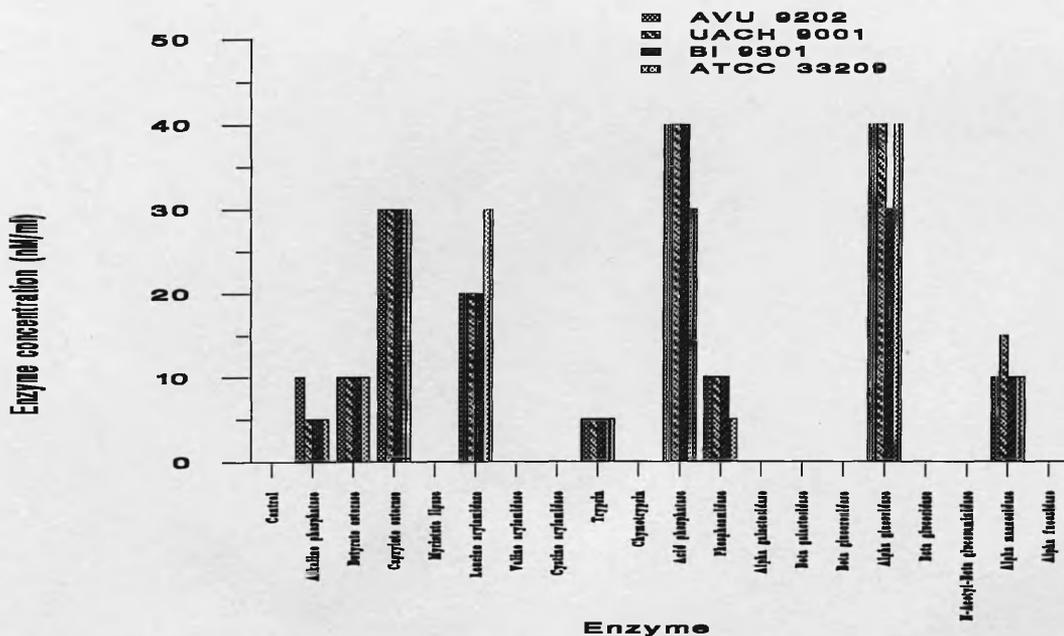
Table 4.5 ENZYMIC ANALYSIS OF *R. salmoninarum* ISOLATES (Cont.)\*

ENZYME	1111	1112	1113	1114	1115	1116	9201
Control	--	--	--	--	--	--	--
Alkaline phosphatase	5	5	5	5	5	5	5
Butyrate esterase (C4)	10	5	5	5	5	5	10
Caprylate esterase (C8)	40	30	30	40	40	40	30
Myristate lipase (C14)	--	--	--	--	--	--	--
Leucine arylamidase	10	10	20	10	10	10	20
Valine arylamidase	--	--	--	--	--	--	--
Cystine arylamidase	--	--	--	--	--	--	--
Trypsin	5	5	5	5	5	5	5
Chymotrypsin	--	--	--	--	--	--	--
Acid phosphatase	30	30	30	40	30	30	40
Phosphoamidase	10	10	10	10	10	10	10
$\alpha$ -galactosidase	--	--	--	--	--	--	--
$\beta$ -galactosidase	--	--	--	--	--	--	--
$\beta$ -glucuronidase	--	--	--	--	--	--	--
$\alpha$ -glucosidase	20	40	40	40	30	30	40
$\beta$ -glucosidase	--	--	--	--	--	--	--
$\beta$ -glucosaminidase	--	--	--	--	--	--	--
$\alpha$ -mannosidase	5	5	5	5	5	5	10
$\alpha$ -fucosidase	--	--	--	--	--	--	--

**TABLE 4.5 API ZYM profile of *R. salmoninarum* isolates (Cont.)\***

ENZYME	92256	33209	94166	92235	9401
Control	--	--	--	--	--
Alkaline phosphatase	<5	5	10	5	5
Butyrate esterase (C4)	10	10	10	5	10
Caprylate esterase (C8)	30	20	40	30	40
Myristate lipase (C14)	--	--	--	--	--
Leucine arylamidase	30	30	20	20	20
Valine arylamidase	--	--	--	--	--
Cystine arylamidase	--	--	--	--	--
Trypsin	5	5	5	5	5
Chymotrypsin	--	--	--	--	--
Acid phosphatase	30	30	40	30	40
Phosphoamidase	10	10	10	10	10
α-galactosidase	--	--	--	--	--
β-galactosidase	--	--	--	--	--
β-glucuronidase	--	--	--	--	--
α-glucosidase	40	40	40	30	30
β-glucosidase	--	--	--	--	--
β-glucosaminidase	--	--	--	--	--
α-mannosidase	10	10	10	5	10
α-fucosidase	--	--	--	--	--

\* Figure s represents nM ml-1



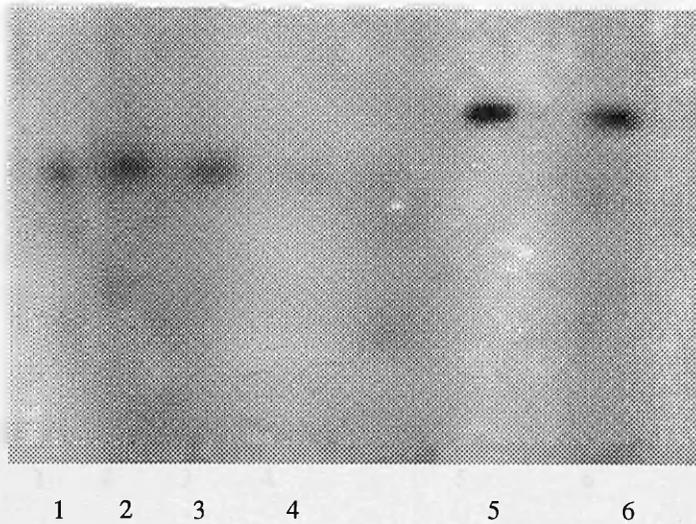
**Figure 4.3** API ZYM profile of *R. salmoninarum* isolates from four different countries. Chile (UACH 9001, Japan (BI 9301), USA (ATCC 33209) and U.K. (AVU 9202)

#### 4.3.5 Multilocus enzyme electrophoresis (MLEE) analysis

The presence of seven enzymes was investigated. Only four were detected, namely glucose-6-phosphate dehydrogenase (G6P), 6-phosphogluconic acid dehydrogenase (6PG), mannose phosphate isomerase (MPI), and alanine dehydrogenase (ALD). No reaction was obtained for leucylglycineglycine peptidase (LGG),  $\alpha$ -Esterase (EST  $\alpha$ ) and  $\beta$ -Esterase (EST $\beta$ ), even after trying different buffer systems.

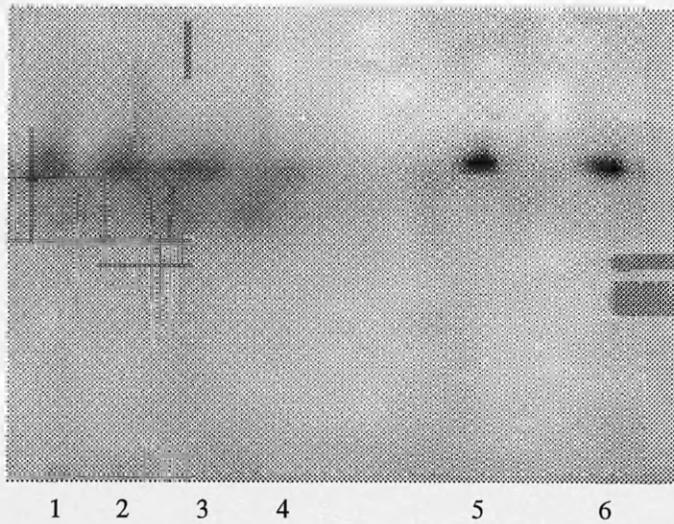
The results from G6P analysis shown a clear difference between the electrophoretic mobility of this enzyme in *R. salmoninarum* and *L. monocytogenes* where renibacterial G6P migrated further. No differences in the mobility of this enzyme were detected between the *R. salmoninarum* isolates tested; however, isolate BI 9301 showed very poor reaction (see Figure 4.4).

The electrophoretic mobility of 6PG, MPI, and ALD from *R. salmoninarum* and *L. monocytogenes* was identical. However the intensity of the reaction was greater for *L. monocytogenes* in some of the gels. The ET of these enzymes were identical in the seven renibacterial isolates tested (Figure s 4.5 and 4.6).



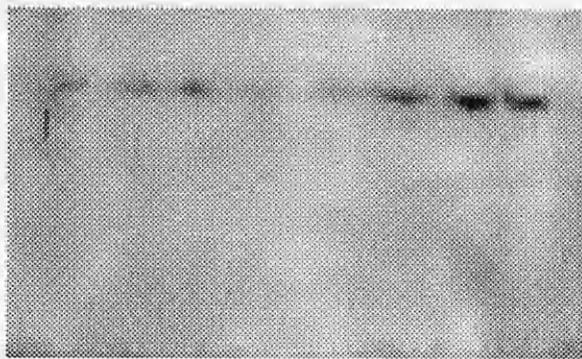
**Figure 4.4** MLEE of Glucose 6 phosphate dehydrogenase (G6P)

- 1.- *R. salmoninarum* ATCC 33209 ((USA)
- 2.- *R. salmoninarum* AVU 9202 (UK)
- 3.- *R. salmoninarum* UACH 9101 (Chile)
- 4.- *R. salmoninarum* BI 9301 (Japan)
- 5.- *L. monocytogenes* isolate 262
- 6.- *L. monocytogenes* isolate 924



**Figure 4.5** MLEE of 6-phosphogluconic acid dehydrogenase (6PG)

- 1.- *R. salmoninarum* ATCC 33209 ((USA)
- 2.- *R. salmoninarum* AVU 9202 (UK)
- 3.- *R. salmoninarum* UACH 9101 (Chile)
- 4.- *R. salmoninarum* BI 9301 (Japan)
- 5.- *L. monocytogenes* isolate 262
- 6.- *L. monocytogenes* isolate 924



1 2 3 4 5 6 7 8

**Figure 4.6** MLEE of 6-phosphogluconic acid dehydrogenase (6PG) of seven different isolates of *R. salmoninarum* cultured in KDM-2

- 1.- *L. monocytogenes* 924 (Scotland)
- 2.- *R. salmoninarum* NCIMB 1112 (Scotland)
- 3.- *R. salmoninarum* AVU 9202 (Scotland)
- 4.- *R. salmoninarum* BOG 9206 (Japan)
- 5.- *R. salmoninarum* UACH 9001 (Chile)
- 6.- *R. salmoninarum* ATCC 33739 (USA)
- 7.- *R. salmoninarum* BI 9301 (Japan)
- 8.- *R. salmoninarum* ATCC 33209 (USA)

#### 4.4 Discussion

The growth pattern of *R. salmoninarum* in all the culture media tested (e.g. KDM-2, SKDM, KDM-charcoal, and MH-C) was characterised by a prolonged lag phase followed by continuous but very slow growth. These findings are in agreement with Sander and Fryer, (1980) and Fryer and Lannan, (1993) who also reported the slow growing nature of this pathogen. Faster growth of the bacterium has been obtained by Dheur *et al.*, (1993) who cultured the type strain (ATCC 33209) using a commercial fermentor at 15 °C. They observed a lag phase of only 48 hrs followed by a relatively rapid growth until the culture reached a plateau at day 10. The slow growth obtained in the present study could be due to the fact that cultures were incubated in a static incubator rather than a rotary one.

The API ZYM profile of *R. salmoninarum* (ATCC 33209) showed some differences depending on the culture media used. KDM-2 and SKDM were found to be better than KDM-Ch and MH- for this type of enzymatic study. Bacteria cultured in KDM-Ch showed a notable reduction in the production of butyrate esterase, caprilate esterase and  $\alpha$ -glucosidase while bacteria grown in MH-C failed to produce acid phosphatase. This finding is in disagreement with Bruno and Munro, (1986) who using the same culture medium managed to detect this enzyme, although at very low level ( $<5 \text{ nM ml}^{-1}$ ). In addition the same authors failed to detect  $\alpha$ -glucosidase activity in their isolates, however this enzyme was detected in relatively high concentration ( $10\text{-}40 \text{ nM ml}^{-1}$ ) in all four culture media tested here and was also detected by Austin and Austin, (1987) and Sakai *et al.*, (1993).

The concentration of leucine arylamidase was considerably increased in the present study (30 nM ml<sup>-1</sup>) in comparison with the report of Bruno and Munro (1986) (5 nM ml<sup>-1</sup>). Phosphoamidase was detected in lower concentration (5 nM ml<sup>-1</sup>) compared with the same authors (20 nM ml<sup>-1</sup>).

The profile obtained using bacteria grown in KDM-2 showed almost the same pattern as described by Austin and Austin,(1993) and Sakai *et al.*,(1993), except for alkaline phosphatase and butyrate esterase where some small differences were observed. There is no reference in the literature regarding the use of SKDM and KM-Ch media for API ZYM characterisation.

This study showed that the concentration of certain enzyme activities was considerably increased when the bacteria was grown in liquid media. The presence of false positive reaction for alkaline phosphatase, butyrate esterase, caprylate esterase and phosphoamidase in the control group suggest that the higher concentration observed in broth cultures may be due, at least in part, to the non specific reaction developed by some of the components of the culture medium.

Comparative analysis of 18 *R. salmoninarum* isolates indicated identical API ZYM profiles, however, minor differences with reference to the intensity of the reaction for the same enzyme were observed. This is apparently the first time that Chilean and Japanese isolates have been included in a comparative study using API ZYM. The results of this study are in complete agreement with Austin and Austin, (1987) and Bruno and Munro,(1986) but slightly differ from the results of Sakai *et al* (1993) who described a novel character (e.g. butyrate esterase activity) in Japanese isolates, probably related to a geographic difference of the pathogen. This activity was also found in the present study in concentrations ranging from 10 to 30 nM ml<sup>-1</sup>, however the present results suggest that this new character could be more related to the false positive reaction (observed when the API ZYM was performed in non inoculated media) rather than the bacterium itself.

The results of the MLEE analysis of seven *R. salmoninarum* isolates showed positive reaction for four out of seven enzymes tested. They were glucose 6-phosphate (G6P), 6-phosphoglucuronic acid (6PG), mannose phosphate isomerase (MPI) and aldose (ALD). Each of these enzyme showed the same electrophoretic type in all the isolates tested, which were obtained from USA (ATCC 33209, ATCC 33739), Japan (BOG 9206, BI 9301), Chile (UACH 9001) and Scotland (AVU 9202, NCIMB 1112). As the electrophoretic types (mobility variants, electromorphs or allozymes) of an enzyme can be directly equated with alleles at the corresponding structural gene locus (Selander *et al.*, 1986), it is possible that all *R.s* isolates tested are genetically closely related and that the distribution of this pathogen could have a clonal nature.

Electrophoretic studies of bacterial populations have revealed that most bacterial species are clonal in structure, implying low rates of recombination between chromosomal genes in nature (Musser *et al.*, 1987; Ochman *et al.*, 1984; Pinero *et al.*, 1988; Denny *et al.*, 1988; Gargallo-Viola, 1989). Clonal structure is characterised by the existence of the same multilocus genotype (or electrophoretic type, ET) from temporally and geographically unassociated hosts (Selander and Musser, 1990). Isolates that have the same multilocus genotype are considered to be members of the same clone and, as expected, typically exhibit identity in other characters including ribotyping (Arthur *et al.*, 1990), RFLP profiles (Denny *et al.*, 1988) and metabolic characters (Selander *et al.*, 1990 and Miller and Hartl, 1986).

The reports of McLellan, (1984); Ramshaw Coyne and Lewontin, (1979); Shumaker, Allard and Kahler,(1982), who studied several proteins of known sequence indicate that gel electrophoresis can detect a large portion (80-90%) of amino acid substitutions. However, because some substitutions do not affect electrophoretic mobility, electromorph profiles may be sequentially heterogeneous (Ayala, 1982; Coyne, 1982; Milkman, 1973), and at the level of the nucleotide sequence of the gene itself, there is even greater

heterogeneity, owing primarily to silent substitution. Although electromorph profiles over loci can be equated with multilocus genotypes and electromorph frequencies can be equated with allele frequencies, it is with the understanding that the alleles recognised may actually be groups of isoalleles (Selander *et al.*, 1986)

The fact that the *R.s* isolates tested belonged to the same ET does not mean categorically that genetic variation, expressed as amino acid substitutions does not occur. Furthermore, these results support the previously reported antigenic and biochemical analysis (Bullock *et al.*, 1974; Paterson *et al.*, 1981; Getchel *et al.*, 1985; Bruno and Munro, 1986; Austin and Austin, 1987; and Hsu *et al.*, 1991) suggesting the homogeneity of the *R. salmoninarum* isolates from different geographic areas.

## CHAPTER 5

### PRODUCTION OF POLYCLONAL AND MONOCLONAL ANTIBODIES AGAINST *R. salmoninarum* AND THEIR USE IN AN ANTIGENIC ANALYSIS OF THE BACTERIUM.

#### 5.1 Introduction

The antigenic structure of *R. salmoninarum* isolates have been studied by numerous researchers. Getchell *et al* (1985) first described the presence of a common antigen (F antigen) in several isolates of this bacterium. Further analysis using sophisticated biochemical and immunological techniques showed that this antigen is the major extracellular product of this pathogen and was termed p57.(Dubreuil *et al*, 1990; Kaatari *et al*, 1989; Wiens and Kaatari, 1989; Griffiths and Lynch, 1991).

Within the renibacterial cell wall, polysaccharides are the major components and their immunogenic nature for rabbits has been demonstrated by Kusser and Fiedler, (1983); Fiedler and Draxl, (1986) and Sørum and Robertsen, (1994). Furthermore, the studies of Arakawa *et al*, 1987 suggest that they are also immunogenic in mice. As occurs in many bacterial species where serotypes are defined, the type of polysaccharide plays an important role in determining serological differences between serotypes. The analysis of Fiedler and Draxl, (1986) suggests that the quantity in the cell and biochemical composition of the renibacterial polysaccharide appear to be similar, at least in the 13 isolates tested by the authors. However, no serological studies with reference to polysaccharide differentiation between isolates has been performed to date.

The second main component of renibacterial cell wall is peptidoglycan. This macromolecule possesses highly immunogenic properties in the majority of gram positive bacteria. The studies of Krausse (1975); Schleifer and Seidl (1977) have led to the discovery of at least five independent antigenic epitopes in the peptidoglycan molecule. Although crossreactivity between peptidoglycan from different gram positive bacteria have been reported (Karakawa *et al.*,1968), highly specific antisera against the particulate interpeptide bridges of the peptidoglycan molecule have been raised and allowed to differentiate serologically between *Staphylococcus* and *Micrococcus* (Seidl and Schleifer, 1978a). The same authors concluded that a difference of even one amino acid (alanine or glycine) in the interpeptide bridge of peptidoglycan could be serologically detected.

Fiedler and Draxl (1986) reported that renibacterial peptidoglycan contains a peptide bridge containing an amino acid sequence which is unique among the gram positive bacteria. This particular section of the renibacterial PG is composed of glutamic acid, lysine, alanine and glycine in a molar ratio of 1:1:4:1. Based on this finding, the development of monoclonal or polyclonal antibodies specific for this amino acid sequence could result in the production of a specific probe for the identification of *R. salmoninarum*.

A number of polyclonal antibodies (PABs) against renibacterial cells have been reported to date (Bullock and Stuckey, 1975; Paterson *et al.*, 1981; Austin and Rayment, 1985; Getchell *et al.*, 1985; Hsu *et al.*,1991) and have been used mainly for diagnostic purposes. They recognise primarily the 57 kDa antigen and its breakdown products but usually crossreact with other gram positive bacteria, particularly coryneform species, fish pathogenic *Mycobacterium spp.* and *Rothia dentocariosa* (Bullock *et al.*,1980; Austin and Rayment, 1985; Austin and Austin, 1987).

To date , a few MAbs against *R. salmoninarum* have been developed. The first was reported by Arakawa *et al.*,(1987); however, they crossreacted with other bacterial species and failed to recognise some of the renibacterial isolates used in the study.

MAbs against p57 were developed by Wiens and Kaatari, (1989). They did not crossreact with any of the gram positive and negative fish pathogens tested. Eight MAbs were obtained and classified on the basis of their differential recognition of p57 proteolytic breakdown products. Group I MAbs recognise p34 and p20, group II MAbs. recognised p36 and p34 fragments and group III MAbs bound proteolytic fragment p45 and two fragments p36 and p25.

MAbs against heat extracted antigens of the bacterium, produced by Sakai *et al.*, ( 1991b), recognised a 60 kDa protein and several lower molecular weight protein antigens, probably p57. A monoclonal antibody against p57 was produced by Dr. Fred Markham, at the Prince Edward Island University, Canada, (personal communication) and it is included in this study for comparative purposes.

This chapter describes the production of polyclonal and monoclonal antibodies against extracellular and cell wall antigens of *R. salmoninarum*, the effect of different culture media on the expression of antigens and the immunogenic characterisation of 18 isolates of *R. salmoninarum*.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Library of anti *R. salmoninarum* antibodies

A library of PAbs (PVIR, PKPL) and MAbs (B6G8, D11A) were obtained from external sources. Their name, specificity, type, host and origin are given in Table 5.1.

PAb PVIR was produced in rabbits by Dr. Frerichs, Virology Unit, Institute of Aquaculture using whole bacterial cells as the inoculum. Polyclonal antibody PKPL was purchased from Kingergaar Perry Lab, USA (KPL). It was produced in goats using whole bacteria and further adsorbed with several fish pathogens to increase its specificity. Anti-p57 MAb (B6G8) was obtained from Dr. Fred Markham, Prince Edward Island University. Freeze dried antibodies were reconstituted in 1 ml of PBS and considered as neat. They were aliquoted and stored at -70 °C until use.

**Table 5.1 Polyclonal and monoclonal antibodies obtained from external sources and used in the present study**

<b>Name</b>	<b>Immunogen</b>	<b>Type</b>	<b>Host</b>	<b>Origin</b>
PKPL	Whole bacteria	Polyclonal	Goat	KPL, USA
PVIR	Whole bacteria	Polyclonal	Rabbit	Scotland
B6G8	57 kDa protein	Monoclonal	Mouse	Canada

### 5.2.2 Production of polyclonal antibodies (PAbs).

Polyclonal antibodies were produced using the methods described by Harlow and Lane (1988). Two New Zealand albino rabbits and 6 Balb/c female mice six weeks old were obtained and maintained at the Animal House, University of Stirling. Animals were inoculated with different renibacterial antigens, including cell wall, polysaccharide, 57 kDa protein and 70 kDa protein. The antigens were obtained as described in section 2.2. The primary inoculation protocol is shown in Table 5.2.

The antigens (1 ml each) were emulsified separately with equal volume of Freund's complete adjuvant (FCA, Sigma Chemical Company) by using two glass syringes connected through one 18 Gauge double hub emulsification needle. To do so, the antigen suspension was pushed into the adjuvant syringe first and then mixed by forcing the material back and forth through the double hub needle for approximately five minutes or until a whipped-cream like water in oil emulsion was formed which is signalled by the sudden increase in viscosity. To test the stability of the emulsion, one drop was placed on water, it should hold together on the surface of water.

**Table 5.2 Primary immunisation of rabbit and mouse for polyclonal antibody production**

Antibody	Antigen	Adjuvant	Route	Dose(ml)	Host(No)
155/p57	P57 (140 µg)	FCA	SC	1.0	Rabbit(1)
158/p70	P70 (80 µg)	FCA	SC	1.0	Rabbit(1)
CW3	Cell wall (40 µg)	FCA	IP	0.2	Mouse(3)
PS	Polysaccharide (20 µg)	FCA	IP	0.2	Mouse(3)

A blood sample was collected from each animal before injection and use as pre-immune sera. Animal were boosted 3 times with 30 days between inoculations using Freund's incomplete adjuvant (FIA) and the same inoculation route.

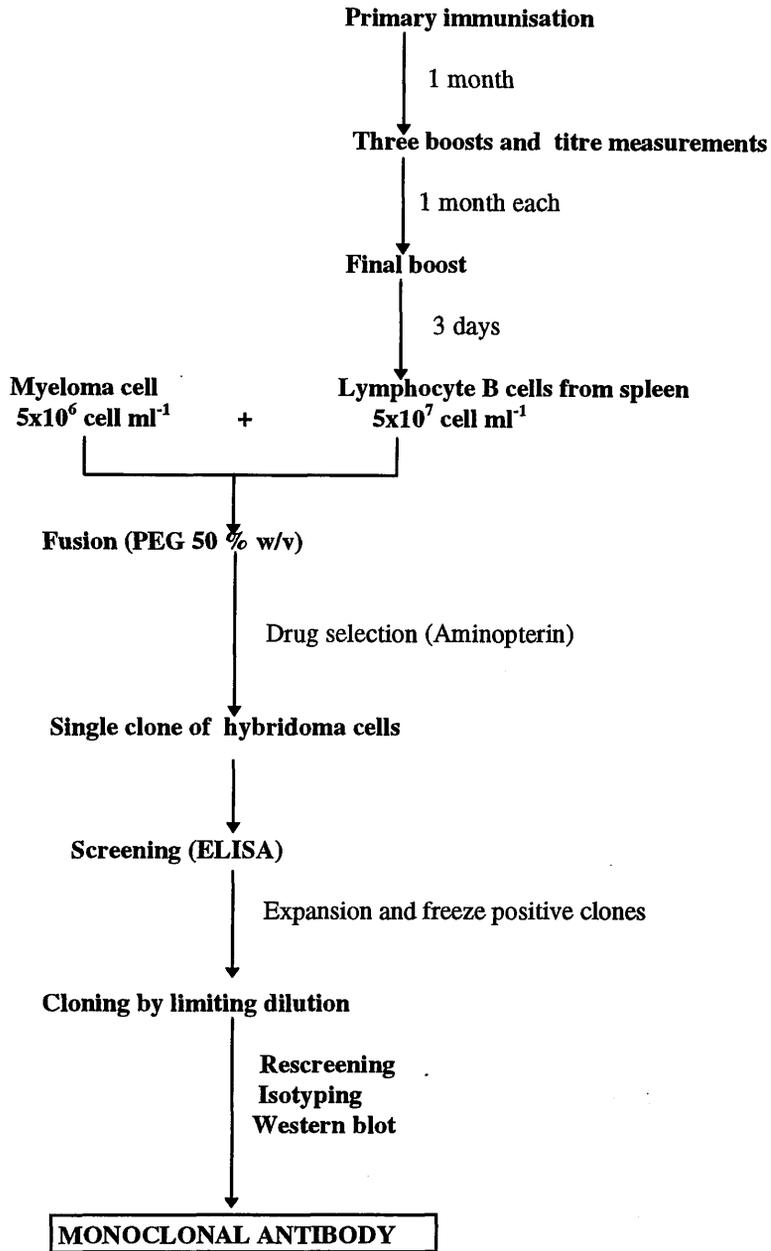
The last boost (boost 4) was given i.v. using 0.4 ml (rabbit) and 0.1 ml (mouse). Serum samples were collected 7-10 days after each boost and the antibody titre determined by ELISA using the methods described in sections 2.3.1 and 2.3.2. Pre-immune sera (rabbit or mouse) diluted at 1:1,000 were used as negative control. Animal were bled out by cardiac puncture and the sera collected and stored at -70 °C until use.

### **5.2.3 Production of monoclonal antibodies (MAbs)**

MAbs against extracellular and cell surface antigens of *R. salmoninarum* were developed using the methods described by Campbell (1984); Harlow and Lane (1988) and Adams *et al.*, (1993). The main steps involved in the process are shown in Figure 5.1.

#### **5.2.3.1 Cell lines and culture conditions**

Myeloma cell line (SP2/0-Ag-14) was purchased from ICN Flow Laboratories, UK. Myeloma and hybridomas cells were cultured, unless specified, in the Dulbecco's modification of Eagles medium (DMEM) with 3.7 g l<sup>-1</sup> sodium bicarbonate and supplemented with L-glutamine (200 mM stock solution, 10 ml l<sup>-1</sup>), Penicillin/Streptomycin (5000 IU of each, 10 ml l<sup>-1</sup>), heat inactivated FCS (20 % v/v) and sodium pyruvate (100 mM x200 stock solution, 5 ml l<sup>-1</sup>). Cells were incubated at 37 °C in a CO<sub>2</sub> atmosphere (5 %) and maintained around 1-2 x10<sup>5</sup> cells ml<sup>-1</sup>. They were passaged every 2-3 days by diluting the culture 1/10 or 1/20 in fresh medium.



**Figure 5.1** Diagram showing the major steps in the production of monoclonal antibodies to *R. salmoninarum* antigens

### **5.2.3.2 Immunisation protocol**

Six Balb/c female mice, six to eight weeks old, were inoculated i.p. with 0.2 ml of sonicated bacterial cells (isolate AVU 9202 cultured in KDM-2 without FCS) prepared in section 2.2.2. The antigen suspension was mixed with Freund's complete adjuvant (1:1 v/v) as described in section 5.2.2. Mice were boosted three times (1 month between each inoculation) with the same antigen in Freund's incomplete adjuvant. Serum samples were collected before the first inoculation and 7-10 days after each boost. The antibody titre of sera were determined by ELISA using extracellular proteins (ECP) and cell wall (CW) as described in section 2.3.1 and 2.3.2. Three days before fusion, mice with the higher antibody titre were boosted i.v. with 0.1 ml of sonicated cells in PBS.

### **5.2.3.3 Preparation of myeloma cells**

Myeloma cells were prepared seven days before the fusion. FCS (Sigma Chemical Company, 1 ml) was placed carefully in the bottom of a 10 ml conical tube containing nine ml of DMEM and allow to warm at 37°C for 15 minutes. A vial of frozen non-secreting myeloma cells containing  $1 \times 10^5$  cells  $\text{ml}^{-1}$  were remove from the liquid nitrogen and thawed in a water bath at 37° until little pieces of ice were left and then transferred to a sterile hood where the vial was wiped with 70% alcohol. Cells were then transferred to the culture medium previously made up and centrifuged at 1000 rpm (150xg) for 10 minutes in a Wifug Centrifuge. The supernatant was carefully removed and the cell pellet transferred to a small tissue culture flask containing 10 ml of complete tissue culture media DMEM. The culture was incubated as described in section 5.2.3.1 until the fusion day.

#### 5.2.3.4 Fusion

On the fusion day, the myeloma cells grown at miolog phase were centrifuged at 1000 rpm for 10 minutes. The cell pellet was resuspended in 20 ml of prewarmed serum-free DMEM in a conical universal tube and left at 37°C. Meanwhile the viability percentage and the total number of viable cells per ml were obtained. To do this, 50 µl of the cell suspension were mixed with the same volume of Trypan blue dye in an Eppendorf tube. One drop (20 µl) of this mixture was loaded on an improved Neubauer chamber and at least 200 cells were counted using a light microscope at 40x. Dead cells appeared blue in colour while viable cells remain clear. The following formula were used to determine both parameters:

$$\text{N}^\circ \text{ viable cells/ml} = \text{N}^\circ \text{ viable cells} \times 2 \times 10^4 / \text{N}^\circ \text{ squares counted}$$

$$\% \text{ Viability} = \text{N}^\circ \text{ viable cells} \times 100 / \text{total N}^\circ \text{ cells}$$

The previously boosted mouse was killed using CO<sub>2</sub>, bled out by cardiac puncture, and immersed in 70% ethanol. The spleen was removed using sterile instruments and placed in a small petri dish containing 5 ml of prewarmed DMEM medium without FCS at 37°C. The fatty tissue was carefully removed and the spleen cells teased from the capsule using two 21G needles. Cells were then taken up into 10 ml syringe and gently pushed through a 21G needle. The procedure was repeated once more with a 21G needle and twice with 25G needles. Cells were observed under the microscope to check that they were dispersed. If there were many clumps the pipetting was repeated using 25G needle. The cell suspension was then put in a conical universal tube and allowed to stand for two to three minutes to allow any remaining clumps to settle out. Spleen cells were then transferred to a universal tube and centrifuged at 1000 rpm for 10 minutes. The cell pellet was washed twice by resuspension in 10 ml of DMEM without FCS and further centrifugation. Spleen cells were then counted by mixing 50 µl of cell suspension with 300 µl of ammonium chloride which causes the cells to swell making them easier to see under the microscope.

Five ml of myeloma cells ( $5 \times 10^6$  cells  $\text{ml}^{-1}$ ) were mixed with 5 ml of spleen cells ( $5 \times 10^7$  cells  $\text{ml}^{-1}$ ) in a conical universal and centrifuged at 800 rpm for 10 minutes. The supernatant was removed to leave as dry a pellet as possible and 1 ml of 50% PEG (prewarmed at  $37^\circ\text{C}$ ) added to the pellet over 30 seconds, flicking the tube all the time, then pipetted up and down over 30 seconds, then allowed to stand for further 30 seconds. Two ml of DMEM without FCS was added dropwise over two minutes shaking the cells gently all the time. Finally, another five ml of DMEM without FCS was added and allowed to stand for three minutes before centrifugation at 800 rpm for ten minutes at room temperature. The supernatant was carefully removed and the cell pellet diluted in 50 ml of HAT medium containing 1.5 ml of red blood cells as feeder cells to a final dilution of  $1 \times 10^6$  cells  $\text{ml}^{-1}$ . Five 96 well plates were loaded with 100  $\mu\text{l}$  well $^{-1}$  of this cell suspension. Control myeloma and non fused spleen cells were diluted at  $1 \times 10^6$  cells  $\text{ml}^{-1}$  and plated out in a separate 96 wells plate labelled as Control. The plates were incubated for seven days at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  and checked every day using an inverted microscope.

#### 5.2.3.5 Screening of hybridomas

Seven days after the fusion, all the wells containing single hybridoma clones were screened by ELISA using the methods described in sections 2.3.1 and 2.3.3. Due to the difficulties in culturing *R. salmoninarum in vitro*, the amount of antigens such as peptidoglycan, polysaccharide, fimbrial protein, and cell wall available was very small, and thus a continuous limitation. Therefore, the first screening was performed by ELISA using fresh harvested heat killed whole bacteria which contained all the antigens needed. Positive clones ( $\text{OD}_{450} < 0.6$ ) were selected and rescreened using ECP and cell wall (second screening). Positive clones with the higher optical density were rescreened using peptidoglycan and polysaccharide. Selected clones were subcloned by the limiting dilution technique described by Campbell (1984) and rescreened by ELISA until the clones became monoclonal. MAbs were further characterised

by Western blot (see section 2.3.5) and isotyped using Sigma Isotyping Kit™ following the instructions provided by the manufacturers.

#### **5.2.4 Effect of different culture media on the expression of renibacterial antigens**

The effect of four common culture media on the expression of extracellular and cell surface antigens was studied. Four *R. salmoninarum* isolates (ATCC 33209, NCIMB 1111, AVU 9202 and B88151) were analysed. They were grown in KDM-2, SKDM, KDM-Charcoal and MH-C agar (see section 2.1). After incubation for 18 days at 15 °C, they were harvested and heat inactivated as described in section 2.5.1. Cells were assayed by ELISA using the method described in section 2.3.1. Different renibacterial antigens were identified using antibodies against whole bacteria (PAb PVIR), ECP proteins (MAb 1C7/1E1), p57 antigen (MAb B6G8), cell wall (PAb CW3) and peptidoglycan (MAb 12B7).

The production of antigens was also analysed by SDS-PAGE. Bacteria (ATCC 33209) were collected from each culture media and suspended in sterile saline solution so that the OD<sub>610</sub> of a 1/10 aliquot of this bacterial suspension was equal to 1.0. This is equivalent to 10<sup>10</sup> cells ml<sup>-1</sup>. The cell suspension was then mixed 1:1 with Laemmli sample buffer and stored at -70 °C until use. Gels were prepared, run and stained with Coomassie blue as described in section 2.3.4.

### 5.2.5 Crossreaction of anti *R. salmoninarum* monoclonal and polyclonal antibodies

The immunoreactivity (IR) of 31 different bacterial species, mainly fish pathogens, to a pool of monoclonal and polyclonal antibodies against *R. salmoninarum* was studied

The name, origin and cultural characteristics for each bacterial species used in the present study is given in table 5.4. Bacteria were harvested and heat inactivated as described in section 2.2.1. Each microplate was coated with all bacterial species. Three wells per bacterial species per plate (approximately  $10^7$  cell well<sup>-1</sup>) were used. Each plate were assayed by ELISA (see section 2.3.1) using one particular antibody.

The averaged OD<sub>450 nm</sub> of the positive control (*R. salmoninarum* ATCC 33209, cultured in MH-C) was designated a 100 % of IR. A reaction was considered negative when less than 11 % of IR was found (based on the averaged optical density of preimmune sera). Wells coated with buffer without bacteria were used as the negative control. All determinations were performed in triplicate and the average was then taken. The results were coded as none, low, moderate, and strong crossreactivity depending on the percentage of crossreactivity obtained (see Table 5. 3).

**Table 5.3 Codification for the crossreaction assay results**

<b>Symbol</b>	<b>Immunoreactivity (%)</b>	<b>Crossreactivity</b>
-	< 11	none
x	12-20	low
xx	21-40	moderate
xxx	>41	strong

**Table 5.4 Bacterial species used for crossreaction assay**

BACTERIAL SPECIES	ORIGIN	MEDIUM	TEMPERATURE
<i>Arthrobacter aureus</i>	NCIMB 8912	TSA	22°C
<i>Arthrobacter globiformis</i>	NCIMB 8907	TSA	22°C
<i>Nocardia asteroides</i>	NCIMB 1290	TSA	22°C
<i>Carnobacterium piscicola</i>	NCIMB 12234	TSA	22°C
<i>Micrococcus luteus</i>	NCIMB 570	TSA	22°C
<i>Mycobacterium fortuitum</i>	NCIMB 1294	SB	37°C
<i>Mycobacterium marinum</i>	NCIMB 1298	SB	37°C
<i>Corynebacterium aquaticum</i>	NCIMB 9460	TSA	22°C
<i>Streptococcus faecalis</i>	IOA	TSA	22°C
<i>Bacillus cereus</i>	IOA	TSA	22°C
<i>Bacillus subtilis</i>	IOA	TSA	22°C
<i>Lactobacillus plantarum</i>	IOA	TSA	22°C
<i>Aeromonas hydrophila</i>	NCIMB 87	TSA	22°C
<i>A. salm. achromogenes</i>	NCIMB 1110	TSA	22°C
<i>A. salmonicida masoucida</i>	NCIMB 2020	TSA	22°C
<i>A. sal. salmonicida</i>	IOA	TSA	22°C
<i>Edwardsiella tarda</i>	NCIMB 2034	TSA	22°C
<i>Cytophaga columnaris</i>	NCIMB 2246	CA	22°C
<i>Flexibacter marinus</i>	NCIMB 2154	MCA	22°C
<i>Haemophilus piscium</i>	NCIMB 1952	TSA*	22°C
<i>Pseudomonas aeruginosa</i>	NCIMB 8295	TSA	22°C
<i>Pseudomonas anguilliseptica</i>	NCIMB 1455	TSA	22°C
<i>Pseudomonas fluorescens</i>	NCIMB 1283	TSA	22°C
<i>Serratia sp</i>	IOA	TSA	22°C
<i>Vibrio anguillarum</i>	NCIMB 571	TSA	22°C
<i>Vibrio ordalii</i>	NCIMB 2167	TSA	22°C
<i>Vibrio salmonicida</i>	NCIMB 2262	MA	15°C
<i>Yersinia ruckeri</i>	IOA	TSA	22°C
<i>Escherichia coli</i>	IOA	TSA	22°C
<i>Pasteurella piscicida</i>	IOA	TSA**	22°C
<i>Renibacterium salmoninarum</i>	ATCC 33209	MH-C	15°C

TSA : Tryptone soy agar

MA : Marine agar

MCA : Marine cytophaga agar

TSA\* : TSA + 1% NaCl + cocarboxylase

TSA\*\* : TSA + 1.5% NaCl

SKDM : Selective kidney disease medium

MH-C : Mueller Hinton cysteine

NCIMB: National Collection of Industrial and Marine Bacteria. Scotland. U.K.

ATCC : American Type Culture Collection USA

IOA : Institute of Aquaculture. University of Stirling. Scotland. U.K.

TSB : Tryptone soy broth

CA : Cytophaga agar

BA : Blood agar

SM : Soutlon medium

KDM-2 : Kidney disease medium 2

KDM-C : Kidney disease medium charcoal

### 5.2.6 Antigenic analysis of 18 *R. salmoninarum* isolates using monoclonal and polyclonal antibodies

The immunoreactivity of a library of *R. salmoninarum* isolates to several monoclonal and polyclonal antibodies was investigated. *R. salmoninarum* isolates were obtained as described in section 5.2.1. They were grown on MH-C agar for 18 days at 15 °C, harvested and heat inactivated as described in section 2.2.1. All the analysis was performed by ELISA using whole cells (See section 2.3.1). *Aeromonas salmonicida salmonicida* and Rs ATCC 33209 were used as negative and positive control respectively.

## 5.3 RESULTS

### 5.3.1 Production of polyclonal and monoclonal antibodies

High polyclonal antibody levels were obtained when animals were inoculated with renibacterial p57 and cell wall (titres 1:500,000 and 1:250,000, respectively). However, no immunoresponse was observed in animals immunised with polysaccharide and p70. Figures 5.2 and 5.3 shows the titration curves of rabbit and mouse antisera after final boost and bleed. The titre and working dilution of all polyclonal antibodies used in this chapter is given in table 5.5.

**Table 5.5 Antibody titre and working dilution of polyclonal and some of the monoclonal antibodies used in this study.**

Name	Titre	Working dilution
PKPL	1:2,000,000	1:20,000
PVIR	1:1,000,000	1:20,000
B6G8	1: 500,000	1:20,000
155/P57	1: 500,000	1:20,000
158/P70	1: 60,000	1: 5,000
CW3	1:250,000	1:20,000

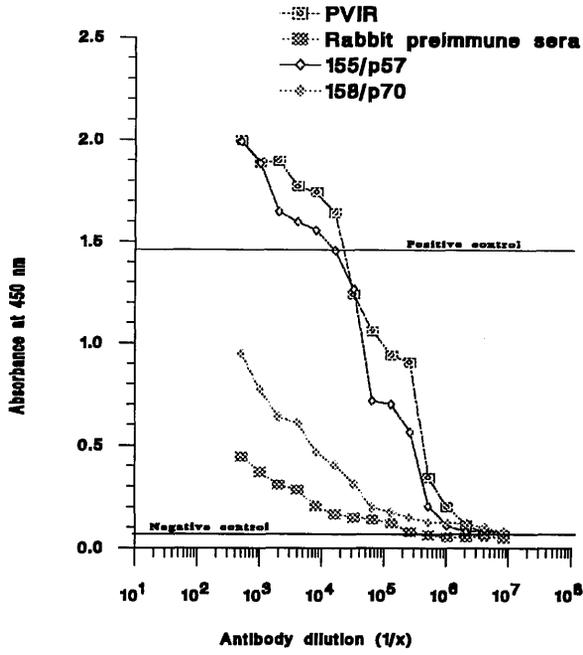


Fig. 5.2 Titration curve for rabbit polyclonal antibodies after final boost and bleed.

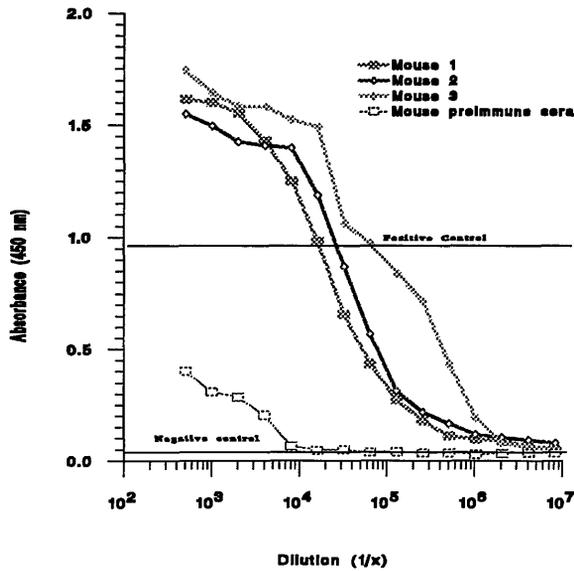


Fig. 5.3 Titration curves of the serum from three mice inoculated with renibacterial cell wall (After final boost and bleed).

All mice immunised for the production of monoclonal antibodies showed almost similar antibody titres. The titre of the best responding mouse was 1:500,000 (for cell wall) and 1:1,000,000 (for ECP). The titration curve is given in Figure 5.4.

The first screening of hybridoma cells (using whole cells) showed 405 positive wells with  $OD_{450} > 0.6$ . They all were rescreened using ECP and cell wall, the results are given in Table 5.6. Positive clones ( $OD > 0.6$ ) to ECP proteins (106) and to cell wall (6) were found. One clone (IB3) was found to recognise both subunits. Three clones producing antibodies against ECP proteins (2H8, 4H2, 4A3), 2 clones recognising cell wall (2C9, 5D1) and one clone recognising both subunits were selected and rescreened by ELISA using peptidoglycan and polysaccharide. The results are given in table 5.7.

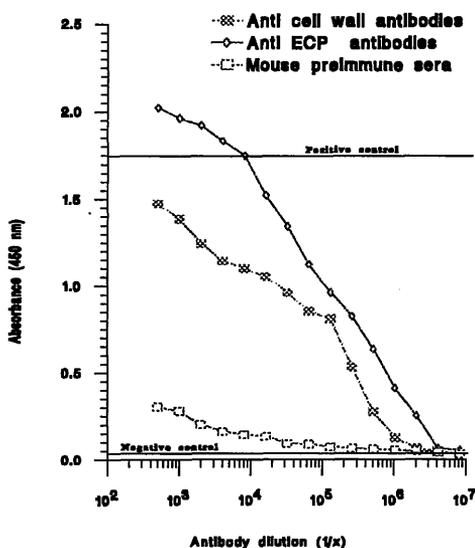


Figure 5. 4 Graph showing the anti cell wall and anti ECP antibody titration curves for serum obtained from the fusion mouse

**Table 5.6a Identification of single clones of hybridoma cells producing antibodies against whole cell, cell wall and ECP proteins by ELISA \***

Parent cell line	Whole bacteria	Cell wall	ECP
1A1	0.998	0.294	1.061
1B1	0.691	0.823	0.969
1D1	0.774	0.275	1.088
1E1	0.635	0.273	0.884
1F1	1.401	0.292	1.445
1G1	0.843	0.355	1.113
1H1	0.607	0.356	1.022
1A2	1.097	0.327	1.396
1D2	0.711	0.319	1.034
1E2	0.705	0.348	1.304
1F2	0.601	0.061	0.792
1G2	0.631	0.257	0.817
1A3	0.806	0.299	0.791
1B3**	0.891	1.057	1.385
1A4	0.634	0.272	0.381
1D4	0.751	0.328	1.312
1E4	0.712	0.292	1.203
1F4	0.656	0.371	0.841
1G4	0.669	0.319	0.362
1A5	0.725	0.311	0.828
1B5	0.611	0.298	0.826
1C5	0.618	0.257	0.785
1E5	1.099	0.303	1.387
1F5	0.654	0.247	0.826
1G5	0.822	0.277	0.753
1D6	0.621	0.257	0.985
1G6	0.658	0.416	0.837

\* The values given represent the average absorbance measured at 450 nm from triplicated assays

\*\* MAb selected for further characterisation and subcloning

**Table 5.6b Identification of single clones of hybridoma cells producing antibodies against whole cell, cell wall and ECP proteins by ELISA\* (Cont.)**

Parent cell line	Whole bacteria	Cell wall	ECP
1A7	0.975	0.306	1.344
1C7	0.811	0.271	1.589
1E7	0.649	0.271	0.826
1H7	1.321	0.299	1.583
1D8	0.706	0.273	1.068
1C12	0.821	0.276	1.309
1F12	0.608	0.258	1.107
2A1	1.149	0.308	0.729
2C1	0.606	0.269	0.249
2E1	0.639	0.253	0.803
2G1	0.869	0.328	0.889
2C2	1.029	0.258	1.186
2H2	0.763	0.303	0.852
2C5	0.831	0.336	0.846
2E5	0.783	0.286	0.581
2F5	0.981	0.275	1.331
2A6	0.627	0.344	0.999
2E6	0.863	0.308	1.725
2B7	0.612	0.251	1.001
2A8	1.266	0.337	1.561
2H8 **	1.422	0.176	1.751
2C9 **	1.137	1.680	0.457
AE9	1.266	0.273	1.725
2F10	0.978	0.384	1.194
2E11	0.793	0.316	1.217
3F1	1.571	0.278	1.694
3G1	1.717	0.277	1.642
3B2	0.749	0.331	1.092

\* The values given represent the average absorbance measured at 450 nm from triplicated assays

\*\* MAb selected for further characterisation and subcloning

**Table 5.6c Identification of single clones of hybridoma cells producing antibodies against whole cell, cell wall and ECP proteins by ELISA\* (Cont.)**

Parent cell line	Whole bacteria	Cell wall	ECP
3D2	1.094	0.392	0.753
3E2	0.703	0.487	1.246
3C4	0.631	0.324	1.255
3C6	0.842	0.327	1.623
3A7	1.378	0.287	1.688
3G9	0.668	0.301	0.585
3G10	0.715	0.297	0.561
3H10	1.192	0.279	1.671
3F11	0.602	0.284	1.366
3H11	1.016	0.287	1.693
3A12	0.662	0.291	1.237
3B12	1.135	0.253	0.938
4D1	1.244	0.326	1.139
4F1	0.613	0.338	0.751
4H1	0.657	0.302	0.917
4A2	0.753	0.295	0.698
4B2	0.667	0.266	1.002
4E2	0.657	0.256	0.944
4H2**	1.173	1.374	0.512
4A3**	1.762	0.265	1.731
4F3	0.795	0.312	1.476
4G3	0.646	0.013	0.744
4A4	0.648	0.047	0.785
4A7	1.158	0.272	0.60
4C9	0.686	0.251	1.737
5C1	0.601	0.043	0.471
5D1**	1.241	1.643	0.434
5E1	0.728	0.033	0.777
5G1	0.834	0.276	0.706

\* The values given represent the average absorbance measured at 450 nm from triplicated assay

\*\* MAb selected for further characterisation and subcloning

**Table 5.6d Identification of single clones of hybridoma cells producing antibodies against whole cell, cell wall and ECP proteins ELISA\*(Cont.)**

Parent cell line	Whole bacteria	Cell wall	ECP
5A2	0.639	0.221	0.199
5D2	0.625	0.002	0.652
5E2	1.591	0.065	0.835
5G2	0.775	0.029	0.706
5F3	0.718	0.077	0.964
5G3	0.874	0.067	1.231
5A4	0.909	0.031	1.266
5E4	0.768	0.068	0.197
5F4	0.771	0.242	1.532
5H4	0.816	0.244	0.183
5B5	0.701	0.039	1.015
5E5	0.969	0.082	0.993
5G5	1.063	0.015	1.422
5H5	0.931	0.026	1.101
5A6	0.655	0.272	0.774
5B6	0.794	0.315	0.697
5E6	0.706	0.032	0.492
5G6	0.715	0.071	0.897
5A7	0.946	0.289	1.625
5E7	0.797	0.316	0.966
5F7	0.622	0.275	0.824
5G7	0.623	0.282	1.111
5C8	0.629	0.427	1.305
5D8	0.887	0.452	1.341
5E8	0.661	0.421	0.771
6G8	0.892	0.291	1.149
5H8	0.654	0.288	0.739
5A9	0.713	0.307	1.022

\* The values given represent the average absorbance measured at 450 nm from triplicated assay

\*\* MAb selected for further characterisation and subcloning

**Table 5.7 Immunoscreening of selected hybridoma cells to renibacterial antigens\***

Parent cell line	MAb name	p57	Cell wall	Peptidoglycan	Polysaccharide
4H2	9F5**	0.062	1.368	1.305	0.118
4H2	9C8	0.064	1.348	1.099	0.073
5D1	8C10	0.164	1.333	1.033	0.118
5D1	8A10**	0.138	1.327	1.057	0.092
5D1	8B9	0.139	1.293	0.981	0.096
5D1	8D7	0.148	1.314	0.987	0.116
5D1	7B8	0.154	1.321	0.977	0.096
5D1	7E6	0.121	1.356	1.217	0.122
2C9	14C2**	0.096	1.404	1.212	0.086
2C9	13C7	0.229	1.762	1.172	0.093
2C9	13G2	0.152	1.441	1.143	0.085
4A3	5A11**	1.578	0.007	0.449	0.260
4A3	6H4	1.561	0.027	0.693	0.317
2H8	2E6	1.726	0.004	0.133	-----
2H8	1C7**	1.716	0.017	0.165	-----
1B3	11D11**	1.223	1.768	1.501	1.478
1B3	11F8	1.291	1.832	1.511	0.436
1B3	11E12	1.292	1.750	1.445	1.197
1B3	11G2	1.202	1.695	1.244	0.126
1B3	12B7**	1.160	1.480	1.333	1.439
1B3	12E4	1.019	1.133	1.422	1.282

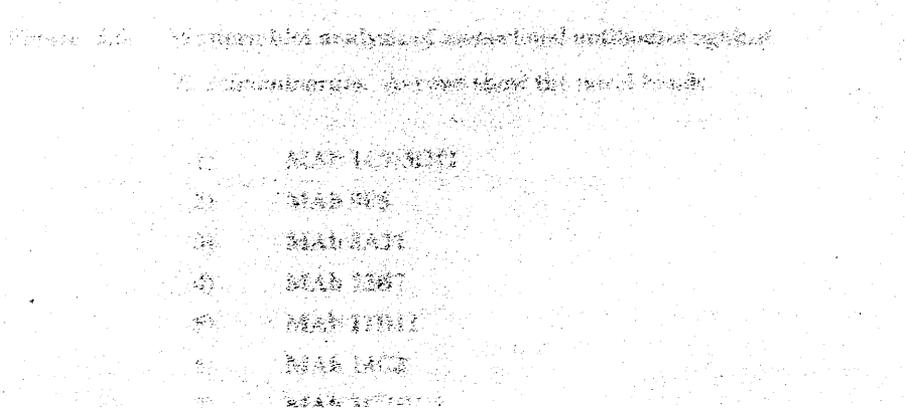
\* The values given represent the average absorbance measured at 450 nm from triplicated assay

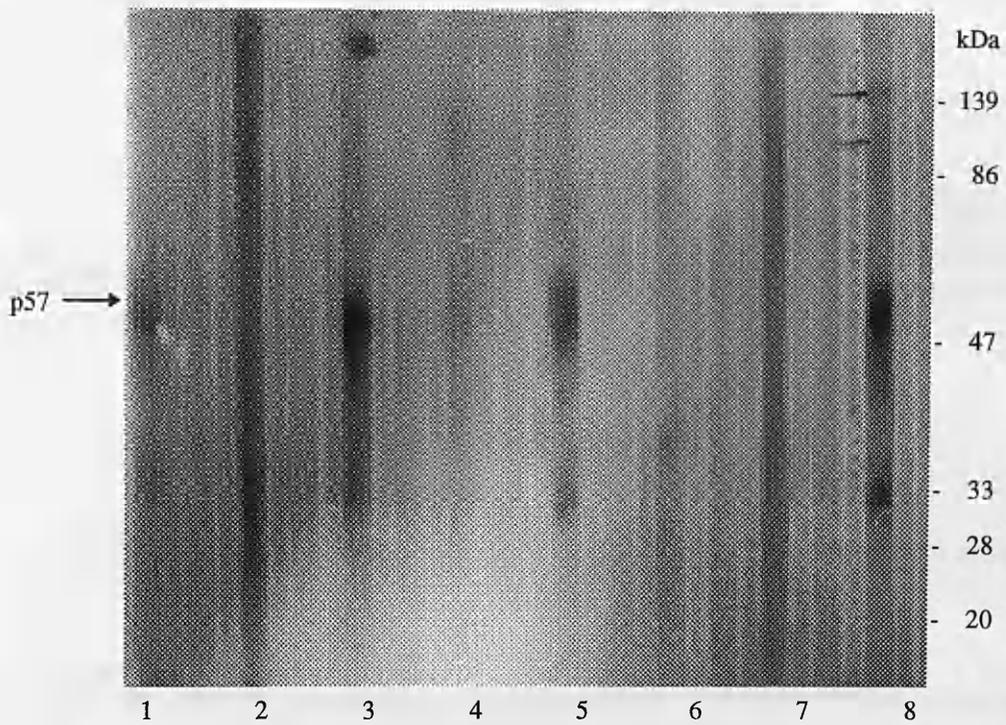
\*\* MAb selected for further characterisation and subcloning

One single clone from each parent cell line was selected, expanded and subcloned by limiting dilution (3-4 times) until they became monoclonal. In this process, cell line 8A10 lost its ability to secrete antibodies.

The Western blot analysis of the MAbs showed p57 antigen and its breakdown products (57, 40, 30 kDa proteins) were identified by MAb 1C7/3D11, 11D11, 5A11 and 1C7/1E1. MAb 1C7/1E1 also recognised two unknown proteins of MW approximately higher than 100 kDa. MAb 9F5 gave strong background and no identifiable band was observed. MAb 14C2 and 12B7 gave no reaction at all (Figure 5.5).

MAbs were isotyped using Sigma Isotyping Kit™ following the instructions provided by the manufacturers. Table 5.8 shows the full characterisation of the monoclonal antibodies produced in the present study.





**Figure 5.5** Western blot analysis of monoclonal antibodies against *R. salmoninarum*. Arrows show the novel bands.

- 1) MAb 1C7/3D11
- 2) MAb 9F5
- 3) MAb 5A11
- 4) MAb 12B7
- 5) MAb 11D11
- 6) MAb 14C2
- 7) MAb 1C7/2C2
- 8) MAb 1C7/1E1

**Table 5.8 Characterisation of monoclonal antibodies to renibacterial antigens**

<b>MAB</b>	<b>Parent cell line</b>	<b>Isotype</b>	<b>Antigen recognised</b>	<b>Detection system</b>
<b>14C2</b>	2C9	IgM	Peptidoglycan	ELISA, IHC
<b>9F5</b>	4H2	IgG1	Peptidoglycan	ELISA, IHC
<b>5A11</b>	4A3	IgG1	p57 antigen	ELISA,IHC, WB
<b>1C7/3D1</b>	2H8	IgM	p57 antigen	ELISA,IHC, WB
<b>1C7/1E1</b>	2H8	IgM	p57 antigen, and two unknown proteins (MW > 100 kDa)	ELISA,IHC, WB
<b>11D11</b>	1B3	IgM	Peptidoglycan, p57 antigen	ELISA, IHC
<b>12B7</b>	1B3	IgM	Peptidoglycan	ELISA, IHC

ELISA: enzyme-linked immunosorbent assay

IHC: immunohistochemistry

WB: Western blot

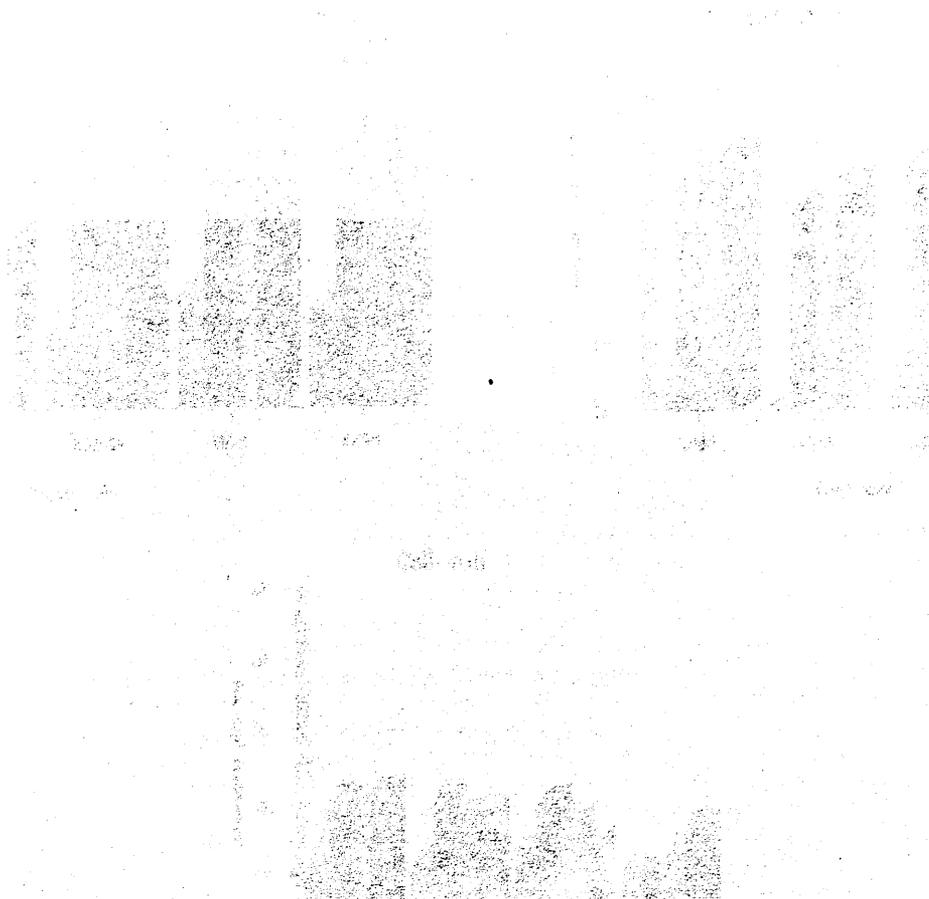
### **5.3.2 Effect of different culture media in the expression of renibacterial antigens**

No significant differences were observed between the isolates or between the different culture media when the bacteria were tested using PAb anti whole renibacterial cells (PVIR). Cell wall antigens showed small differences depending on the culture media and the isolate analysed. Isolate B88151 and NCIMB 1111 showed similar results, however, isolates ATCC 33209 and AVU 9202, showed a 50 % of reduction in the immunoreactivity when those isolates were cultured in SKDM

The production of ECP antigens showed no differences between the culture media analysed. However, isolate B88151 showed a decrease of 66 % compared with the other isolates. The production of p57 antigen showed no differences between the culture media. However, once again, the isolate B88151 showed a reduction ranging from 87.5 % and 100 %. This finding was especially evident in the bacterial sample cultured in KDM-Charcoal where no antigen was detected. Furthermore, the intensity of

the reaction in all the isolates and culture media showed almost 50 % of the immunoreactivity obtained for other renibacterial fractions.

No significant differences were found in the production of peptidoglycan between culture media and isolates. The SDS-PAGE analysis showed that the production of ECP proteins was significantly decreased when the bacteria were cultured in KDM-Ch and increased in MH-C. KDM-2 and KDM showed similar banding patterns (Figure 5.7).



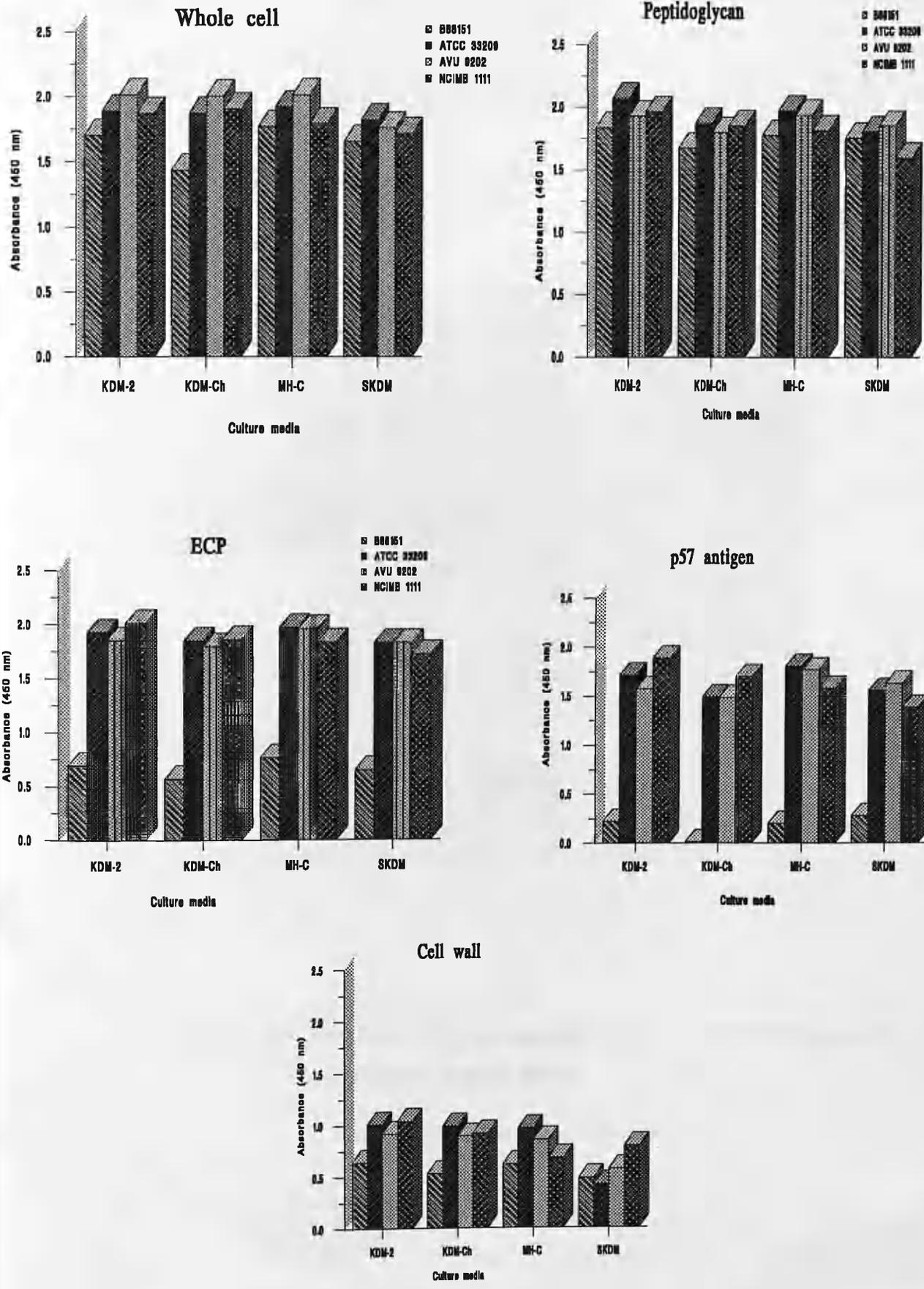
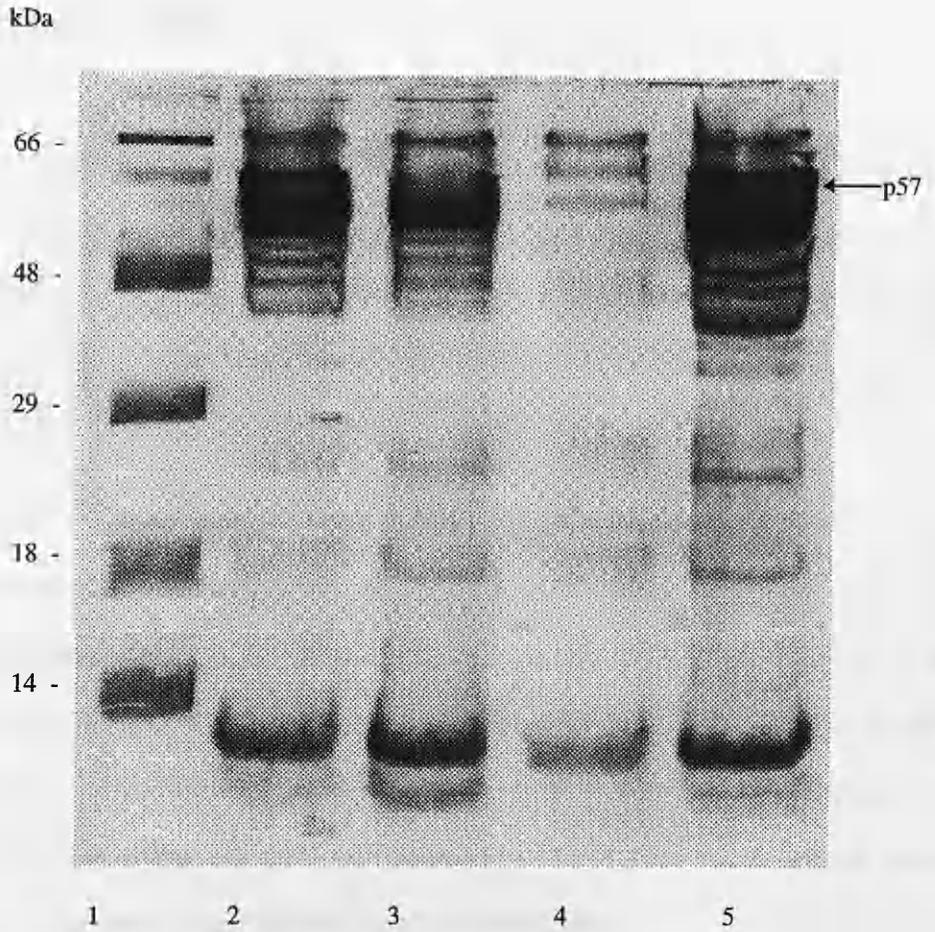


Figure 5.6 Effect of different culture media in the production of renibacterial antigens



**Figure 5.7 SDS-PAGE of whole bacteria (isolate ATCC 33209) grown in four different culture media.**

1. Standard MW marker (Sigma, low range)
2. KDM-2
3. SKDM
4. KDM-Charcoal
5. MH-C

### 5.3.3 Crossreaction of anti *R. salmoninarum* monoclonal and polyclonal antibodies

The crossreactivity of all polyclonal and monoclonal antibodies tested in the present study is shown in tables 5.9 (PAbs) and 5.10 (MAbs).

PAb PKPL and MAbs B6G8, 1C7/3D1 and 1C7/1E1 showed no crossreaction with any of the bacterial species tested. The OD of the positive control was  $1.467 \pm 0.021$  (for PKPL),  $1.260 \pm 0.017$  (for B6G8),  $1.231 \pm 0.026$  (for 1C7/3D1) and  $1.168 \pm 0.008$  (for 1C7/1E1). No reactivity was observed in the negative control (wells containing no bacteria)

PAb CW3 (anti-cell wall) crossreacted with many gram positive and negative bacteria, particularly with *Arthrobacter aureescens* (58 %), *Streptococcus faecalis* (44.8 %), *Mycobacterium marinum* (43.9 %), *Mycobacterium fortuitum* (40.8 %), *Carnobacterium piscicola* (39.1 %), *Vibrio ordalii* (35.3 %), *Arthrobacter globiformis* (32.3 %), *Micrococcus luteus* (28 %), *Nocardia asteroides* (25.5 %), *Yersinia ruckeri* (22.6 %), *Corynebacterium aquaticum* (21.3 %), *Bacillus subtilis* 17.5 %, *Flexibacter marinum* (16.5 %), *Escherichia coli* (13.7 %), *Edwardsiella tarda* (15.1 %). In addition, strong crossreaction (52 %) of poly-L-lysine (negative control) was also observed.

PAb PVIR (against whole bacteria) showed crossreaction with only gram positive bacteria, including *Arthrobacter aureescens* (39.8 %), *Micrococcus luteus* (20.5 %), *Mycobacterium marinum* (20.2 %), *Mycobacterium fortuitum* (20 %), *Arthrobacter globiformis* (19.6 %), *Streptococcus faecalis* (16.9 %), *Corynebacterium aquaticum* (16.2 %), *Bacillus subtilis* (12.4 %) and *Nocardia asteroides* (12.3 %). Negative reaction (<11% IR) was obtained for all gram negative bacteria tested. A moderate crossreaction (24.3 %) was found in the negative control which was due to nonspecific binding to poly-L-lysine.

PAb 155/p57 (anti SDS-PAGE purified p57) crossreacted only with *Arthrobacter aureescens* (55.8%) and *Micrococcus luteus* (36.2%).

MAbs 11D11 and 12B7 reacted, at very low level, with *Micrococcus luteus* (13.9 % and 14.4 % respectively). MAb 14C2 showed very low level of crossreaction (<14 %) with *Carnobacterium piscicola*, *M. luteus*, and *M. fortuitum*

MAb 9F5 showed strong crossreaction with *Mycobacterium sp.* (60% ) and several and several other gram positive bacteria (20-30%). MAb 5A11 showed strong crossreaction with poly-L-Lysine in the negative control and also with almost all bacterial species tested.

**Table 5.9 Crossreactivity of polyclonal antibodies to different bacterial species**

Bacterial species	Polyclonal antibodies			
	CW3	PVIR	155/P57	PKPL
<i>Renibacterium salmoninarum</i>	xxx	xxx	xxx	xxx
<i>Arthrobacter aureus</i>	xxx	xx	xxx	-
<i>Arthrobacter. globiformis</i>	xx	x	-	-
<i>Nocardia asteroides</i>	xx	x	-	-
<i>Carnobacterium piscicola</i>	xx	x	-	-
<i>Micrococcus luteus</i>	xx	x	xx	-
<i>Mycobacterium marinum</i>	xxx	x	-	-
<i>Mycobacterium fortuitum</i>	xx	x	-	-
<i>Corynebacterium aquaticum</i>	x	x	-	-
<i>Streptococcus faecalis</i>	xxx	x	-	-
<i>Bacillus cereus</i>	x	-	-	-
<i>Bacillus subtilis</i>	x	x	-	-
<i>Lactobacillus plantarum</i>	-	-	-	-
<i>Aeromonas hydrophila</i>	-	-	-	-
<i>A. salmonicida achromogenes</i>	-	-	-	-
<i>A. salmonicida masoucida</i>	-	-	-	-
<i>A. salmonicida salmonicida</i>	-	-	-	-
<i>Flexibacter columnaris</i>	x	-	-	-
<i>Flexibacter maritimum</i>	-	-	-	-
<i>Haemophilus piscium</i>	-	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-
<i>Pseudomonas anguilliseptica</i>	-	-	-	-
<i>Pseudomonas fluorescens</i>	-	-	-	-
<i>Serratia marcerens</i>	-	-	-	-
<i>Vibrio anguillarum</i>	-	-	-	-
<i>Vibrio ordalii</i>	xx	-	-	-
<i>Vibrio salmonicida</i>	-	-	-	-
<i>Yersinia ruckeri</i>	xx	-	-	-
<i>Escherichia coli</i>	x	-	-	-
<i>Pasteurella piscicida</i>	-	-	-	-
<i>Edwardsiella tarda</i>	x	-	-	-
Negative control*	xxx	xx	-	-

- : Negative reaction (IR <11 %)

x : 12-20 % IR;

xx : 21-40 % IR;

xxx : >41 % IR

\*Positive reaction is due to nonspecific binding to poly L-lysine

**Table 5.10(a) Crossreactivity of monoclonal antibodies to different bacterial species**

Bacterial species	Monoclonal antibody		
	5A11	9F5	14C2
<i>Renibacterium salmoninarum</i>	xxx	xxx	xxx
<i>Arthrobacter aurescens</i>	xx	x	-
<i>Arthrobacter globiformis</i>	xx	x	-
<i>Nocardia asteroides</i>	xx	xx	-
<i>Carnobacterium piscicola</i>	xx	xx	x
<i>Micrococcus luteus</i>	xx	xx	x
<i>Mycobacterium marinum</i>	xxx	xxx	-
<i>Mycobacterium fortuitum</i>	xxx	xxx	x
<i>Corynebacterium aquaticum</i>	xx	x	-
<i>Streptococcus faecalis</i>	xx	xx	-
<i>Bacillus cereus</i>	xx	-	-
<i>Bacillus subtilis</i>	-	-	-
<i>Aeromonas hydrophila</i>	x	-	-
<i>A. salmonicida achromogenes</i>	xx	-	-
<i>A. salmonicida masoucida</i>	-	-	-
<i>A. salmonicida salmonicida</i>	x	-	-
<i>Flexibacter columnaris</i>	x	-	-
<i>Flexibacter marinum</i>	x	-	-
<i>Haemophilus piscium</i>	-	-	-
<i>Pseudomonas aeruginosa</i>	x	-	-
<i>Pseudomonas anguilliseptica</i>	-	-	-
<i>Pseudomonas fluorescens</i>	x	-	-
<i>Serratia marcerens</i>	x	-	-
<i>Vibrio anguillarum</i>	x	-	-
<i>Vibrio ordalii</i>	x	-	-
<i>Vibrio salmonicida</i>	-	-	-
<i>Yersinia ruckeri</i>	xx	-	-
<i>Escherichia coli</i>	x	-	-
<i>Pasteurella piscicida</i>	x	-	-
<i>Edwardsiella tarda</i>	xx	-	-
Negative control*	xxx	-	-

- : Negative reaction (IR <11 %)

x : 12-20 % IR;

xx : 21-40 % IR;

xxx : >41 % IR

\* Positive reaction is due to nonspecific binding to poly L-lysine

**Table 5.10(b) Crossreactivity of monoclonal antibodies to different bacterial species**

Bacterial species	Monoclonal antibody				
	11D11	1C7/1E1	1C7/3D1	B6G8	12B7
<i>Renibacterium salmoninarum</i>	xxx	xxx	xxx	xxx	xxx
<i>Arthrobacter aurescens</i>	-	-	-	-	-
<i>Arthrobacter globiformis</i>	-	-	-	-	-
<i>Nocardia asteroides</i>	-	-	-	-	-
<i>Carnobacterium piscicola</i>	-	-	-	-	-
<i>Micrococcus luteus</i>	x	-	-	-	x
<i>Mycobacterium marinum</i>	-	-	-	-	-
<i>Mycobacterium fortuitum</i>	-	-	-	-	-
<i>Corynebacterium aquaticum</i>	-	-	-	-	-
<i>Streptococcus faecalis</i>	-	-	-	-	-
<i>Bacillus cereus</i>	-	-	-	-	-
<i>Bacillus subtilis</i>	-	-	-	-	-
<i>Lactobacillus plantarum</i>	-	-	-	-	-
<i>Aeromonas hydrophila</i>	-	-	-	-	-
<i>A. salmonicida achromogenes</i>	-	-	-	-	-
<i>A. salmonicida masoucida</i>	-	-	-	-	-
<i>A. salmonicida salmonicida</i>	-	-	-	-	-
<i>Flexibacter columnaris</i>	-	-	-	-	-
<i>Flexibacter marinum</i>	-	-	-	-	-
<i>Haemophilus piscium</i>	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-
<i>Pseudomonas anguilliseptica</i>	-	-	-	-	-
<i>Pseudomonas fluorescens</i>	-	-	-	-	-
<i>Serratia marcerens</i>	-	-	-	-	-
<i>Vibrio anguillarum</i>	-	-	-	-	-
<i>Vibrio ordalii</i>	-	-	-	-	-
<i>Vibrio salmonicida</i>	-	-	-	-	-
<i>Yersinia ruckeri</i>	-	-	-	-	-
<i>Escherichia coli</i>	-	-	-	-	-
<i>Pasteurella piscicida</i>	-	-	-	-	-
<i>Edwardsiella tarda</i>	-	-	-	-	-
Negative control*	-	-	-	-	-

- : Negative reaction (IR <11 %)

x : 12-20 % IR;

xx : 21-40 % IR;

xxx : >41 % IR

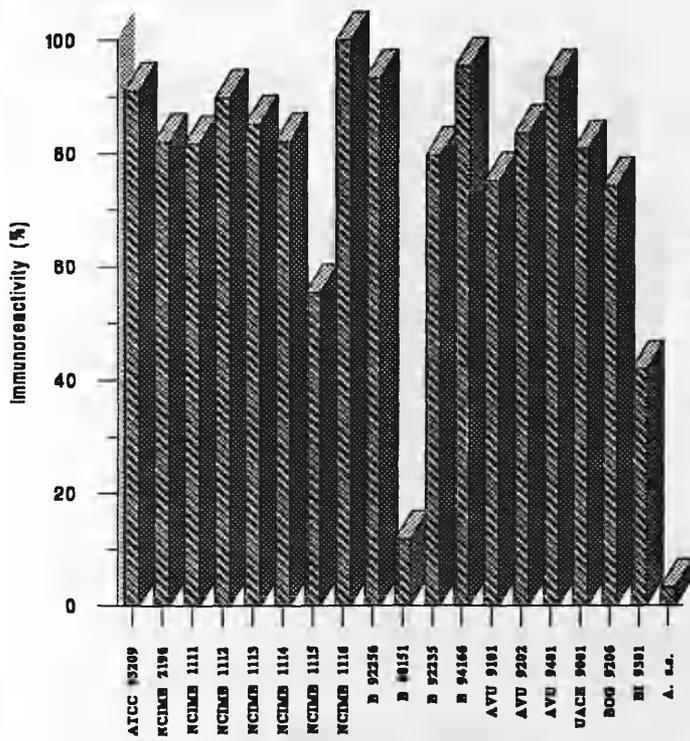
\* Positive reaction is due to nonspecific binding to poly-L-lysine

### 5.3.4 Antigenic analysis of 18 *R. salmoninarum* isolates using monoclonal and polyclonal antibodies

The IR of monoclonal antibodies against extracellular antigens (5A11, 1C7/3D11, 1C7/1E1 and B6G8) showed similar results. Immunoreactivity higher than 80 % was found in 83.3 % of the isolates tested (15 out of 18). The poorest responder was isolate B88151 which showed 19.2% IR (using MAb 5A11), 34.4 % IR (with MAb 1C7/3D11) and 11.9 % IR (with MAb 1C7/3D11). Isolate UACH 9001, and BI 9301 also showed lower responses (43.2 % and 41.9 % IR, respectively). See figures 5.8 to 5.10.

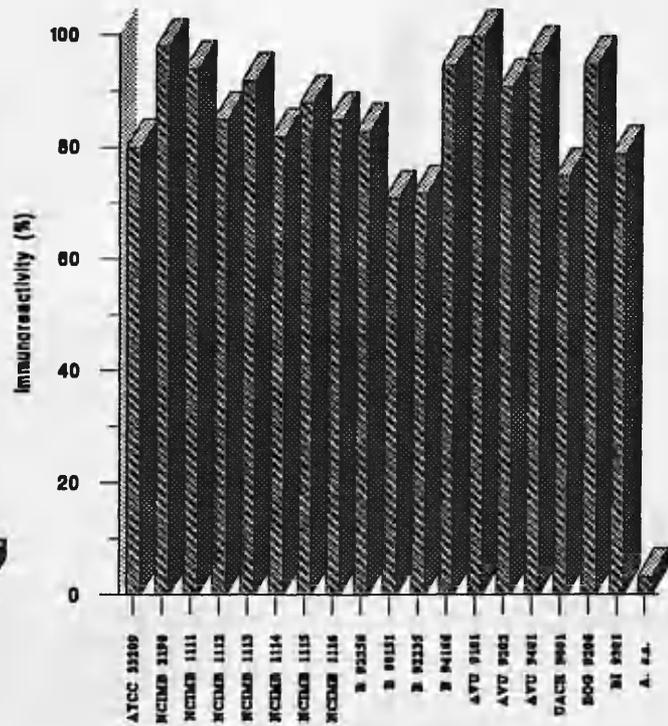
All the isolates showed high immunoreactivity to anti- cell wall MAb (11D11, 12B7 and 14C2), with IR ranged between 70% to 100 %. However, using MAb 11D11, 61.1 % (11 out of 18) isolates showed immunoreactivity higher than 70 %, 33.3 % (6 out of 18) isolates with IR between 44 % to 69 % and one isolate UACH 9001 showed IR as low as 19.6 %). Using polyclonal antibodies, all the isolates reacted similarly with IR between 70 to 100 %.

**MAb B6G8**



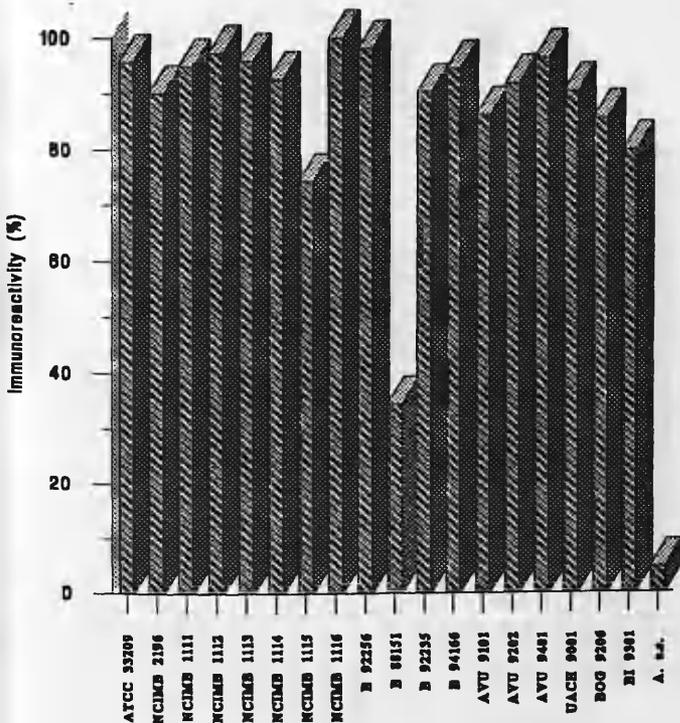
**R. salmoninarum isolates**

**MAb 9F5**



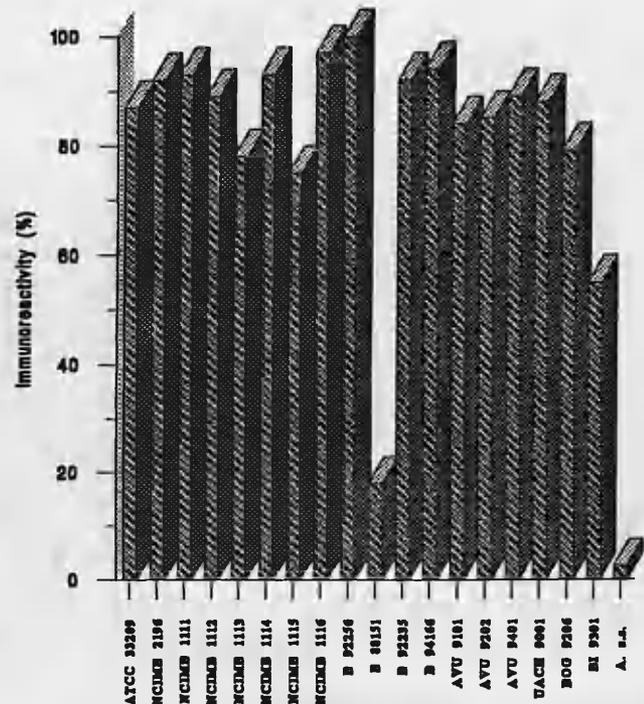
**R. salmoninarum isolates**

**MAb 1C7/3D11**



**R. salmoninarum isolates**

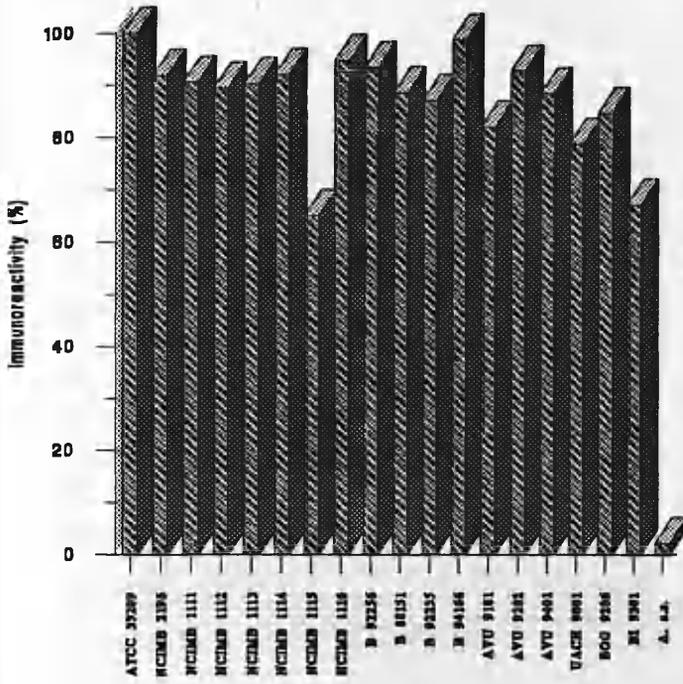
**MAb 1C7/1E1**



**R. salmoninarum isolates**

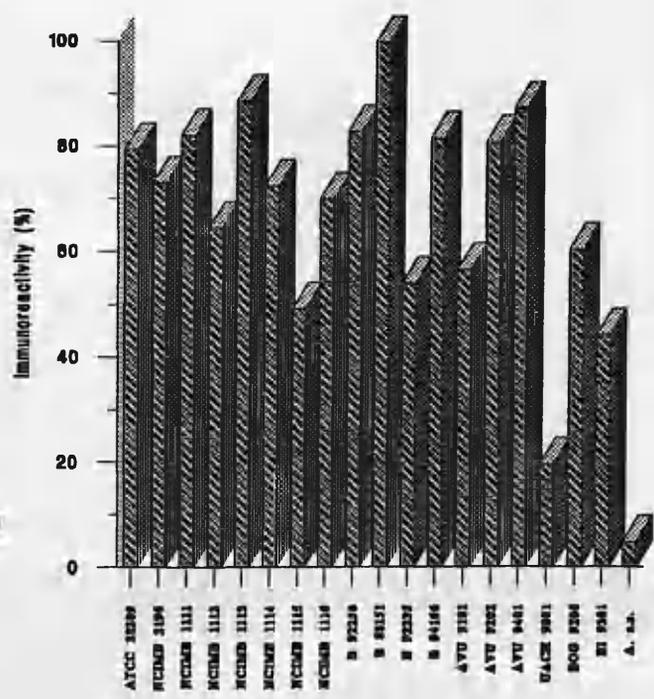
**Fig.5.8 Immunoreactivity of monoclonal antibodies B6G8, 9F5, 1C7/1E1 and 1C7/3D11 to 18 isolates of *R. salmoninarum* .**

**MAb 12B7**



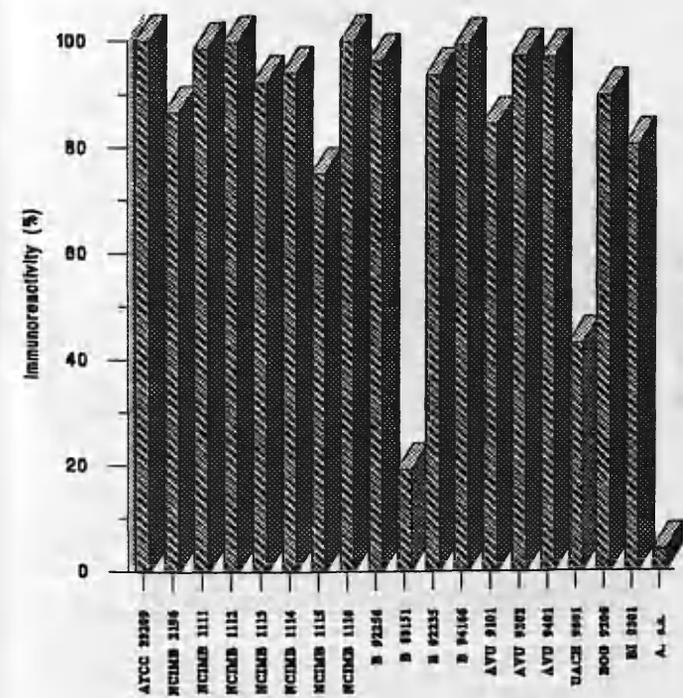
**R. salmoninarum isolates**

**MAb 11D11**



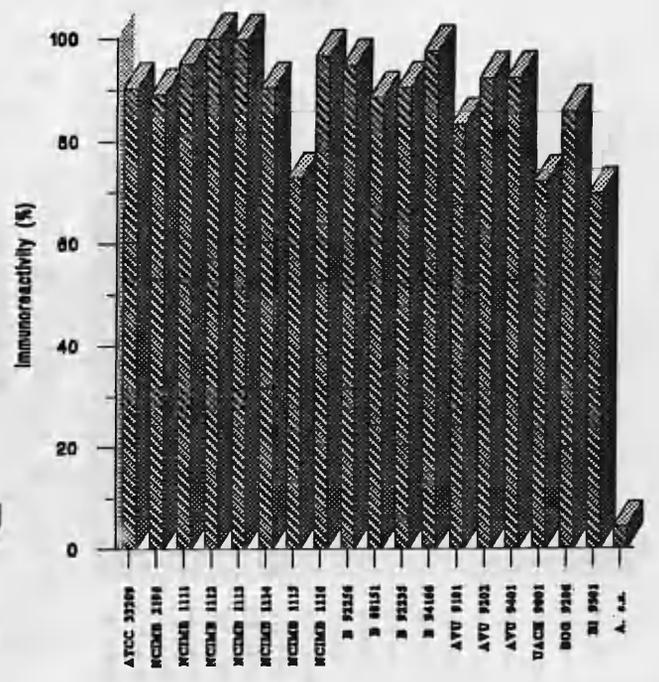
**R. salmoninarum isolates**

**MAb 5A11**



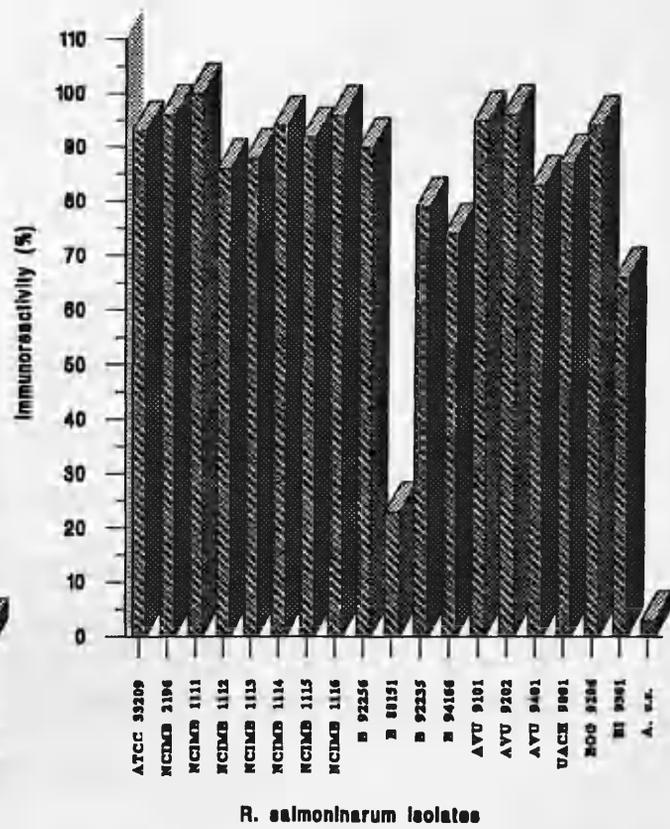
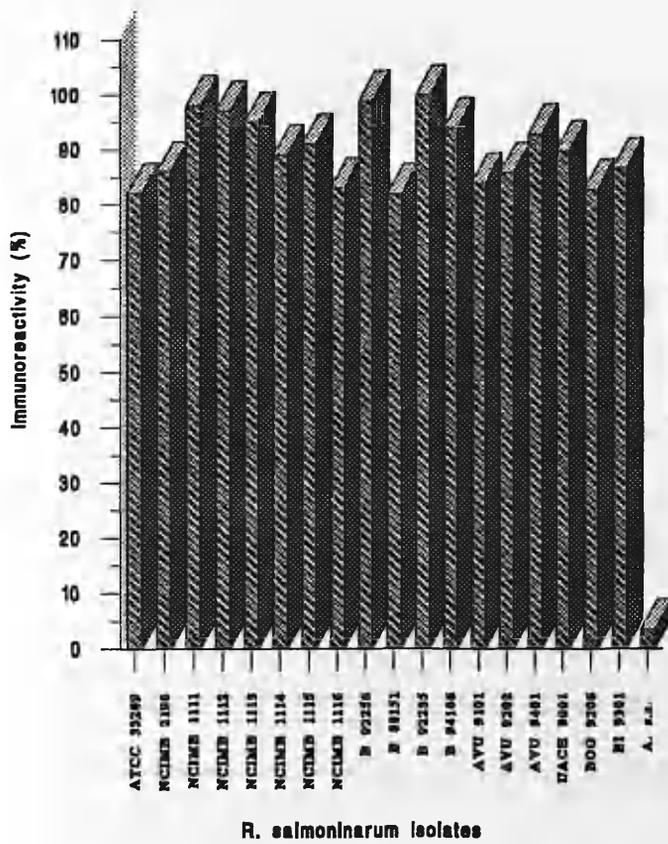
**R. salmoninarum isolates**

**MAb 14C2**



**R. salmoninarum isolates**

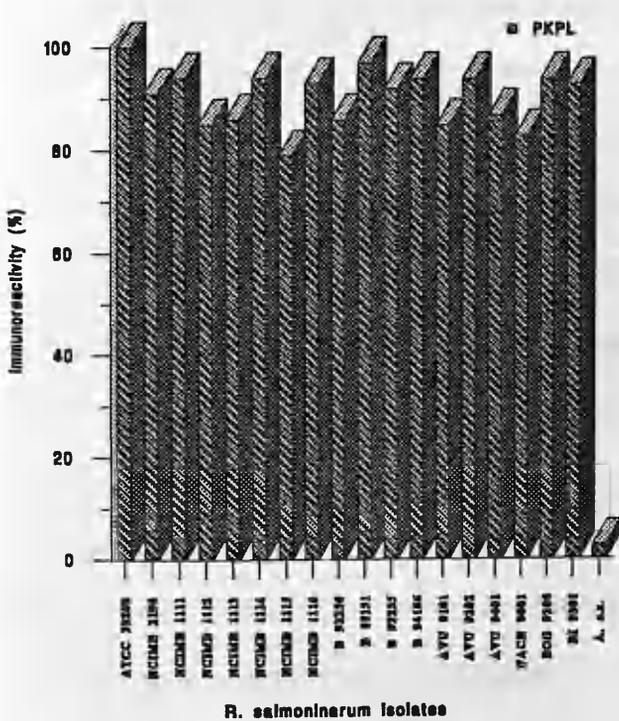
**Figure 5.9 Immunoreactivity of monoclonal antibodies 12B7, 11D11, 5A11 and 14C2 to 18 isolates of *R. salmoninarum*.**



*R. salmoninarum* isolates

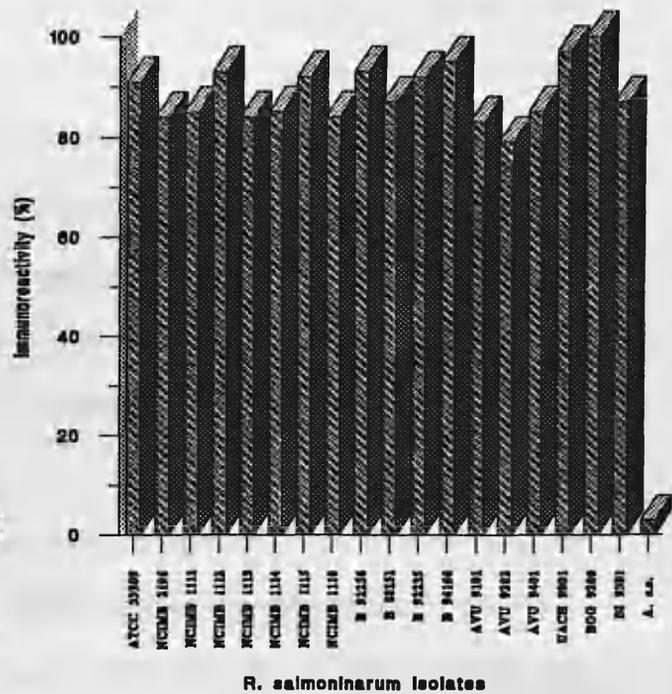
*R. salmoninarum* isolates

PAb PKPL



*R. salmoninarum* isolates

PAb PVIR



*R. salmoninarum* isolates

Figure 5.10 Immunoreactivity of monoclonal antibodies CW3, 155/p57, PKPL and PVIR to 18 isolates of *R. salmoninarum*.

## 5.4 DISCUSSION

In the present study, seven MAbs against extracellular and cell surface antigens of *R. salmoninarum* were produced. Three of these recognised only peptidoglycan epitopes (14C2, 9F5, and 12B7), two recognised only epitopes present in the p57 antigen and its breakdown products (5A11 and 1C7/3D1). MAb 1C7/1E1 recognised p57 antigen and two other ECP proteins of MW higher than 100 kDa (probably 110 and 140 kDa). MAb 11D11 recognised a common epitope present both in p57 and peptidoglycan antigens. The two novel bands recognised by MAb 1C7/1E1 were hardly visible in SDS-PAGE analysis but tiny bands were obtained using Western blot. The presence of these proteins could be related to the serine protease (MW higher than 100 kDa) detected by Rockey *et al* , (1991).

MAb anti ECP antigens (B6G8, 1C7/3D1, 1C7/1E1) and MAb anti cell wall antigens (12B7, 11D11) showed no crossreaction with any of the 30 different bacterial species tested nor the poly-L-lysine present in the negative control, except MAb 12B7 and 11D11 which showed a very low level of crossreaction (<14.5 %) with *Micrococcus luteus*. Therefore they could be used successfully as a diagnostic tools for *R. salmoninarum*. Anti-cell wall MAb could be particularly useful for the quantification of renibacterial cells and also for immunocytochemical studies where more accurate results could be obtained using these MAbs rather than those recognising soluble antigens. This is apparently the first study where specific monoclonal antibodies to cell wall antigens have been developed

Isotyping characterisation showed that five of the seven MAb belonged to the class IgM and two to the class and subclass (IgG<sub>1</sub>). Some of the MAbs also showed moderate to strong crossreaction with poly-L-lysine (9F5 and 5A11 respectively). L-lysine is a common component of the p57 antigen (Dubreuil *et al*, 1990) and it is also present in the interpeptide bridge of the renibacterial peptidoglycan (Kusser and Fiedler, 1983). Based on the fact that these MAbs gave strong IR to whole cells, renibacterial cell wall and

peptidoglycan it seems likely that they recognised a common epitope where poly-L-lysine is acting as immunogenic determinant.

The production of ECP antigens, in particular p57 showed differences depending on the culture media and the isolate analysed. Old isolates such as B88151 showed a considerable reduction in the IR of ECP which gave only 66% of IR compared with the other isolates tested. The production of p57 antigen was even more affected in isolate B88151 which showed 80 % reduction when it was grown in KDM-2, SKDM and MH-C. No p57 antigen was detected when the same isolate was cultured in KDM-Charcoal. This finding is in agreement with the reports of Bruno, (1988) who described the presence of avirulent isolates of R.s which lacked of ECP protein, particularly p57. The same author also reported that old cultures tend to lose p57 and become avirulent.

No significant differences in the IR were found when 18 renibacterial isolates were assayed using polyclonal and monoclonal antibodies and ELISA plates coated with whole cells, except isolate B88151 which showed only 25 % of the IR obtained in the other isolates.

The production of cell wall and peptidoglycan antigens showed very little differences between isolates and culture media. However, isolates ATCC 33209 and AVU 9202 showed a 50 % of reduction in the IR when cultured in SKDM. These findings could be due to the effect of the antibiotics added to the culture media as some of these antibiotics inhibit the synthesis of cell wall, particularly D-cycloserine. Based on this fact, it is likely that the differences in the production of cell wall and peptidoglycan antigens observed when the bacteria were grown in SKDM could be due to the effect of antibiotics.

Fiedler and Draxl,(1986) reported that the composition of the peptidoglycan and the structure of the cell wall from bacteria cultured in KDM-2 or SKDM was identical; however no immunogenic analysis was performed. Monoclonal antibodies to an unknown cell wall antigen (Arakawa *et al*, (1987), showed that

immunogenic differences between isolates do exist. These differences were suggested to be due to differences between the PS fraction; however no further analyses were performed to confirm this.

P70 and polysaccharide preparations failed to induce specific antibodies in the experimental animals. Immunoreactivity of p70 protein was first reported by Getchell *et al.*,(1985) using polyclonal antibodies against whole bacterial cells. P70 appears to be present only in fresh isolates and tends to disappear after prolonged artificial culture as suggested in Chapter 3. The reason why this protein failed to induce an immune response is unknown, perhaps the antigen was destroyed during extraction or is poorly immunogenic. No previous report on the immunogenicity of this component has been reported to date and so no comparison can be made.

In the present study, different techniques to coat PS to the ELISA microplate were used, including polymyxin B, lectins, antibodies, and poly-L-lysine, however inconsistent results were obtained. Preliminary results using Ouchterlony technique ( using both polyclonal and monoclonal antibodies) also failed to detect specific antibodies to PS. However, immunisation experiments using trypsinized renibacterial cells (Sørum and Robertsen, 1994) and trypsinized cell walls (Fiedler and Draxl, 1986) showed the formation of antibodies to PS in rabbits (using immunodiffusion technique).

The MW of renibacterial PS has been estimated to be 10 kDa (Fiedler and Draxl, 1986). Antigens with a molecular mass lower than 10 kDa are usually poor immunogens (Harlow and Lane, 1988). This fact could explain why the PS fraction failed to induce specific antibodies in the present study. Low MW antigens can be coupled to haptens to increase their immunogenicity. Renibacterial PS is originally linked (covalently) to peptidoglycan, whose immunogenic nature was demonstrated in this chapter. Therefore, it seems likely that renibacterial peptidoglycan is playing the role of hapten for the production of anti-PS

antibodies. This suggestion could explain the formation of specific antibodies to PS obtained by Fiedler and Draxl, (1986) and Sørum and Robertsen, (1994) using whole cells and cell wall.

PABs (PVIR, 155/p57 and CW3) showed crossreaction with other bacteria, particularly *Arthrobacter sp*, *Micrococcus luteus*, *Mycobacterium sp*, *Corynebacterium aquaticum*, *Streptococcus faecalis* and also poly L-lysine. In contrast, PAB PKPL showed no crossreaction with any of the bacteria tested, possible because this commercial antisera has been adsorbed with several common fish pathogens. These findings are in agreement with Bullock *et al.*,(1980); Austin and Rayment, (1985); Austin and Austin, (1993); Bandin *et al*, (1993) who also detected crossreaction of PABs, particularly with coryneform species, a fish pathogenic *Mycobacterium spp.* and *Rothia dentocariosa*, *Corynebacterium aquaticum* and *Carnobacterium piscicola*.

## CHAPTER 6

### IMMUNISATION AND PASSIVE IMMUNIZATION OF RAINBOW TROUT WITH SELECTED *R. salmoninarum* ANTIGENS AND ANTIBODIES.

#### 6.1 Introduction

Vaccination of fish against *R. salmoninarum* has been studied by numerous researchers and excellent general reviews have been published (Elliot *et al.* 1989; Evelyn , 1988, Munro and Bruno, 1988 and Evenden *et al.*, 1993).

Evelyn (1971) first showed the production of agglutinating antibodies against Rs. in 1-3 year old sockeye salmon held in fresh water at 12-15 °C. After 30 days 1/10 fish responded, after 60 days 5/10 fish and after 90 days 8/10 fish, indicating a very slow response. In a separate experiment using Freund's complete adjuvant (FCA) and 200 µg antigen/g of fish, the same authors reported that a response was still detectable after 16 months. When fish were given a secondary antigenic stimulation at 13 months their response three months later was far greater than the primary at 3 months indicating a strong memory response. However, these fish were not challenged with live bacterial cells, and it is unknown whether they had developed protective immunity.

The most promising anti-BKD vaccine studies were those using Atlantic salmon (Paterson *et al.*, 1981) and rainbow trout (McCarthy *et al.*, 1984). These species produced high titres of agglutinins in under yearling and post yearling parr following a single injection of killed R.s cells in FCA. The humoral immune

response of post yearling parr lasted to the smolt stage and appeared to be protective from natural challenge. Using Pacific salmon (sockeye and coho salmon), Evelyn *et al.*, (1984) evaluated various vaccine preparations and methods of presentation. The potential vaccines tested were all derived from formalin-killed R.s cultures and included cell associated and extracellular antigens, whole cultures, intact cells and various crude cell fractions. The preparations were administered *i.p* with or without FCA by feeding, spraying, or by the two step hyperosmotic infiltration method. The efficacy of the vaccines was determined by comparing mortalities in vaccinated and unvaccinated fish following natural and experimental (injection) challenge with live pathogen. None of the vaccinated fish showed any evidence of protection from BKD.

Munro and Bruno (1988) suggested that the results of experimental vaccination in all species indicated a significant but slow to develop, non protective agglutinating antibody response and concluded that the immunising preparations are inadequate perhaps because they contain the wrong antigen, insufficient of those necessary to confer protection, or perhaps the important antigens are too weakly immunogenic or response to them is suppressed by other antigens. It has been reported that fish are more restricted in their ability to produce antibodies with a wide variety of specificities, when compared to mammals (Hastings and Ellis, 1988). The use of purified renibacterial subunits or isolated antigens has provided useful information for the development of vaccines. Some favourable results have been already obtained using novel purified antigens obtained from *R. salmoninarum* cultured under restricted conditions (Thiry, Personal Communication).

Passive immunisation has been used successfully to identify protective antigens for a number of fish pathogens. Using this approach, Ellis *et al.*, (1988) identified the extracellular protease of *A. salmonicida* as a protective antigen. This approach is recently being used with *R. salmoninarum* using serum from challenge survivors and the results obtained indicated that the antibodies produced may indeed be protective (Campbell, Personal Communication).

This chapter describes the ability of experimentally immunised and naturally infected rainbow trout to induce a specific humoral immune response to *R. salmoninarum* antigens. Fish antisera were screened by ELISA using microplate coated with normal ECP, induced ECP, p57, low MW protein, cell wall, peptidoglycan, and whole cells. The presence of protective antigens was investigated by passively immunising fish with rabbit, mouse or fish antibodies followed by a challenge with live *R. salmoninarum* cells.

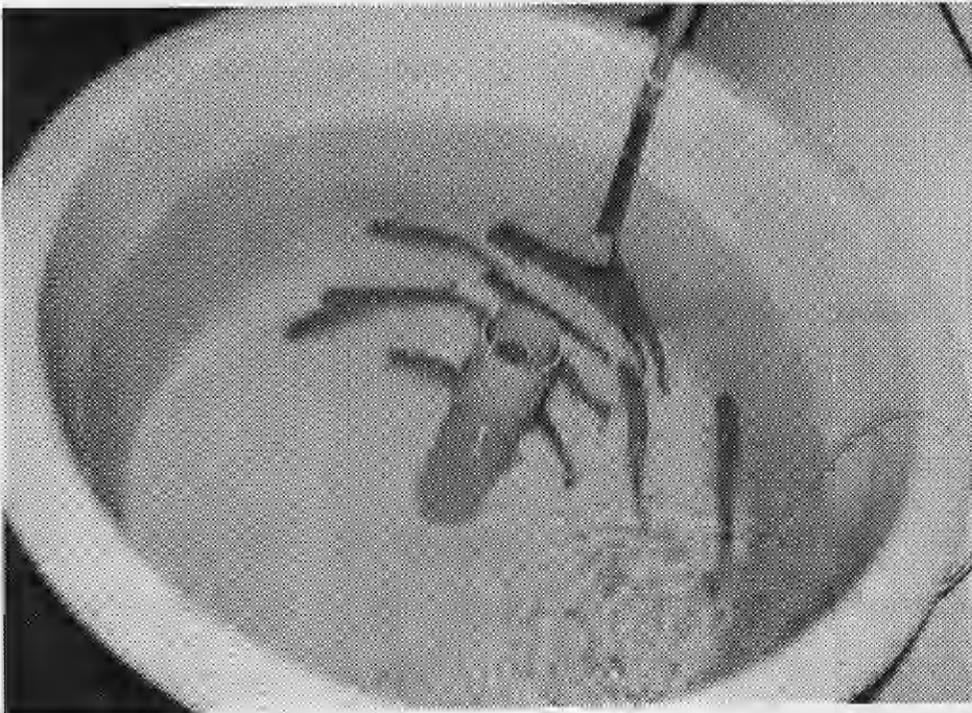
## **6.2 MATERIALS AND METHODS**

### **6.2.1 Experimental fish and maintenance conditions**

Rainbow trout (30 to 1200 g) were purchased from a local trout farm and acclimatized for a minimum of 3 weeks before commencing experimentation. Covered fibreglass tanks containing 150 L of flow through water were used to accommodate the fish (see plate 6.1). Mains supply water was treated by passing it through a charcoal filter (WTS, Cumbernauld, Scotland) which was backwashed once a day. Chlorine levels of the purified water were monitored periodically and did not exceed acceptable levels. Water circulated through tanks at a flow rate of  $1.5 \text{ L min}^{-1}$  and was aerated by compressed air delivered through pumice air stones. Water temperature followed seasonal fluctuations and illumination was set at cycles of 13 hours of daylight (provided by banks of 40 W fluorescent tubing), 11 hours of darkness. Stock fish were maintained on commercial diets, taking into consideration the size of the fish, with respect to the pellet size and composition.

Although a flow through water supply removed excess diet and debris, stand pipes had to be cleared daily, and tank walls wiped weekly. Bacterial and fungal infection were minimised by periodic treatment with chloramine T and malachite green both at a dose of 2 ppm respectively, especially in the summer time. Fish were starved for 24 hours prior to treatment and anaesthetized with benzocaine ( $50 \text{ mg l}^{-1}$ ) just before treatment.

Experimental infection of fish with live *R. salmoninarum* cells was performed in an isolation room which was exclusively used for this purpose. The effluent was disinfected by iodine treatment.



**Figure 6.1 Tank system used to maintain fish**

## **6.2.2 Immunisation of rainbow trout with *R. salmoninarum* preparations.**

The ability of rainbow trout to raise antibodies against selected *R. salmoninarum* antigens was studied. Low MW antigen and ECP from iron restricted culture medium (termed induced ECP or ECP<sup>+</sup>) was obtained from Pharos S.A. Belgium. Other antigens (e.g. p57, normal ECP, peptidoglycan), bacterial preparations (e.g. cell wall, live cell, heat killed cells) and stock fish used in the present study were obtained as described in sections 2.2 and 6.2.1). The antibody titres were measured by ELISA using the protocol described in section 2.3.1 and 2.3.2 but with an additional incubation for 2 hours at room temperature with Mab anti salmon IgM (4C10) between the fish antisera and the conjugated antibody. Preimmune fish sera was included as negative control. The end point titre of the antisera was determined as the dilution where the absorbance of the sample antisera was at least twice of the absorbance obtained in the preimmune sera at the same dilution. All samples were tested in triplicate and the mean absorbance determined. Three different experiments were set up as described below.

### **Experiment 1: Immunisation of rainbow trout with *R. salmoninarum* cell wall**

Five rainbow trout (200-300 g) were inoculated *i.p.* with 0.2 ml fish<sup>-1</sup> of cell wall suspension in FCA (1:1), containing 500 µg cell wall. A second group of 5 fish were inoculated with the same antigen but using PBS instead of FCA. A boost was given to the fish at the 9<sup>th</sup> week. A blood sample was collected from each fish before inoculation (preimmune sera), and 3, 6, 8 and 11 weeks post immunisation. The sera was collected, pooled (per group) and stored at -70 °C. The antibody titre to cell wall antigens was measured by ELISA as described previously.

**Experiment 2: Immunisation of rainbow trout with normal ECP , ECP<sup>+</sup>, live and , killed cells.**

This experiment was performed in collaboration with Dr. Ruth Campbell at Stirling University. Experimental fish were divided in four different groups (A to D), two fish (800-1200 g each) per tank and per group. They were inoculated *i.m.* with 0.2 ml of the following antigens:

- Group A: Induced ECP (20 mg ml<sup>-1</sup>) in sterile saline solution plus FCA (1:1)
- Group B: Normal ECP (20 mg ml<sup>-1</sup>) in sterile saline solution plus FCA (1:1)
- Group C : *R. salmoninarum* isolate AVU 9202 lived cells (4x10<sup>7</sup> cells per fish) in 0.1 ml sterile saline plus FCA (1:1)
- Group D: *R. salmoninarum* (AVU 9202) heat killed (4x10<sup>7</sup> cells per fish) in 0.1 ml sterile saline plus FCA (1:1).

Fish were bled every 2-3 weeks and serum was collected and stored at -70°C. The level of specific antibodies in the vaccinated fish sera was determined by ELISA using soluble antigens which included: normal ECP (80 µg ml<sup>-1</sup>), p57 antigen (80 µg ml<sup>-1</sup>), induced ECP (10 µg ml<sup>-1</sup>), and Pharos' Low MW antigen (10 µg ml<sup>-1</sup>). ELISAs were also performed using particulate antigens such as whole cells (AVU 9202, cultured in SKDM, 4x10<sup>7</sup> cells/ml), cell wall (100 µg ml<sup>-1</sup>) and peptidoglycan (100 µg ml<sup>-1</sup>). Fish antisera were diluted 1:20 before use.

### **Experiment 3: Detection of specific antibodies in the sera collected from naturally infected fish**

Pooled antisera from moribund fish naturally infected with *R. salmoninarum* (collected in section 3.2.1) and from survivor fish from experimental challenges (obtained from Dr. Ruth Campbell) were pooled and assayed by ELISA using microplates coated with induced ECP, normal ECP and Pharos' low MW antigen. Rabbit polyclonal antibody (S7.1, against induced ECP, obtained from Dr. M. Thiry, PHAROS, Belgium) and normal fish sera were used as positive and negative control respectively. All fish antisera were used at 1:20 and rabbit PAb at 1:1000 dilution.

#### **6.2.3 Passive immunization and challenge of rainbow trout.**

The production of a protective immune response in rainbow trout against selected *R. salmoninarum* was studied. Rainbow trout (30-50 g) were injected *i.p.* with 0.1 ml of antisera, monoclonal antibody or saline solution as shown in table 6.1. Monoclonal antibodies (prepared in chapter 5) were dialysed against PBS overnight before use in order to removed the antibiotic contained in the hybridoma culture medium.

Fish were challenged *i.m.* with 0.1 ml (approximately  $1 \times 10^8$  cells) of live *R. salmoninarum* (isolate B94166 cultured on MH-C) 24 hours after the inoculation of the antibodies. The mortality rate and the water temperature were monitored daily. Kidney and muscular tissue samples of dead fish were collected and processed for bacteriology, Gram's stain (see Appendix 1) and IFAT (performed by Dr Campbell) in order to confirm the presence of the pathogen in tissue.

**Table 6.1 Antibodies used for passive immunization of rainbow trout**

Group	Antibody	N° of fish	Antigen recognised	Titre	Host	Type
1	Challenged fish	15	Whole bacteria	-----	R. trout	PAb
2	155/p57	15	p57	1:1x10 <sup>6</sup>	Rabbit	PAb
3	1C7/IE1	15	p57,100, 140 kda	1:16,000	Mouse	MAB
4	Anti-FCA	9	FCA	-----	R. trout	PAb
5	1C7/3D11	15	p57	1:16,000	Mouse	MAB
6	14C2	15	Cell wall	1:8,000	Mouse	MAB
7	CW3	15	Cell wall	1:5x10 <sup>5</sup>	Mouse	PAb
8	Saline	15	None	-----	-----	-----

## 6.3 RESULTS

### 6.3.1 Immunisation of rainbow trout

The results from the immunisation of rainbow trout with renibacterial cell wall (experiment 1) is shown in Figures 6.2 and 6.3. The highest fish antibody level was found in the group where the antigen was inoculated with FCA, especially in the sample collected at the 3rd week post vaccination where the titre was 1/256, This titre dropped to 1/128 in the sample collected at the 6th week. No specific antibody level was detected in the sample collected at week 8.

A very poor humoral immune response was observed in the group where vaccination was carried out without adjuvant. In this group, the highest antibody level was found in the sample collected in the 3rd week. Undiluted preimmune sera was found to produce strong background in all the experiments; however it was considerably reduced by diluting it to 1/128.

The fish immune response to cell wall antigens during the 11 weeks of the immunisation experiment is shown in Figure 6.3. The antibody level reached a peak at the 3rd week but almost disappeared by the 8th week. A second dose of antigen given at the 9th week increased the levels of antibodies again but only moderately. The group of fish vaccinated without adjuvant showed only 50 % of the immune response obtained in the group where FCA was used.

The results from experiment 2 are shown in Figure 6.4 (soluble antigens) and Figure 6.5 (particulate antigens). The ELISA screening of fish antisera using soluble antigens (normal ECP, induced ECP, p57, and low MW antigen) showed that the highest immune response was obtained by the group of fish injected with induced ECP. Within this group, the highest antibody levels ( $OD_{450}$ ) corresponded to anti-LMWp antibodies ( $0.92 \pm 0.017$ ), followed by anti-induced ECP ( $OD 0.58 \pm 0.021$ ), anti-normal ECP

(OD  $0.55 \pm 0.039$ ), and anti-p57 (OD  $0.298 \pm 0.014$ ). Very low levels of anti p57 antibodies were found in all the experimental groups which ranged from OD  $0.071 \pm 0.014$  (group D) and  $0.298 \pm 0.012$  (group A), Negative control (preimmune sera) showed no reaction (OD  $0.032 \pm 0.008$ ).

Using normal ECP antigens, the highest response was obtained in group A (OD  $0.55 \pm 0.013$ ) and group C (OD  $0.43 \pm 0.011$ ) with only OD  $0.051 \pm 0.05$  in the negative control. Surprisingly, the screening of anti-induced ECP and LMWp showed strong background in the negative control (OD  $0.34 \pm 0.015$  and  $0.42 \pm 0.17$ , respectively).

In the group of particulate antigens (Figure 6.5), very high levels of anti-whole bacteria ( $1.22 \pm 0.014$ ) and cell wall ( $1.077 \pm 0.027$ ) antibodies were obtained in all the groups. In contrast, anti-peptidoglycan antibodies levels showed a variable response which ranged from  $0.35 \pm 0.011$  (group D) and  $0.71 \pm 0.034$  (group A). In addition, a strong background ( $0.38 \pm 0.019$ ) was detected using preimmune sera.

The results from experiment 3 (figure 6.6) showed that the sera from naturally infected fish contain moderate antibody levels which recognised induced ECP, normal ECP and LMWp (OD  $0.65 \pm 0.016$ ,  $0.82 \pm 0.004$  and  $0.62 \pm 0.019$ , respectively). However, the background of the assayed (preimmune sera) was relatively high varying from OD 0.37 and 0.42, while the positive control (anti-ECP<sup>+</sup>) showed OD ranged from 1.92 and 2.21 for the three antigens tested.

### 6.3.2 Passive immunization and challenge

The passive immunization and challenge experiment showed 100 % mortality in all the experimental groups (Figure 6.7). In the control fish (group 9), mortality began at day 18 (6.7 %) and reached a peak at day 32 (100 %). Almost the same trend was found in groups 1, and 4 (injected with fish antisera), groups 3, 5 and 6 (injected with monoclonal antibodies) and groups 2, and group 7 (injected with rabbit/mouse polyclonal antibodies). However, mortality was delayed to day 42 for group 6 and to day 48 for group 4. Fish from groups 1, 2, 5 and 8 showed 100 % mortality by day 34 while only 57 % mortality was reached by groups 3 and 7 and 60 % by group 6.

*R. salmoninarum* was identified in the kidney and muscular tissue in all the groups by reisolation in SKDM or IFAT. Non characterised gram negative bacteria were also recovered from muscular tissue.

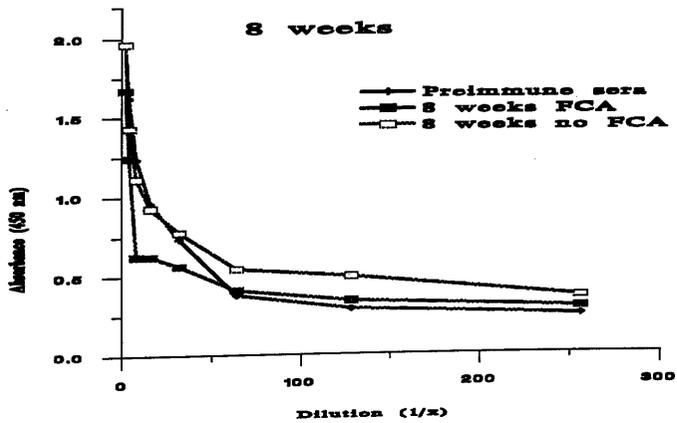
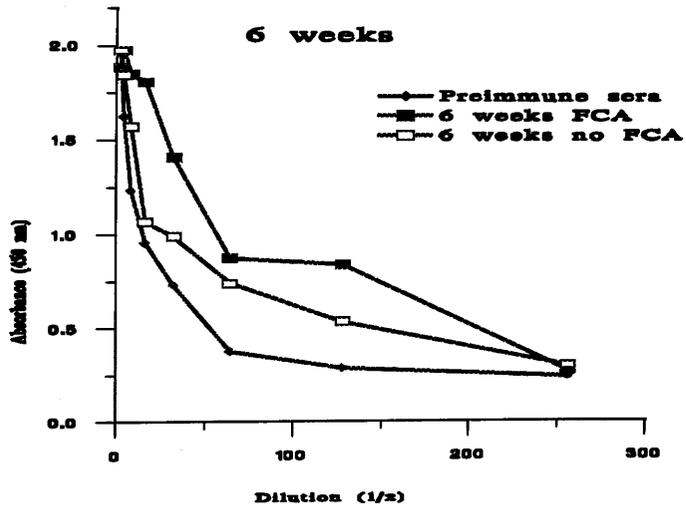
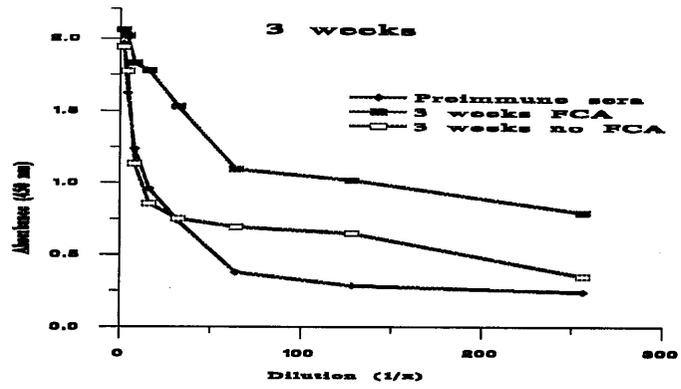
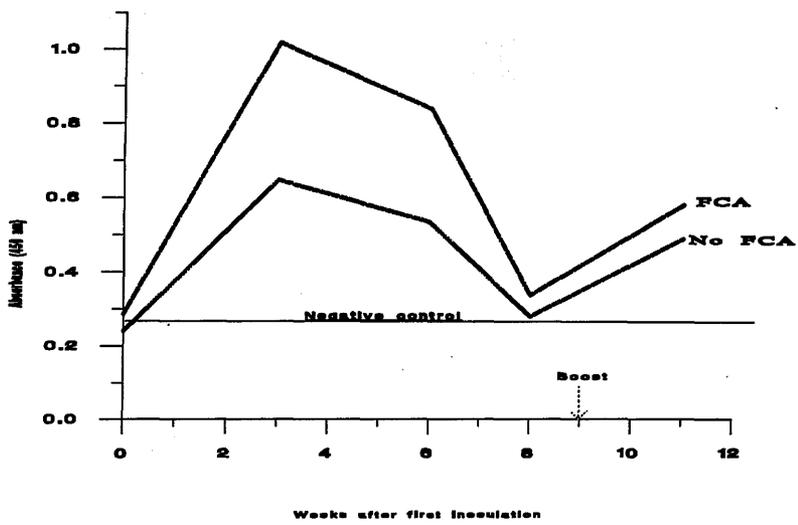


Fig. 6.2 Titration curves for pooled rainbow trout sera vaccinated with *R. salmoninarum* cell wall



**Fig. 6.3** Immune response of rainbow trout to *R. salmoninarum* cell wall. Pooled fish antisera were diluted 1/128.

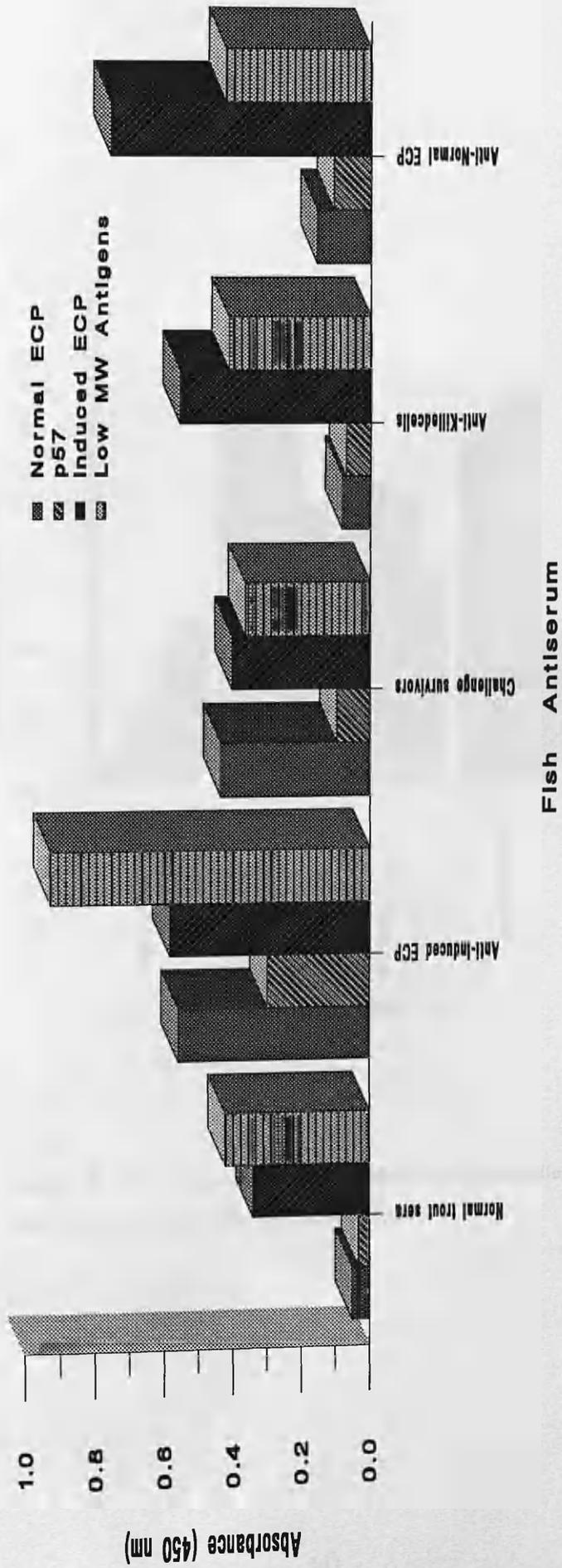


Fig 6.3 Graph showing the production of fish antibodies anti-soluble antigens (Normal ECP, p57, induced ECP and low MW antigen).

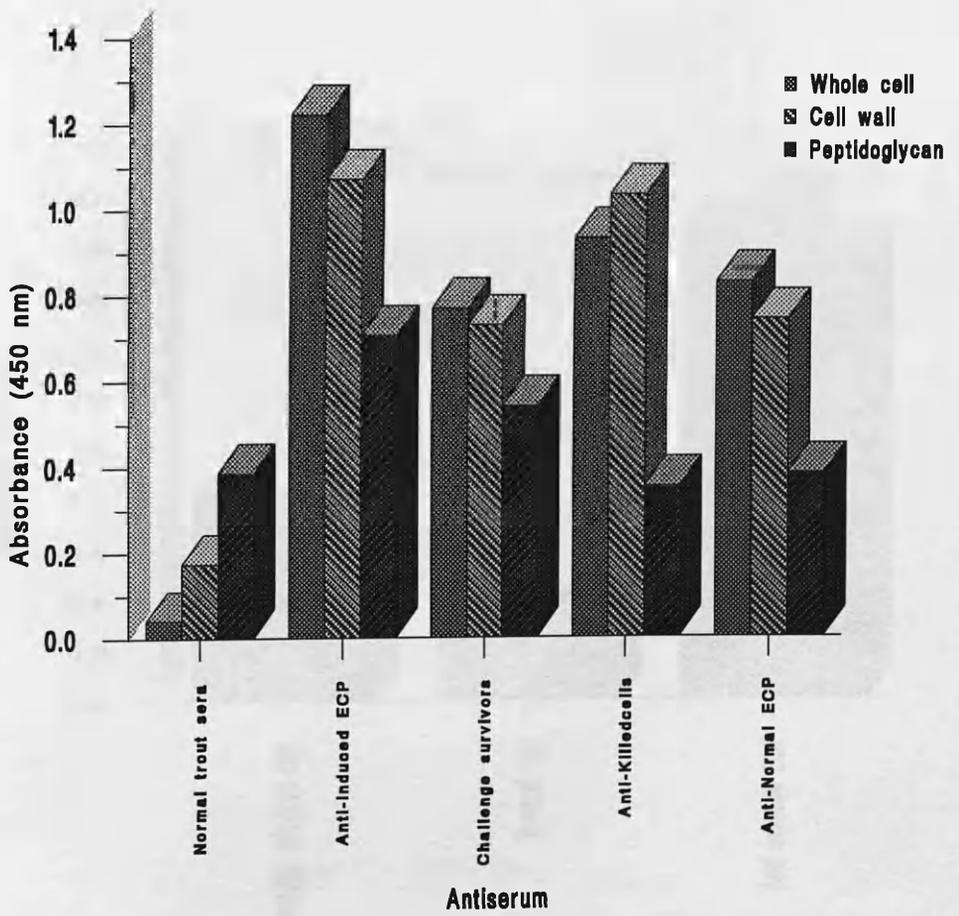


Figure 6.5 Graph showing the production of rainbow trout antibodies to particulate antigens (e.g. whole cells, cell wall, and peptidoglycan)

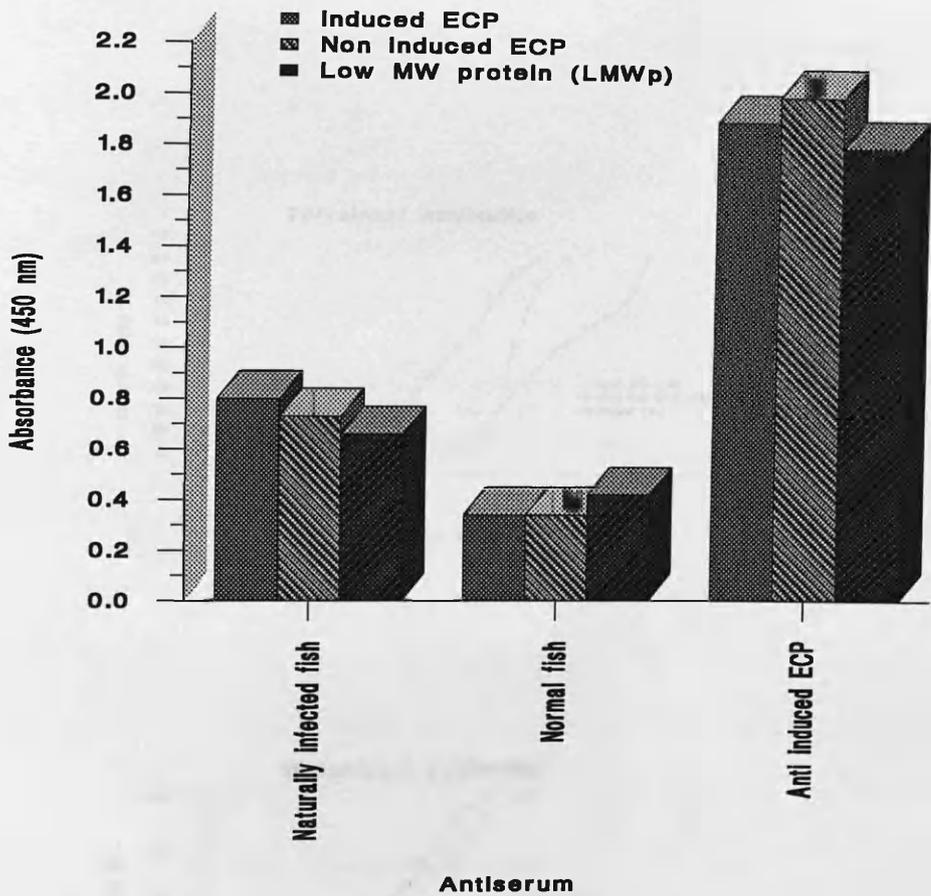


Figure 6.6 Graph showing the production of antibodies to normal ECP, induced ECP and LMWp in rainbow trout naturally infected with *R. salmoninarum*

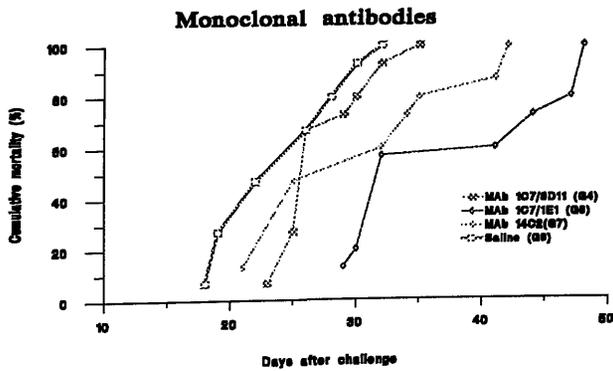
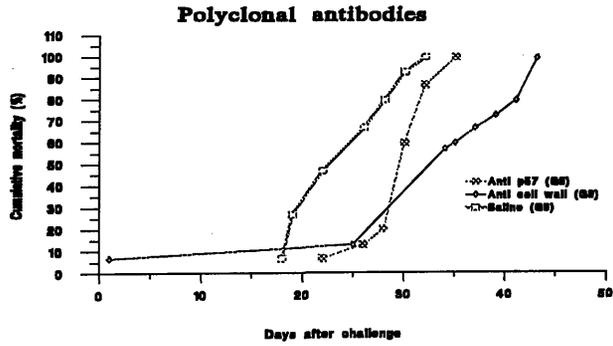
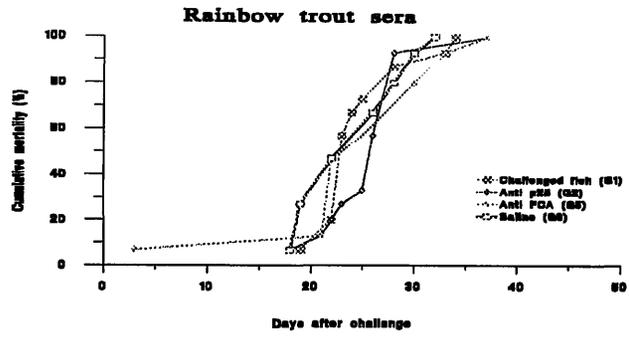


Fig 6.7 Graphs showing the passive immunisation of rainbow trout followed by challenge with *R. salmoninarum*

## 6.4 DISCUSSION

The present study demonstrated that rainbow trout are able to induce a moderate to strong humoral immune response to particulate and soluble antigens of *R. salmoninarum*. These findings are in agreement with previous reports of Evelyn, (1971); Paterson *et al*, (1981); MacCarthy *et al*, (1984) who also found specific immune response in Pacific, Atlantic salmon and rainbow trout, injected with different bacterial and ECP preparations. The highest antibody levels detected in the present study corresponded to anti-cell wall antibodies which showed optical densities, in ELISA, ranging from OD 0.822 and 1.077 in all the experimental groups. The fish immune response to cell wall reached a peak at the third week of inoculation (Experiment 1) and had almost disappeared by the 8th week.

Fiedler and Draxl, (1986), analysed the chemical structure of *R. salmoninarum* cell wall and reported that only two components are characteristic of this bacterium: a unique peptidoglycan and an unusual polysaccharide. The fact that high levels of anti-cell wall antibodies were raised in the injected fish suggests the immunogenic nature of this bacterial fraction. However, in the present study, only low levels of response were detected (OD 0.35 to 0.71) when the fish sera were tested using purified peptidoglycan. This may reflect the fact that the chemical extraction used to purify PG had destroyed important antigens. It could also suggest that polysaccharides need to be covalently linked to peptidoglycan to become potent immunogens for rainbow trout. This suggestion is in agreement with the report of Fiedler and Draxl, (1986); Sørensen and Robertsen, (1994) who were able to raise specific anti-renibacterial polysaccharide antibodies in rabbits using a similar cell wall preparation. The results from chapter 5 suggested that purified polysaccharides are not immunogenic enough to induce an immune response in mice but once linked with peptidoglycan may become potent immunogens.

Surprisingly, antisera from fish immunised with ECP preparations (normal and induced ECP) also reacted with cell wall antigen. As the cell wall preparation was digested with trypsin followed by SDS

treatment, it is unlikely that it could contain any protein components. In addition, no protein was found when this fraction was assayed using biorad kit. Proteins are the major component of the renibacterial ECP; however recent studies showed that it also contains some other components, probably pigments and polysaccharides (Dheur *et al*, 1993). These observations are in agreement with the reports of Collins (1982) who also found large amounts of glycolipids on the surface of the bacterium, probably not covalently linked, which could also be present in the ECP. Other components, such as lipooligosaccharide, have also been reported to be secreted by this bacterium (Kusser, personal communication, taken from Raverty's Thesis 1992). The crossreaction of anti-ECP antisera with cell wall antigens may therefore be due to the presence of common epitopes between cell wall and these non characterised ECP components.

Fish antisera also showed specific antibody responses to some of the soluble antigens tested (normal ECP, induced ECP, p57 and LMWp). The highest antibody levels corresponded to anti LMWp which was found particularly in the group injected with induced ECP. However, both induced ECP and LMWp showed strong background when they were tested using preimmune fish sera. In contrast, anti-p57 antibodies showed almost no background when compared with preimmune sera; however, they were detected only at very low levels in all the experimental groups. As the proteolytic degradation of p57 has been demonstrated to produce a loss of antigenicity (Griffiths and Lynch, 1991), then it is probable that the ECP preparations used to immunise fish contained a high proportion of degradation products. Degradation may also take place once inside the fish as the average water temperature for all the experimental groups was 15.5 °C which appears to be high enough to induce degradation *in vitro* (Griffiths and Lynch, 1991). In addition, the studies of Turaga *et al*, (1987a,b) demonstrated that p57 is immunosuppressive to salmonid lymphocytes *in vitro*. This could explain the low level of antibody response obtained to some of the soluble antigens tested compared with the rabbit antisera (see Figure 6.4). The higher antibody level obtained from experiment 1 (See Figures 6.1 and 6.2), where fish were injected only with cell wall, could support this theory. Another possible explanation for the poor antibody

levels to p57 could be due to the fact that p57 is able to induce the production of immunocomplexes (Ag/Ab) which may reduce the amount of specific antibodies in the serum (Kaattari *et al*, 1989).

Fish naturally infected with *R. salmoninarum* showed low levels of immune response to soluble antigens (normal ECP, induced ECP and LMWp) compared with that obtained using rabbit antisera. These findings are in agreement with the reports of Hastings and Ellis (1988) who suggested that fish may be more restricted in their abilities to produce antibodies, as opposed to mammals. In addition, there are several important differences between mammalian and fish lymphoid systems. Among the fish species examined to date, all appear to produce a single dominant class of immunoglobulin (Ig), a tetrameric molecule similar to IgM of mammals (Lamer, 1985). Though fish respond to immunisation by the production of specific antibodies, they do not have as broad an ability to respond to certain types of antigens. (Lamer,1985). One of the most important differences between mammalian and fish immune systems is the temperature dependent nature of the immune response in fish (Avtalion,1981). Fish immunised at less than physiological optimal temperatures display delayed development of protective immunity and required longer holding periods after vaccination prior to being exposed to the pathogen. There is a strong correlation between water temperature and the intensity of the immune response as well as a potential pathogen's ability to produce disease (Avtalion,1981).

Although none of the polyclonal or monoclonal antibodies used in the passive immunisation experiment showed any evidence in protection against experimental BKD infection, there was a delay in the onset of mortality in some of the groups. By the time 100 % mortality was reached in the majority of the experimental fish, those injected with MAb 1C7/1E1 (group 3), MAb 14C2 (group 6) and mouse polyclonal antibody anti cell wall (group 7) showed 57 %, 57 % and 60 % of mortality respectively. However, Campbell *et al*, 1994, who passively immunised rainbow trout with serum from challenge survivor fish found that after challenge some fish showed evidence of protection. These differences could be due to the fact that in the present experiment, a high challenge dose was used ( $1 \times 10^8$  cell fish<sup>-1</sup>) which

was injected *im*. In the study of Campbell *et al*, (1994) only  $1 \times 10^6$  cells fish<sup>-1</sup> were inoculated *ip*. The results obtained in the present chapter are in agreement with the report of Kaatari *et al* (1988), who challenged coho salmon with a mixture of live *R. salmoninarum* cells and high titre antiserum to homologous *R. salmoninarum* soluble proteins. They found that fish induced a specific but not protective immune response to ECP proteins, although fish took 25 % longer to die compared to control fish.

The results from this study together with Dr Campbell's experiments suggest that fish humoral immune response may play an important role in protecting fish against *R. salmoninarum*. However, further research is required in order to develop a standardised challenge procedure, particularly using a low dose, delivered by a route of infection more natural than *ip* injection, together with long term monitoring of the fish using reliable, sensitive, and accurate methods for detecting the presence of carriers amongst survivors as suggested by Murray *et al*, (1992). In addition, the identification of protective antigens and their production in sufficient amount to allow further biological studies, particularly using genetic engineering, is needed.

## CHAPTER 7

### GENETIC ENGINEERING STUDIES OF *R. salmoninarum*

#### 7.1 Introduction

The potential to vaccinate salmonid fish against *R. salmoninarum* has been investigated using a variety of bacterial preparations including whole cells, ECP, and cell wall products (Evelyn 1971; Paterson *et al.*, 1981; McCarthy *et al.*, 1984; Evelyn *et al.*, 1984). The results suggested that in all species tested a significant but slow to develop, agglutinating antibody response is produced (Munro and Bruno, 1988). In addition, a certain degree of protection appeared to be induced by Atlantic salmon and rainbow trout (Paterson *et al.*, 1981; McCarthy, 1984, Campbell *et al.*, 1995). Munro and Bruno, (1988) suggest that the poor protective immunity observed particularly in Pacific salmon could be due to inadequate immunizing preparations perhaps because they contained the wrong antigen, insufficient of those to confer protection, the important antigens are too weakly immunogenic or because the immune response to them is suppressed by other antigens.

Immunization of fish with selected purified renibacterial antigens is an essential step in the identification of protective antigens. However, due to the difficulties of growing the pathogen in artificial media, is almost impossible to obtain sufficient quantities of purified antigens. Molecular cloning of DNA provides a mechanism for isolating a single discrete segment of DNA

from a population of genes, purifying this segment to homogeneity, and amplifying the DNA segment to produce enough pure material for chemical, genetic or biological analysis. The process of cloning involves performing *in vitro* enzymatic reactions using bacterial DNA cleaving enzymes (restriction enzymes) and modifying enzymes to copy, cut and splice together discrete DNA molecules. DNA molecules are then introduced into bacterial cells after being spliced into autonomous replicating DNA circles (plasmids) or bacterial viruses (bacteriophages). After many rounds of replication, the hybrid molecules are reisolated and purified, yielding sufficient quantities of the cloned DNA segment. Reengineering of the cloned DNA in bacteria or yeast may allow expression of its protein coding sequence, providing an inexpensive and abundant source of otherwise unattainable proteins (Mackett, 1987; Ausbel *et al.*, 1994). This approach has been used successfully with other pathogens which are difficult to culture, such as *Treponema pallidum* (Van Emden *et al.*, 1983), *Mycobacterium leprae* (Ivany *et al.*, 1983 and *M. tuberculosis* (Young *et al.*, 1985).

This chapter describes the screening of a *R. salmoninarum* gene bank, prepared at Pharos S.A. Belgium, using polyclonal and monoclonal antibodies. The gene encoding for 57 Kda protein was isolated, cloned and expressed in *E coli* using the expression vector pBtrp/2.

## 7.2 MATERIALS AND METHODS

### 7.2.1 Immunological screening of a *R. salmoninarum* gene bank

A *R. salmoninarum* gene bank was screened by blotting using PAbs and Mabs. Positive clones were rescreened by dot blot and analyzed by SDS-PAGE and Western blot. Plasmids from positive clones were extracted and analyzed by DNA restriction using Sac I and Sph I restriction enzymes. The resulting fragments were visualized using agarose gel electrophoresis (1 % and 0.8 %).

#### 7.2.1.1 Gene bank construction

The *R. salmoninarum* gene bank was constructed by Mrs. Ingrid Dheur at Pharos, Liège, Belgium. High molecular weight genomic DNA was obtained by growing *R.s* (ATCC 33209) until the stationary phase and then harvesting by centrifugation at 1500 g for 15 minutes. The cell pellet was resuspended in 1/40th of the original volume with TE buffer (50 mM Tris, 10 mM EDTA pH 8.0). Lysozyme was added at 4 mg ml<sup>-1</sup> and incubated at 37°C for 30 minutes with constant agitation. SDS (1%) and Proteinase K (100 µg ml<sup>-1</sup>) were then added and the suspension incubated for 60 minutes at 37°C.

The sample was emulsified by adding the same volume of chloroform/isoamyl alcohol (24:1), mixed gently and centrifuged at 9000 g for 10 minutes. The aqueous phase was recovered and immediately submitted to isopycnic centrifugation in CsCl (1.075g ml<sup>-1</sup>) containing ethidium bromide (0.1mg ml<sup>-1</sup>). The DNA band was identified and collected. Ethidium bromide was removed by isoamyl alcohol extraction. The DNA was then extensively dialysed against TE buffer.

The gene bank was prepared in Lambda phage vector (Lambda GEM 12) and was then transferred to plasmid vector PGem 5Zf(+) (Promega), following the instructions provided by the manufacturer (See Appendix 2). The plasmid gene bank was stored in SOB/Ampicillin medium at 40°C.

### 7.2.1.2 Screening of the gene bank by blotting

Aproximately 1000 clones from the original plasmid gene bank were screened by blotting using pooled MAbs against ECP antigens (group A), MAbs against cel wall antigens (group B) and PAb S7.1 against ECP<sup>+</sup> (group C). PAb (S7.1) was prepared in rabbits using iron restricted ECP and was then adsorbed using *E. coli* cells to reduce background (carried out by PHAROS S.A., Belgium). Mab 1C7/1E1, 1C7/3D11, 14C2, 11D11, and 12B7 were produced at Stirling University as described in chapter 5. Canadian MAb B6G8 (anti p57) was also included in the screening (see table7.1).

**Table 7.1 Polyclonal and monoclonal antibodies used to screen the *R. salmoninarum* gene bank**

Group	Specificity	Antibodies
A	MAb anti ECP antigens	1C7/1E1, 1C7/3D11, B6G8
B	MAb anti cell wall antigens	14C2, 11D11, 12B7
C	PAb anti induced ECP	S7.1

Clones were transferred to fresh SOB/ampicillin plates by using sterile toothpicks in a laminar flow chamber. Plates were incubated at 37°C overnight to reactivate the growth. Each colony was then replicated onto nitrocellulose membranes. These were placed face up onto a fresh SOB/ampicillin plate and incubated at 20°C for 3 days. The master cultures were stored at 4°C.

Bacteria were killed by chloroform vapour for 15 minutes. The membranes were digested overnight at room temperature with constant agitation in lysis solution containing Tris (100 mM), NaCl (150 mM), MgCl<sub>2</sub> (5 mM), BSA (1.5 % w/v), DNase pancreatic (1 mg ml<sup>-1</sup>) and lysozyme (40 µg ml<sup>-1</sup>). Membranes were transferred to a fresh box and incubated three times with 200 ml of TNT solution (10 mM Tris, 150 mM NaCl, 0.05 % v/v Tween-20) for 30 minutes each. In the last change membranes were washed by hand in order to remove any remaining debris and then left incubating for 30 minutes. Membranes were transferred to blocking solution (5 % w/v skimmed milk in TNT) for 2 hrs at room temperature (or 30 minutes at 37°C). After washing in PBS containing 1% of Tween 20 (PBS/T), the membranes were incubated overnight at 4°C in a plastic bag containing 10 ml of the corresponding antibody solution (1/200 in blocking solution for PAb and undiluted for hybridoma supernatants). Membranes were washed then in PBS/T 3 x 5 minutes and incubated for 1 hour with conjugated antibody (anti rabbit IgG-HRP, for PAb, and anti mouse IgG-HRP plus anti mouse IgM-HRP, for hybridoma supernatants). Membranes were washed again as described above plus once with distilled water to remove Tween 20 and then incubated in developer solution (30 mg 4-chloro naphthol in 10 ml methanol plus 40 µl HRP buffer and 30 µl hydrogen peroxide) which was prepared just before use. The reaction was allowed to take place for 5 to 15 minutes and then stopped by washing the membranes with tap water. If the signal was not strong enough the developing solution was mixed with a half volume of tap water and left overnight in the dark.

### 7.2.1.3 SDS-PAGE

Gels for SDS-PAGE (15%) were prepared in batches of ten using the Mighty Small Multi Gel Caster (SE 200 series, Hoefer Scientific Instruments, San Francisco, California, USA). Acrylamide stock solution (30 ml) was mixed with separating buffer (15 ml), SDS 10 % (600  $\mu$ l), nanopure water (14.1 ml), APS 10 % w/v (30  $\mu$ l) and TEMED (20  $\mu$ l). Butanol (300  $\mu$ l) was added on top the gels which were then allowed to polymerize for 1 hour at room temperature. Butanol was removed from the gels by washing with distilled water and then the stacking gel added. Stacking gels were prepared by mixing acrylamide (4.1 ml), stacking buffer (5 ml), SDS 10 % (200  $\mu$ l), nanopure water (12.2 ml), APS 10 % (100  $\mu$ l) and TEMED (20  $\mu$ l). See Appendix 1 for buffer and stock solution preparation. The combs were placed on top and stacking gel left to polymerize at room temperature for 45 minutes. Gels were then stored at 4°C covered with cling film and foil for up to one week.

Gels were allowed to warm at room temperature for 30 minutes and then loaded with samples (10  $\mu$ l well<sup>-1</sup>) and protein standard marker (5  $\mu$ l), using low molecular weight standard (BioRad) if gel was to be stained with Coomassie blue, or Prestained molecular weight marker (IBR) if gels were to be further processed for Western blot. Gels were run at 150 volts for 1.5 hours or until the blue marker reached the bottom of the gel. Gels were stained for 1 hour and then destained overnight as described for SDS-PAGE in section 2.3.4.

#### **7.2.1.4 Western blot**

SDS-PAGE gels were prepared and run as described previously. The gels were then soaked in transfer buffer (see Appendix 1) for 20 minutes at room temperature together with six pieces of Whatman N°3 filter paper. One nylon membrane (Immobilon-P transfer membrane, Millipore) was washed in ethanol (70% v/v) for 1 minute and then in distilled water. The membrane was soaked in transfer buffer just before use. Three pieces of soaked filter paper were placed onto the transfer machine followed by the gel, the nylon membrane and three more pieces of paper filter. All bubbles were carefully removed. The transfer was performed in a Fast blot System for semi dry Transfer (Pharos S.A.) at 120 mA for 1 hour. Membranes were then soaked in PBS containing 5% of powdered skimmed milk and 1 ml of PMSF (100 mM) at 37°C for 2 hours. The membranes were incubated with antibodies and further processed as described for blotting ( see section 7.2.1.2).

#### **7.2.1.5 Kinetic analysis of *E. coli* DH10B containing plasmid PGEM-5Zf(+) plus DNA insert coding for renibacterial antigens**

LB/ampicillin broth (10 ml) was inoculated with a single colony of *E. coli* clone. Cultures were incubated at 20 °C for up to five days with constant agitation. One ml aliquots were taken on a daily basis. An aliquot (100 µl) was mixed with 900 µl of water and the OD at 600 nm measured. The remaining aliquot was centrifuged at 13200 g for 5 minutes and the supernatant carefully transferred to a fresh eppendorf tube. The cell pellet was then resuspended in 100x volume of the real OD of the culture (e.g. if OD<sub>600</sub> of a 1/10 diluted culture was 0.366, then the real OD is 3.66. So the cell pellet should be resuspended in a total volume of 366 µl. This was accomplished, by resuspending the cell pellet in 183 µl of distilled water and

once the cells were well suspended, 183  $\mu$ l of sample buffer was added. Samples were stored at  $-20^{\circ}\text{C}$  until required.

The proteins in the supernatant were precipitated by adding 100  $\mu$ l TCA (100% v/v), mixed gently and left overnight at  $4^{\circ}\text{C}$ . Samples were centrifuged at 13200 g for 5 minutes and the supernatant discarded. The protein pellet was then washed with cold ( $-20^{\circ}\text{C}$ ) acetone and centrifuged again as above. The supernatant was completely removed and carefully discarded. The remaining pellet was redissolved in 25  $\mu$ l of sample buffer (2x). Samples were stored at  $-20^{\circ}\text{C}$  until use. SDS-PAGE was carried out for each sample and the gels were stained with Coomassie blue. Western blot was also performed using pooled MAbs.

#### **7.2.1.6 Extraction and purification of plasmid DNA (Minipreps)**

One transformed bacterial colony was subcultured in 3 ml of LB medium containing 3 $\mu$ l of ampicillin (stock solution, 100 mg/ml) and incubated overnight at  $37^{\circ}\text{C}$  with agitation. A sample of bacterial culture (1.5 ml) was then centrifuged at 13,200 g and the supernatant discarded. The cell pellet was resuspended in 100  $\mu$ l of miniprep sol I (sucrose 50 mM, EDTA 10 mM, Tris-HCl 25 mM pH 8.0) and incubated for 5 min. at room temperature. Then 200  $\mu$ l of miniprep solution II (NaOH 200 mM, SDS 1%), prepared just before use, was added and mixed gently by inversion and incubated for 5 minutes on ice. The genomic DNA and proteins were precipitated by the addition of 150  $\mu$ l of cold ( $4^{\circ}\text{C}$ ) miniprep solution III (K acetate 3 M, pH 5.8, glacial acetic acid). The sample was gently agitated and incubated for 5 minutes on ice. After centrifugation at 13,200 rpm for five minutes, the supernatant was transferred to a fresh eppendorf tube and 450  $\mu$ l of phenol chloroform (v/v) added. The sample was vortexed for 5 seconds and centrifuged at 13,200 g for five minutes to separate the phases. The upper phase (colourless) was carefully collected and transferred to a fresh tube where 1 ml of absolute ethanol was then added to precipitate the

DNA. The tube was mixed gently and left on the bench at room temperature for 5 minutes. After centrifugation at 13,200 g for 5 minutes the supernatant was discarded and the DNA pellet was washed once with 1 ml of cold (-20°C) ethanol 70%. The sample was centrifuged again at 13,200 g and the supernatant discarded completely. The plasmid DNA pellet was then dried out for 10 minutes in a speed vacuum and then redissolved in 20 µl of TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) containing RNase

### **7.2.1.7 Digestion of plasmid DNA by Sac I and Sph I**

Plasmid PGEM 5Zf(+) containing inserts (1 µl) were digested using restriction enzyme Sac I (Pharos S.A., 1 µl) and Sph I (Pharos S.A., 1 µl), blue restriction buffer (Tris-HCl 10 mM, MgCl<sub>2</sub> 10 mM, NaCl 50 mM, DTT 1 mM, pH 7.5, 1 µl), and nanopure water (6 µl). Samples were incubated at 37°C for 2 hours and then submitted to agarose gel electrophoresis (1%).

### **7.2.1.8 Agarose gel electrophoresis (0.8 and 1 %)**

Agarose (0.8 or 1.0 g) was dissolved in 100 ml of TEA buffer (Tris acetate 4 mM, EDTA 1 mM, pH 8.5) containing ethidium bromide to a final concentration of 0.2 mg ml<sup>-1</sup>. The agarose was melted in a microwave oven for 2 minutes and then cooled to 55°C in a water bath before pouring onto the gel platform. The comb was inserted making sure that no bubbles were trapped underneath the combs and all bubbles on the surface of the agarose gel were removed before the gel set. When the gel had hardened, the comb was removed and the gel casting platform containing the gel was placed in the electrophoresis tank containing enough TAE/Eth. bromide buffer. Samples were mixed with 1/10 volume of 10x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll). Sample (10 µl) was placed in each

well plus 5  $\mu$ l of DNA marker. Running was performed at 50 volt for 10-20 minutes using a MUPID 2, Baby gel electrophoresis system (Pharos S.A., Liège, Belgium). The DNA fragments were visualized by placing on a UV light source, and were photographed.

#### **7.2.1.9 Restriction analysis of plasmid PGCS**

The restriction analysis of plasmid pGEM 5Zf(+), probably containing the DNA insert coding for renibacterial cell surface antigens (PGCS) was performed by digesting the plasmid, with Sac I, Sph I, and Sac I plus Sph I separately. The cocktail for digestion with Sac I was prepared by mixing plasmid DNA (1  $\mu$ l), blue buffer (1  $\mu$ l), Sac I (1  $\mu$ l) and nanopure water (7  $\mu$ l). The cocktail for digestion with Sph I was made up using plasmid DNA (1  $\mu$ l), blue buffer (1  $\mu$ l), Sph I (1  $\mu$ l) and nanopure water (7  $\mu$ l). The cocktail for digestion with Sac I plus Sph I was prepared using plasmid DNA (1  $\mu$ l), blue buffer (1  $\mu$ l), Sac I (1  $\mu$ l), Sph I (1  $\mu$ l) and nanopure water (6  $\mu$ l). Samples were incubated at 37°C for 2 hours and then submitted to agarose gel electrophoresis (1 % or 0.8 % w/v) and photographed.

#### **7.2.2 Expression of the gene encoding for the 57 Kda protein in *E. coli***

The *R. salmoninarum* gene encoding for the 57 Kda protein was isolated and amplified from genomic renibacterial DNA using polymerase chain reaction (PCR). The gene was inserted into the expression vector pBTtrp2 within an area controlled by the tryptophan promoter. The recombinant molecule (pBT57) was introduced into *E. coli* cells by electroporation and the resulting clones were screened by PCR, colony hybridisation and DNA restriction. Selected clones containing the pBT57 plasmid were induced to express the p57 gene by activating the tryptophane promoter. A summary of the cloning and expression strategy is presented in Figure 7.2. All the methods used in the present study are described below.

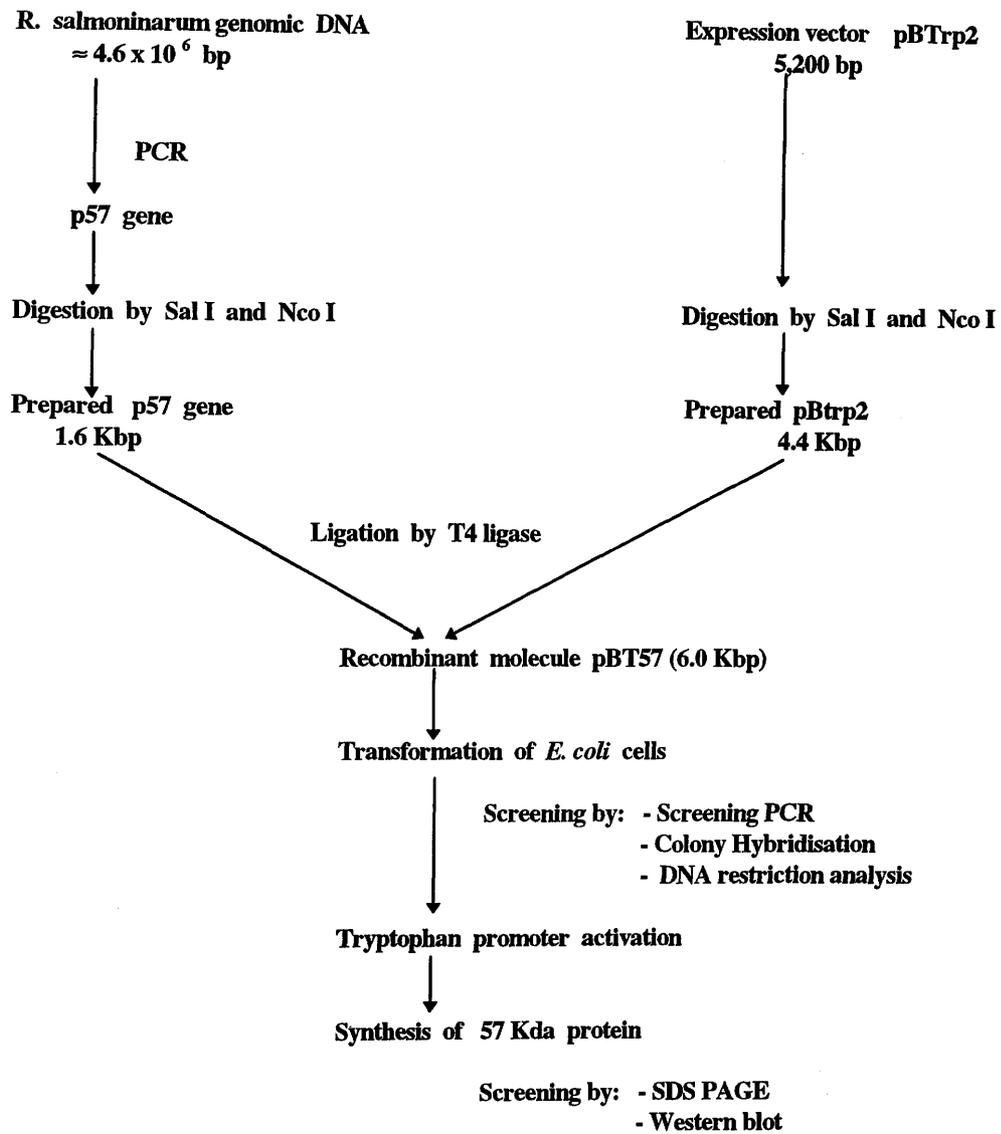
### 7.2.2.1 Amplification of the gene encoding for 57 KDa protein by PCR.

The gene encoding for the production of 57 Kda protein (p57) was isolated and amplified by polymerase chain reaction (PCR) in quadruplicate using specific oligonucleotide synthesized by PHAROS S.A., Belgium. The primers (see Figure 7.1) were constructed based on the sequence of the 57 Kda protein which has been recently published (Chien *et al.*, 1992, see Appendix 2 for the complete sequence). Specific restriction sites for Nco I and Sal I were placed in both ends of the gene in order to be able to isolate and clone the PCR products. The primers were synthesized in 0.2 mmole scale and kept lyophilized. Purification was performed by electrophoresis on polyacrylamide gel followed by elution in sterile water and filtration on a G-25 column

5' > GGC CAT GGA AAT AAA AAA AAT TTT A <3'

5' GGG TCG ACT TAG TTA AAG GTA ATA TC <3'

**Fig.7.1** Nucleotide sequences of the primer used to amplify the p57 gene from genomic DNA (Eurogentec S.A)



**Figure 7.2** Diagram showing the cloning and expression of the renibacterial p57 gene in *E. coli*

Genomic renibacterial DNA (2  $\mu$ l) containing 100 ng/ml was diluted by adding 64  $\mu$ l of pure grade water and kept on ice. Four sets of siliconized eppendorf tubes were prepared and the following mixture was added to each of them: diluted genomic renibacterial DNA (10  $\mu$ l), oligonucleotide 1 (Pharos S.A., 1  $\mu$ l), oligonucleotide 2 (Pharos S.A., 1  $\mu$ l), 10x GoldStar DNA polymerase buffer (Pharos S.A., 10  $\mu$ l), dNTP mixture (Pharos S.A., 1  $\mu$ l), MgCl<sub>2</sub> (Pharos S.A., 6  $\mu$ l), nanopure water (71  $\mu$ l) and GoldStar DNA polymerase (Pharos S.A., 0.5  $\mu$ l). The tubes were centrifuged at 13,200 g for 2 seconds and then 100  $\mu$ l of mineral oil added. PCR was performed in a ThermoJet PCR machine (Pharos S.A., Belgium) for 30 cycles each of them set up for denaturing (1 minute at 94°C), annealing (1.5 minute at 39°C), elongation (2 minutes at 72 °C). After the last cycle, the products were left at 72°C for 5 minutes and then at 4°C overnight. The PCR products were analyzed by agarose gel electrophoresis (1%). PCR products (10 ml) were mixed with 1 ml of loading buffer (10x) and then loaded into the corresponding well. Standard DNA marker (1Kbp) was used as reference.

PCR products were purified from non-reacted PCR mixture (e.g. nucleotides, genomic DNA, polymerases, and primers) using Qiaquick PCR Purification Kit using the method provided by the manufacturers.

### 7.2.2.2 Expression vector pBTrp/2

Expression vector pBTrp2 was purchased from Boehringer Mannheim (Germany). It has a MW of  $3.4 \times 10^6$  daltons and is 5.2 Kbp in size. The expression vector contains a tryptophan promoter, a ribosome binding side, and single cloning sites for *Cla*, *Nco* I, *Bam* H I, *Sph* I and *Sal* I which can be used for the insertion of foreign genes. The *Nco* I site contains the ATG initiation codon. The general structure of this expression vector is shown in Figure 7.3.

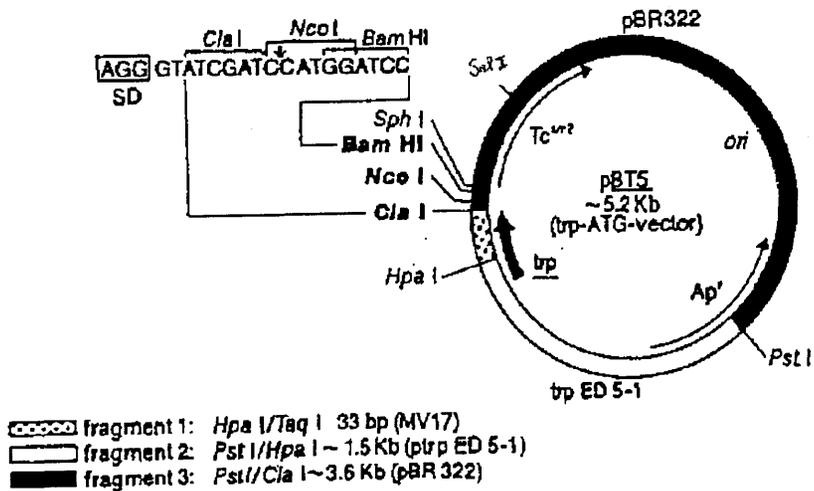


Fig 7.3 pBTrp2 expression vector map

### **7.2.2.3 Digestion of purified PCR product and plasmid pBTrp/2 by restriction enzymes Sal I and Nco I.**

Purified PCR products (20 µl) and whole plasmid pBTrp/2 (20 µl) were double digested by Nco I (5 µl) and Sal I (5 µl) in red buffer (Tris HCl 50 mM, MgCl<sub>2</sub> 10 mM, NaCl 100 mM, DTT 1 mM, pH 7.5, 1µl). Nanopure water was added to a final volume of 100 µl per sample. The tubes were incubated at 37°C overnight and then submitted to agarose gel electrophoresis. Digested PCR products were then purified from enzymes and other impurities by a Chroma spin + TE-100 column following the instructions provided by the manufacturer.

### **7.2.2.4 Ligation**

Digested PCR products and pBTrp/2 plasmid (obtained from Mrs. I. Dheur, Pharos. S.A.) were ligated using T4 DNA ligase. Three different groups were performed as shown in table 7.2.

Samples were incubated at 16°C overnight. Ligated DNA (PCR products plus plasmid pBTrp/2) were then filter dialysed using Millipore 39 VS filter type, pore size 0.025 µm. Filters were placed face up over a petri dish containing pure grade water. Ligated DNA (5 µl) was placed on top of the filter and left to dialyse for 1 hour. The drops were collected and placed in a clean Eppendorf tube and left on ice until use.

**Table 7.2 Ligation protocol**

	Group 1	Group 2	Group 3
pBTrp/2	2.0 µl	2.0 µl	2.0 µl
PCR products	1.0 µl	5.0 µl	-----
Lig. buffer 10x	1.0 µl	1.0 µl	1.0 µl
T4 DNA ligase	0.5 µl	0.5 µl	0.5 µl
Pure grade water	5.5 µl	1.5 µl	6.5 µl
<b>Total volume</b>	<b>10.0 µl</b>	<b>10.0 µl</b>	<b>10.0 µl</b>

### 7.2.2.5 Transformation

*E. coli* cells (DH10B) were partially thawed and kept on ice together with three sterile electroporation cuvettes. Electroporation took place in a laminar flow chamber using a Cellject Basic bacterial electroporation machine (Pharos S.A). Ligated DNA (2 µl) was placed on the electroporation cuvette and mixed with 40 µl of *E. coli* cells. The cuvettes were placed back on ice for 5 minutes making sure that no bubbles were trapped inside the cuvette. After drying the metallic part of the cuvette, it was placed in the electroporation machine and submitted to an electric pulse of 2500 volts. Then, 1 ml of rich broth (SOC, see Appendix 1) was added to the cuvette, mixed well and the total contents of the cuvette transferred to a 10 ml sterile tube with cap and incubated at 37 °C for 1 hour. Culture (100 µl) was then plated out into dried SOB/ampicillin plates using five plates per group except for the control group (group 3) for which only one plate was used. Plates were incubated at 37°C overnight and then the number of colonies was counted.

#### **7.2.2.6 Detection of transformant by screening PCR .**

A PCR cocktail (800  $\mu$ l ) was prepared using siliconised eppendorf tubes. Oligonucleotide 1 (8  $\mu$ l), oligonucleotide 2 (8  $\mu$ l), 10x GoldStar DNA polymerase buffer (80  $\mu$ l), dNTP mixture (8  $\mu$ l), MgCl<sub>2</sub> (48  $\mu$ l), nanopure water (644  $\mu$ l) and GoldStar DNA polymerase (4  $\mu$ l). The tube was centrifuged at 13,200 g for 2 seconds and 20  $\mu$ l of the PCR cocktail was transferred to each well of a polycarbonate V-bottom microtitre plate. One transformed colony was added to each well and the whole plate submitted to lysis protocol (10 minutes at 94°C) using a UNO-THERMOBLOCK PCR Machine (Biometra). The machine was also set up for 30 Cycles as described previously for the standard PCR. Diluted genomic renibacterial DNA was used as a positive control.

The PCR products were analyzed by agarose gel electrophoresis (1%). PCR products (10  $\mu$ l) were mixed with 1  $\mu$ l of loading buffer and then loaded into the corresponding well. Standard DNA markers (1Kbp) was used as the reference.

#### **7.2.2.7 Detection of transformant by colony hybridisation**

All colonies from the transformation plates were replicated onto nitrocellulose filter and hybridised following the method described by Ausbel *et al*, (1994) using p 57 gene labelled with <sup>32</sup>P. The labelling of p57 gene was performed using the Random Primed DNA Labelling Kit (Boehringer manheim) with random oligonucleotides as primers and following the instruction provided by the manufacturers. The p57 gene was obtained from genomic *R. salmoninarum* DNA using PCR as described in section 7.2.2.1.

The method of random primed DNA labelling developed by Feinberg and Vogelstein (1983, 1984) is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labelled. The complementary strand is synthesized from the 3'OH termini of the random hexanucleotide primer using Klenow enzyme, labelling grade. Modified deoxynucleoside triphosphate ( $\alpha$   $^{32}\text{P}$  dATP radioactive) is incorporated into the newly synthesized complementary DNA strand.

#### **7.2.2.8 Digestion of plasmid pBT57 by Sal I, Nco I and Pst I.**

Plasmid pBT57 containing inserts, were obtained by miniprep (section 7.2.1.7) and digested using restriction enzyme Sal I and Nco I and also by Sal I and Pst I. Cocktail for Sal I/Nco I was prepared by mixing red buffer (10  $\mu\text{l}$ ), Nco I (5  $\mu\text{l}$ ), Sal I (5  $\mu\text{l}$ ), nanopure water (60  $\mu\text{l}$ ). Cocktail for Sal I/Pst I contained red buffer (10  $\mu\text{l}$ ), Pst I (5  $\mu\text{l}$ ), Sal I (5  $\mu\text{l}$ ), nanopure water (60  $\mu\text{l}$ ). Samples were incubated at 37°C for 2 hours and then submitted to agarose gel electrophoresis (1%).

#### **7.2.2.9 Overexpression of the gene p57 in *E. coli***

Positive clones containing the plasmid pBT57 were selected and the gene encoding for the renibacterial 57 KDa protein was expressed by activating the tryptophan promoter. One bacterial clone containing the pBT57 plasmid together with a negative clone (containing no plasmid) was selected and cultured overnight in M9 broth (10 ml) containing casamino acid, tryptophan and ampicillin (See Appendix 1). An aliquot of each culture was taken after overnight incubation and labelled as NI (non induced). An aliquot (0.5 ml) of each culture was diluted in 5 ml of M9 broth containing casamino acids and ampicillin (no tryptophan) and incubated at 37 °C for 2 hours. An aliquot was then collected and labelled as T<sub>0</sub>.

Indoleacrylic acid (IAA) was added to the rest of the culture and one aliquot was collected each hour ( $T_1, T_2, T_3, T_4, T_5, T_6$ ) and overnight  $T_{ON}$ . Aliquots (1 ml) from each culture media were collected, the cells were pelleted and then resuspended in LB broth to  $OD_{600} = 1.0$ . An aliquot (10  $\mu$ l) of this suspension was submitted to SDS-PAGE and Western blot analysis as described in section 7.2.1.5. Rabbit Pab (S7.1) was used to identify p57 antigen.

### 7.3 RESULTS

#### 7.3.1 Screening of a *R. salmoninarum* gene bank

The screening of the *R. salmoninarum* gene bank by blotting showed sixty one positive clones to the pooled antibodies against cell wall antigens (group B) and 34 positives clones to induced ECP (group C). No positives clones were found using pooled antibodies against non induced ECP antigens (group A). Figure 7.4 illustrates some of these results.

Sixteen positive clones from the blotting experiment were selected and rescreened by blotting using PAb and MAb (S7.1, 1C7/1E1, 12B7). The results are shown in Figure 7.5. Using PAb S7.1 (Fig. 7.5 A), only one positive clone was found (sample number 7). With MAb 12B7 (Fig 7.5 c), 14 positive clones were detected. The negative control (sample 17) gave a very high background but not as high as in the samples 6, 12 and 13. MAb 1C7/1E1 (fig. 7.5 b) showed no positive reaction.

The clones which showed the best reaction in the blotting experiments (clones 6 and 12) were then submitted to a kinetic analysis (section 7.2.1.5). The results are presented in Figure 7.6. After a 24 hour incubation, a probably novel protein band with MW higher than 70 KDa was observed by SDS PAGE in

the whole cell preparation. This band was not found in the negative control and was still visible in clone 6 after 72 hours but disappeared at 48 hours in clone 12. No clearly visible band was found in Western blot using any of the antibodies tested .

The plasmids from 12 clones which appeared to contain *R. salmoninarum* DNA coding for some antigens were termed PGCS and these were extracted and purified by miniprep ( section 7.2.16). The *R. salmoninarum* DNA inserts were liberated by digesting the plasmid by Sac I and Sph I restriction enzymes (section 7.2.1.9). The resulting fragments were visualised using agarose gel electrophoresis (1 %). The results are presented in Figure 7.7.

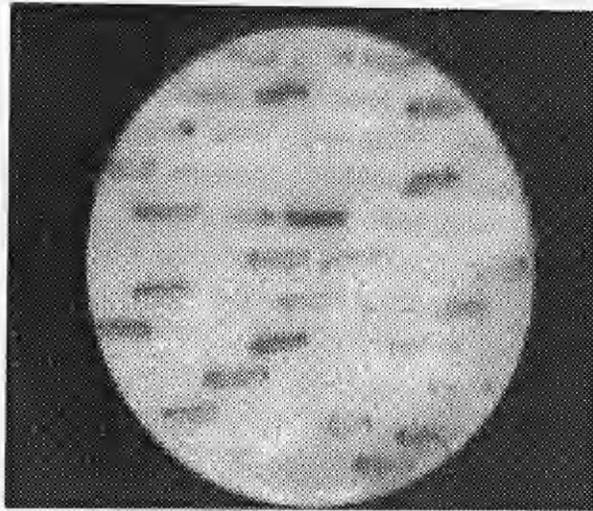
Clone number 12 ( line 3) showed the best digestion. This plasmid produced six fragments of 0.7, 0.8, 1.0, 1.3 1.5 and 2.8 Kbp. The rest of clones analysed show very poor or no digestion at all , while some others have lost the plasmid.

The DNA restriction analysis of the plasmid PGCS (Figure 7.8.) showed that the digestion of Sac I plus Sph I produced five fragments of 0.7, 0.8, 0.9, 1.3, 1.5 and 3.1 Kbp. The digestion with Sac I alone gave also five fragments of 0.8, 1.3, 2.8, 3.2 and probably another fragment of 3.5 Kd which was poorly visible. An additional higher fragment, of approximately 6 Kbp, was also found which corresponded to incomplete digestion of the plasmid. The digestion by Sph I alone showed four fragments of 1.8, 3.2 and 12 Kbp respectively. Due to the poor visualisation of some fragments (which could be duplicated or migrated very close to one another), a second double digestion was performed using 0.8 % agarose and 20x25 cm gel size rather than 1 % and 10x15 cm gel used in the former experiment. The results are presented in Figure 7.9.

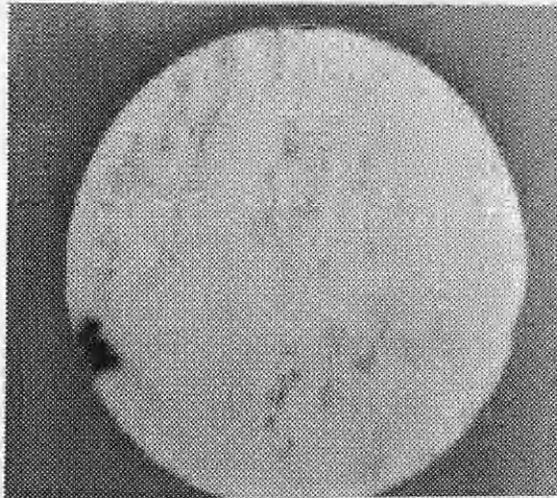
The analysis of Figure 7.9 showed almost the same DNA fragment found in Figure 7.8 but this time the resolution of the fragments was considerably increased. The digestion of the plasmid by Sac I alone produced seven fragments (0.7, 0.8, 0.9, 1.3, 1.5, 2.8 and 3.5 Kbp) plus two additional fragments (4.5 and 6.5 Kbp) that because of their intensity was far below the expected one for this DNA size, it is likely that these fragments are artefacts due to incomplete digestion of the plasmid. For this reason these fragments were not considered.

Using Sph I alone, five fragments were found (0.8, 1.3, 2.5, 3.5 and 3.8 Kbp), while the double digestion by Sac I plus Sph I showed seven fragments (0.7, 0.8, 0.9, 1.3, 1.5, 3.0 and 3.5).





A

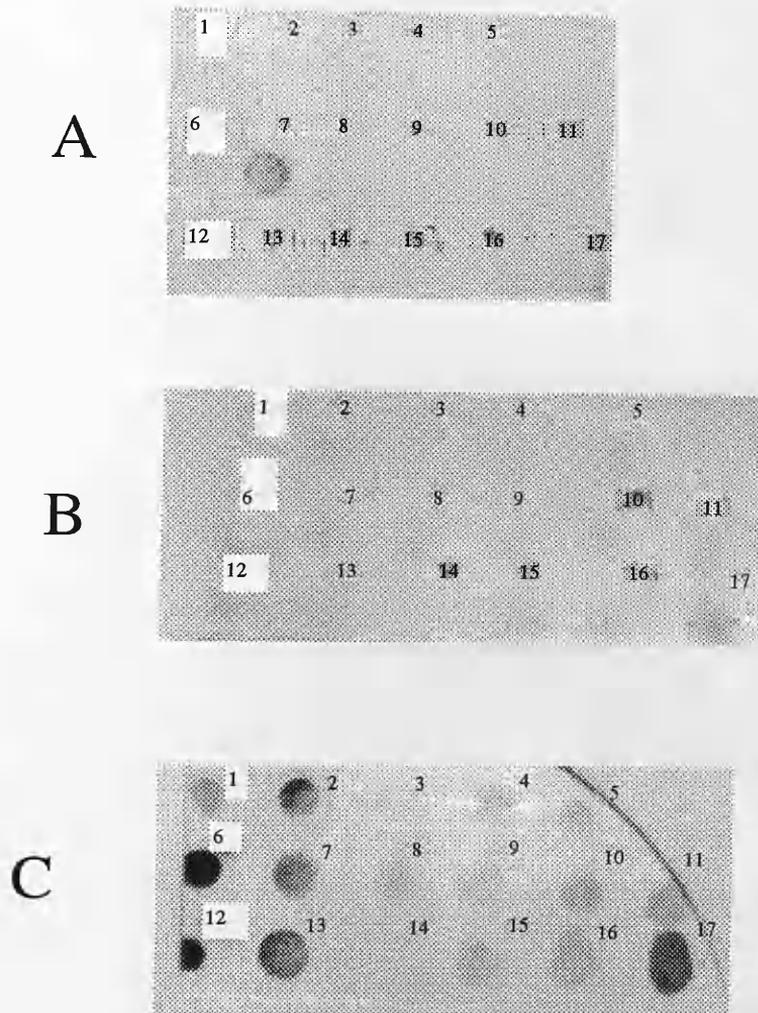


B

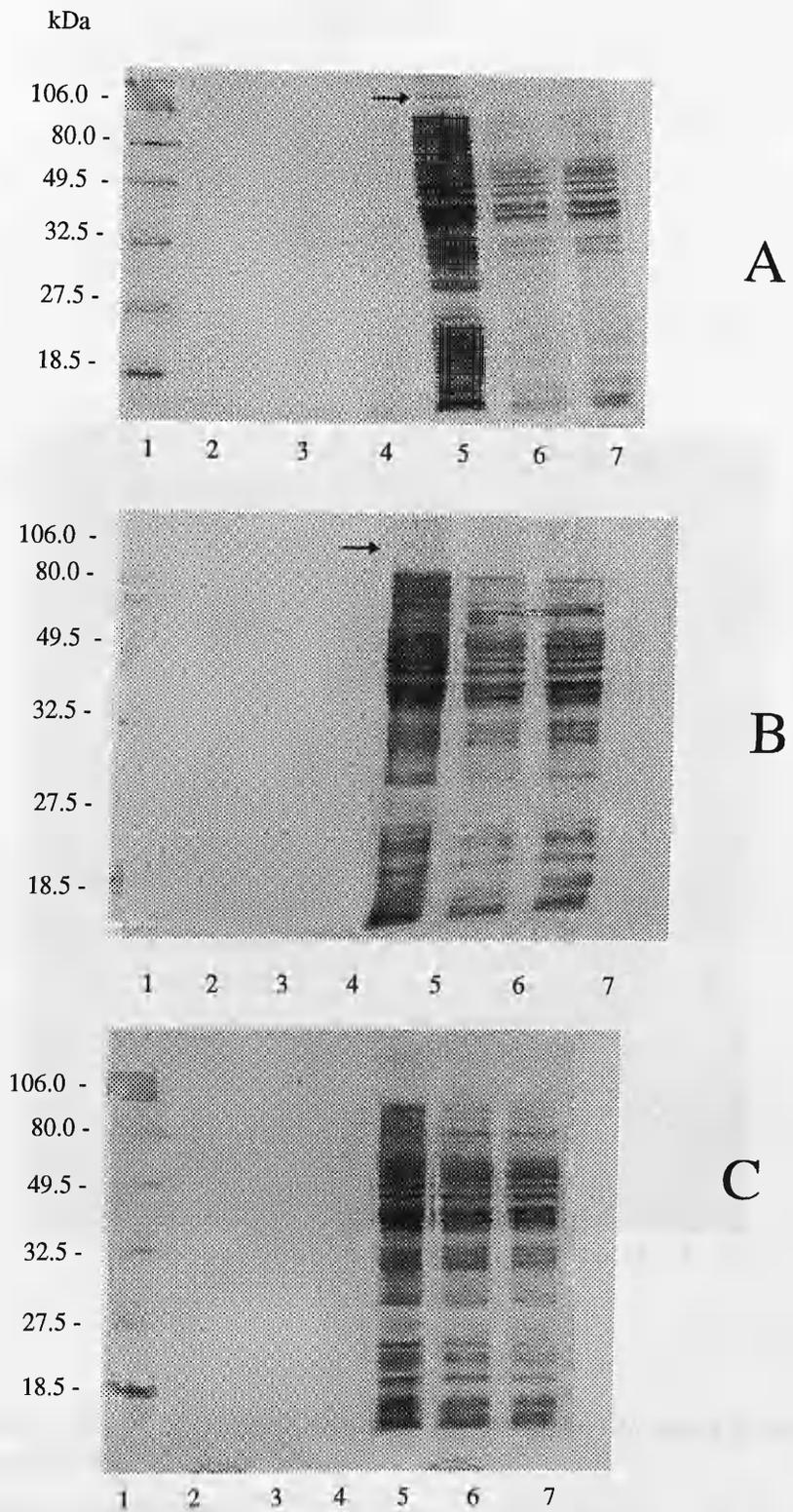
**Figure 7.4** Screening of a *R. salmoninarum* gene bank by blotting using pooled polyclonal and monoclonal antibodies.

**A:** Adsorbed PAb anti ECP<sup>+</sup>

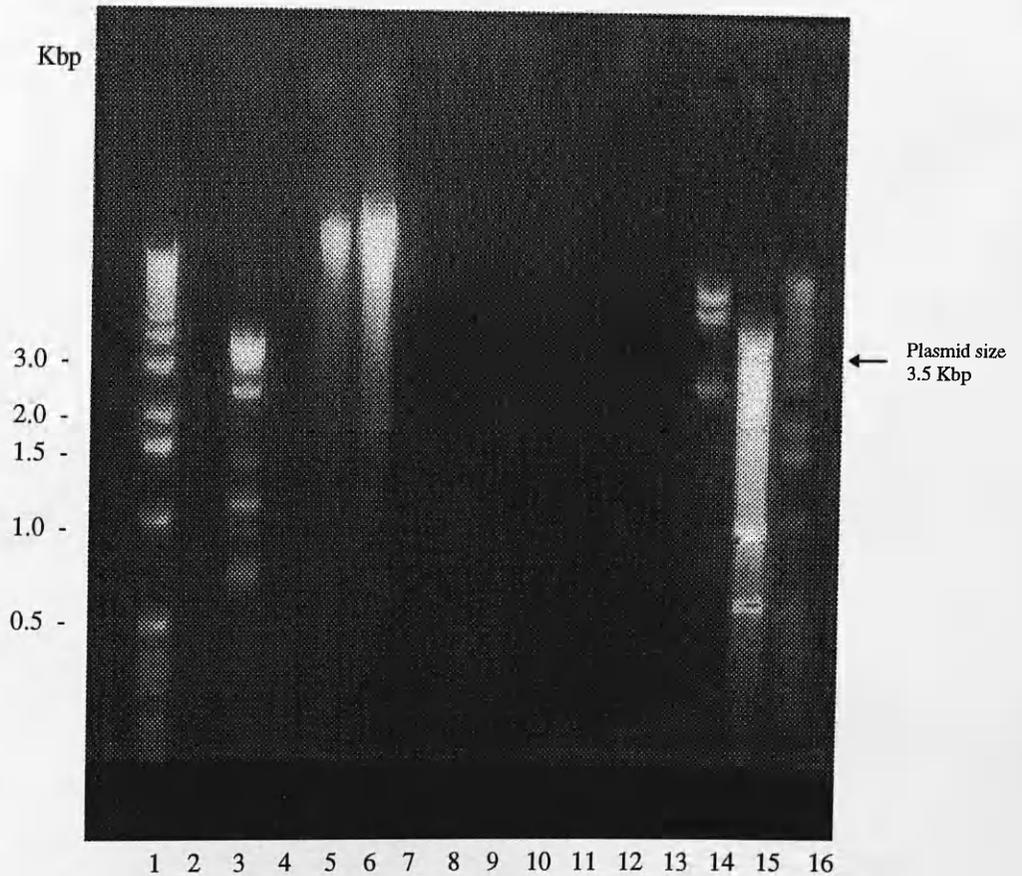
**B:** Pooled Mab anti cell wall antigens



**Figure 7.5** Dot blot of selected clones containing *R. salmoninarum* DNA fragments. The antigens expressed by the clones were detected by A: PAb S7.1, B: Mab 1C7/1E1 (anti ECP) and C: Mab 12B7 (anti cell surface). Sample No 17 corresponds to the negative control (Blue colony).



**Figure 7.6** SDS-PAGE of clone 6 (Figure A), clone 12 (Figure B) and negative control (Figure C) after incubating for 24 to 72 hours at 20 °C. Low MW marker (line 1), ECP 24 h (line 2), ECP 48 h (line 3), ECP 72 h (line 4), whole cells 24 h (line 5), whole cells 48 h (line 6) and whole cells 72 h (line 7). Arrows show the probably novel band.

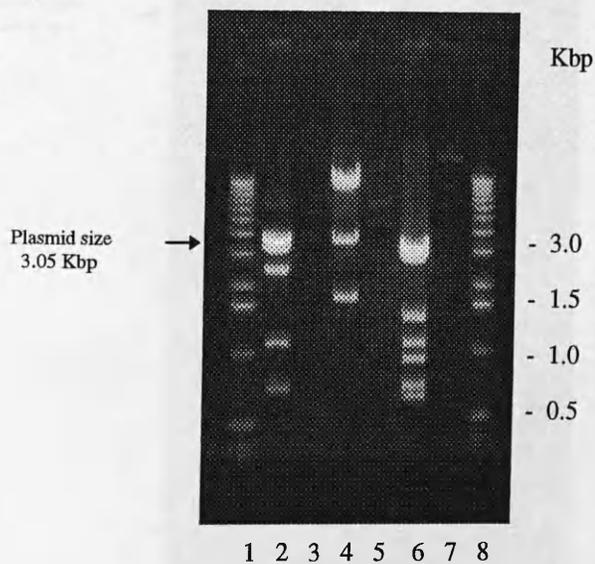


**Fig 7.7**

**Digestion of plasmid PGem 5Zf(+)** containing renibacterial DNA insert by the restriction enzymes **SacI** and **SphI**.

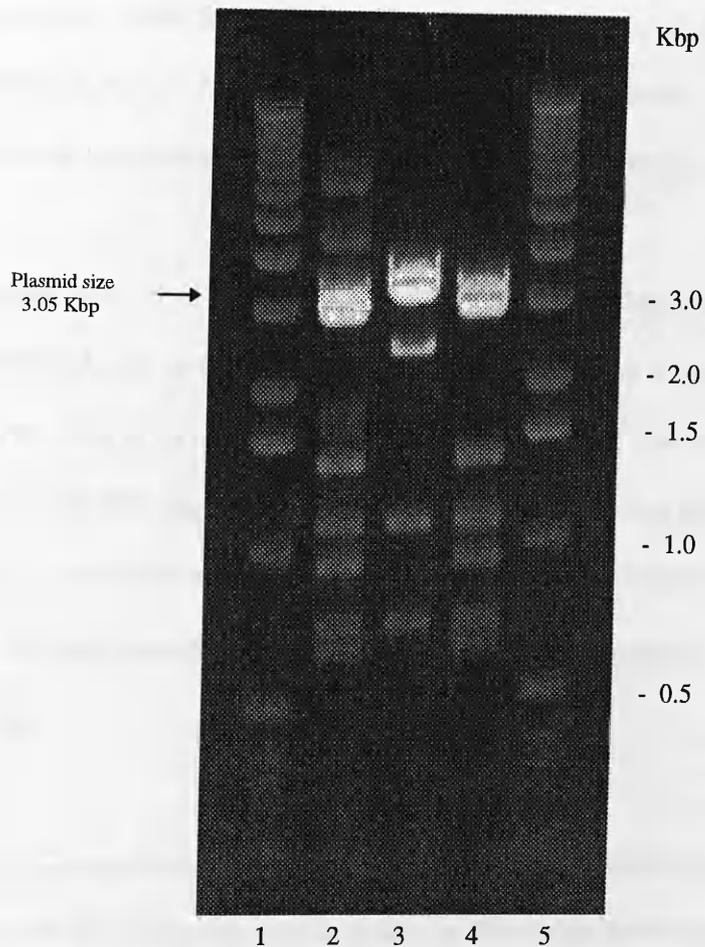
**1 Kbp DNA marker (line 1)**  
**Clone 12 (line 3)**  
**Clone 10 (line 5)**  
**Clone 8 (line 7)**  
**Clone 6 (line 9)**  
**Clone 4 (line 11)**  
**Clone 2 (line 13)**  
**Negative control 2 (line 15)**

**Negative control (line 2)**  
**Clone 11 (line 4)**  
**Clone 9 (line 6)**  
**Clone 7 (line 8)**  
**Clone 5 (line 10)**  
**Clone 3 (line 12)**  
**Clone 1 (line 14)**



**Figure 7.8** Double digestion of plasmid PGem 5Zf(+) containing DNA fragment coding for Renibacterial cell surface antigens (agarose gel 1%, 10 x 15 cm).

1) 1 Kbp DNA marker. 2) clone 12 + Sac I. 3) clone (-) + Sac I. 4) clone 12 + Sph I.  
 5) clone (-) + Sph I 6) clone 12 + Sac I + Sph I. 7) clone (-) + Sac I + Sph I. 8) 1 Kbp  
 DNA marker.



**Figure 7.9** Double digestion of plasmid PGCS by Sac I, Sph I and Sac I plus Sph I using a 20x25 cm agarose gel (0.8 %).

**Lines 1-5:** 1 Kbp DNA marker.

**Line 2:** Clone 12 + Sac I.

**Line 3:** Clone 12 + Sph I.

**Line 4 :** Clone 12 + Sac I + Sph I

### 7.3.2 Expression of renibacterial gene encoding for 57 Kda protein in *E. coli*

The p57 gene was successfully amplified from genomic *R. salmoninarum* DNA in all the samples tested. After purification, the PCR products were visualised using agarose gel electrophoresis (1%). The results are presented in Figure 7.10. A unique fragment of approximately 1.6 Kbp was obtained in all the samples which corresponded to the expected size for this gene (1,648 bp).

The concentration of p57 gene and plasmid pBTrp2 was determined by computerized scanning spectrophotometer set up to a wavelength between 200 to 360 nm (Figure 7.11) Samples were diluted 1/25 (4 ml DNA in 96 ml pure grade water) and their OD at 260 and 280 nm was measured. The concentration of DNA was calculated assuming that 1 ml of a solution with an OD<sub>260</sub> of 1.0 is equivalent to 50 µg. No contamination was detected in either samples as the 260:280 nm absorbance ratio was more than 1.9. The total concentration of p57 gene and pBTrp2 was estimated to be 62 ng µl<sup>-1</sup> and 1.7 µg µl<sup>-1</sup> respectively .

Both, the p57 gene and the pBTrp2 plasmid were submitted to double digestion by Sal I and Nco I in order to produced cohesive ends suitable for ligation. The double digestion protocol is described in section 7.2.2.3. Aliquots (1µl) of the digested DNA were submitted to agarose gel electrophoresis (1%). The results are presented in Figure 7.12.

The digestion of PCR products produced a fragment of 1.6 Kbp and theoretically two other smaller fragments of 8-9 bp which are too small to be visualised. The digestion of pBTrp/2 plasmid should

produce two distinctive fragments of 1.1 and 4.1 Kbp respectively. The 4.1 Kbp fragment should be purified and used for ligation. However, in the present experiment, no fragments were obtained.

After incubation overnight at 37 °C, a mean of 105 and 640 cfu/100 µl of transformed bacteria suspension was obtained in groups A and B, respectively, while the negative control group (group C) showed 38 cfu/100 µl (see Figure 7.13).

Screening PCR was intended to identify the clones containing the p57 gene. Eight clones from group A, 7 from group B and one from group C were selected and screened following the protocol described in section 7.2.2.6. The PCR products were then submitted to agarose gel electrophoresis (1 %). Genomic DNA from *R. salmoninarum* was used as positive control. The results showed no amplification in any of the samples tested, even in the positive control. Because of the poor results obtained by screening PCR, a more sensitive method of detection was employed by using colony hybridization (section 7.2.2.7). Using this technique, a range from 48 to 125 positive clones plate<sup>-1</sup> were detected. The results are shown in Figure 7.14

The plasmid ( pBT57) from ligation group B ( clones 2 and 6) was extracted and submitted to triple digestion by Sal I , Nco I and Pst I as described in section 7.2.2.8. The resulting fragments were submitted to agarose gel electrophoresis (1%). The results are shown in Figure 7.15. The digestion of pBT57 by Sal I + Nco I produced two fragments of approximately 4.6 and 1.6 Kbp, while the digestion by Sal + Pst I showed also two fragments of 2.9 and 3.1 Kbp respectively. A diagram showing the DNA restriction analysis of pBT57 is presented in Figure 7.16.

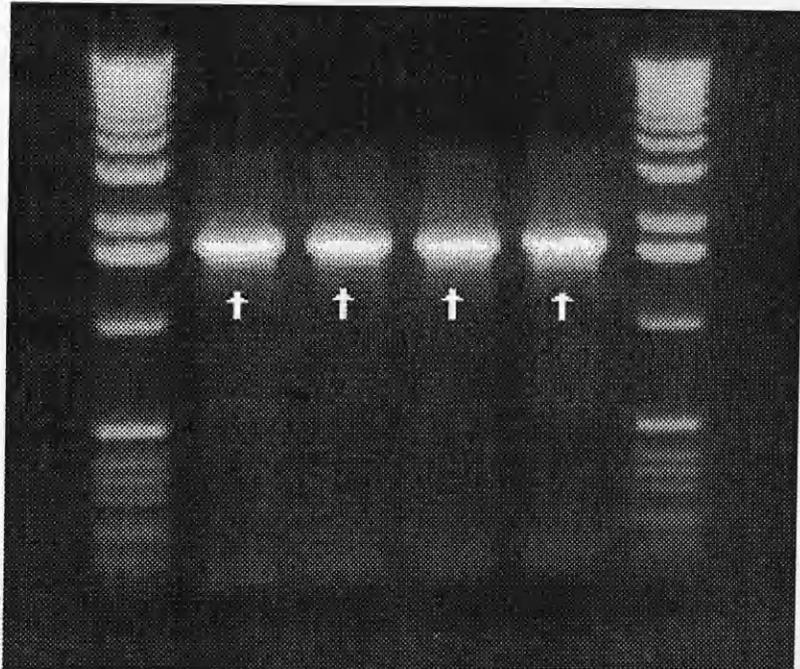
Positive clones containing the plasmid pBT57 were selected and the gene encoding for the renibacterial 57 KDa protein was expressed by activating the tryptophan promoter as described in section 7.2.2.9. The OD<sub>600</sub> was measured in all samples and are shown in Table 7.3. Aliquots (1 ml) from each culture

media was collected, the cells were pelleted and then resuspended in LB broth to  $OD_{600} = 1.0$ . An aliquot (10  $\mu$ l) of this suspension was submitted to SDS-PAGE and Western blot analysis. Rabbit polyclonal antibody against induced ECP (S7.1) was used to identify p57 antigen. The results are presented in Figures 7.18 and 7.19.

No clear differences were observed between the different groups by SDS-PAGE analysis. However, by Western blot a clear band of approximately 57 Kda was found, plus a number of other bands corresponding to degradation products of the expressed protein (Figure 7.18). No bands were found in the negative samples (clone with no plasmid).

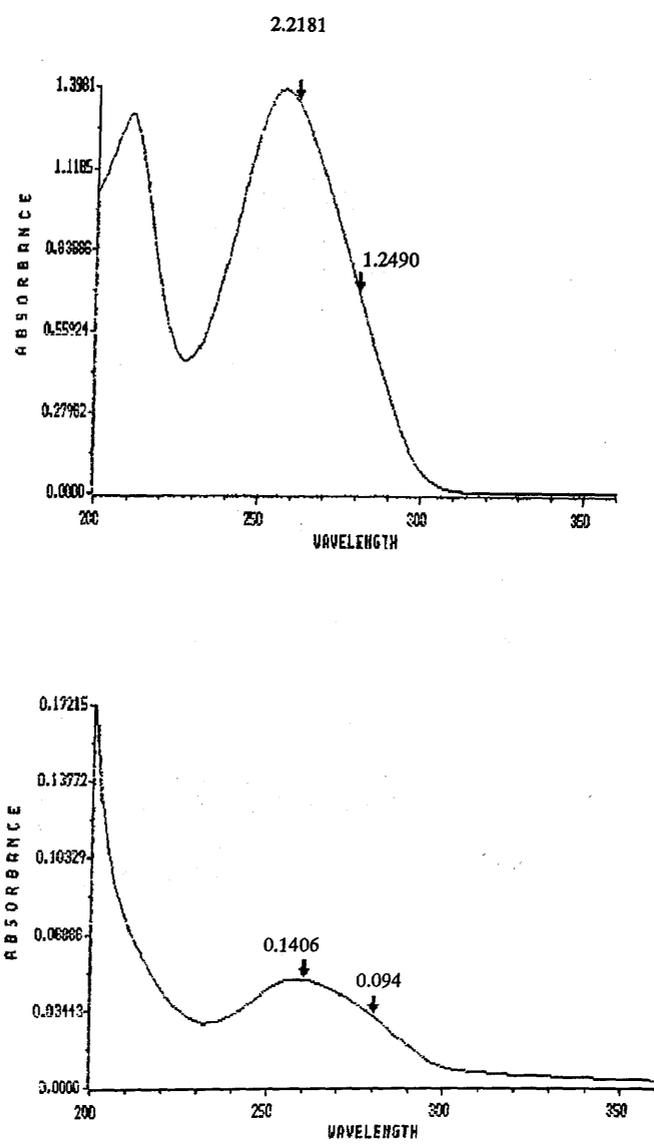


Figure 7.18: Western blot analysis of ECP (S7.1) expression. The blot shows a clear band at approximately 57 Kda, corresponding to the expressed protein, and several smaller bands below it, representing degradation products. No bands were observed in the negative samples (clone with no plasmid).

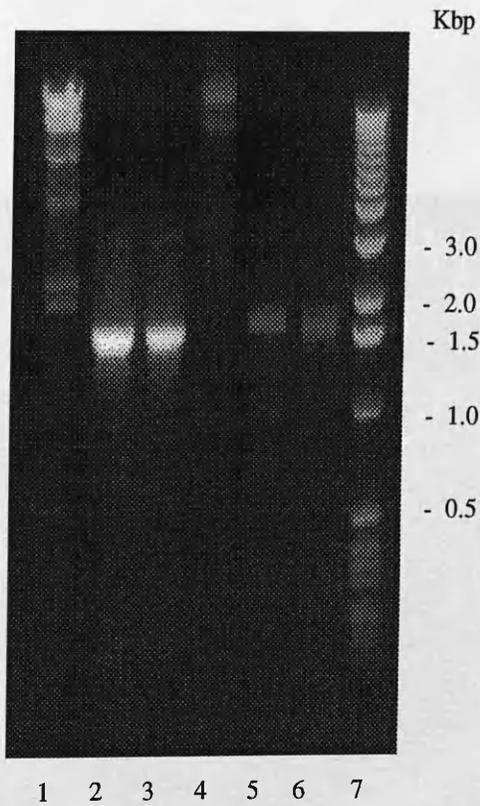


**Figure 7.10**

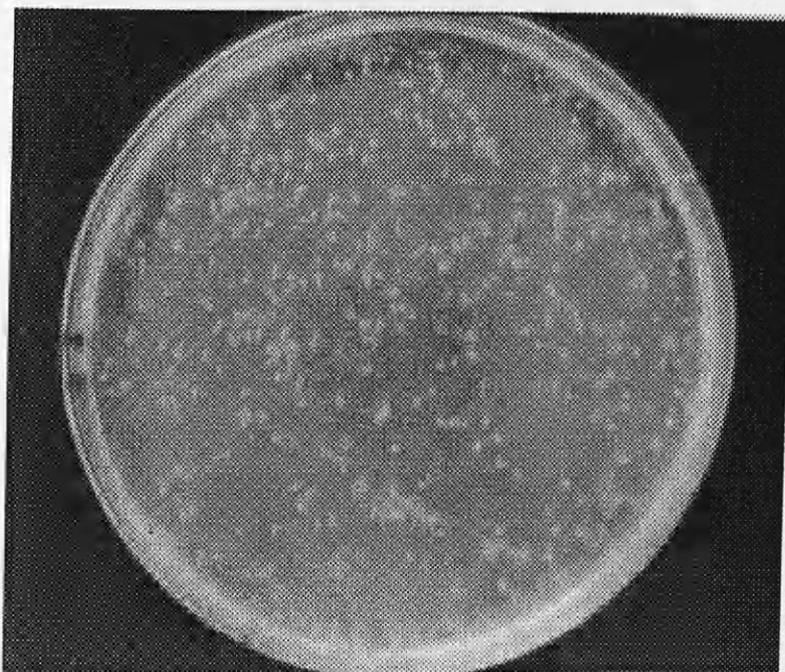
PCR amplification of the gene encoding for the 57 Kda protein from genomic renibacterial DNA. Arrows show the amplified p57 gene. Line 1 and 6 : 1 Kbp DNA marker. Lines 2-5: PCR products (samples 1-4) containing the p57 gene



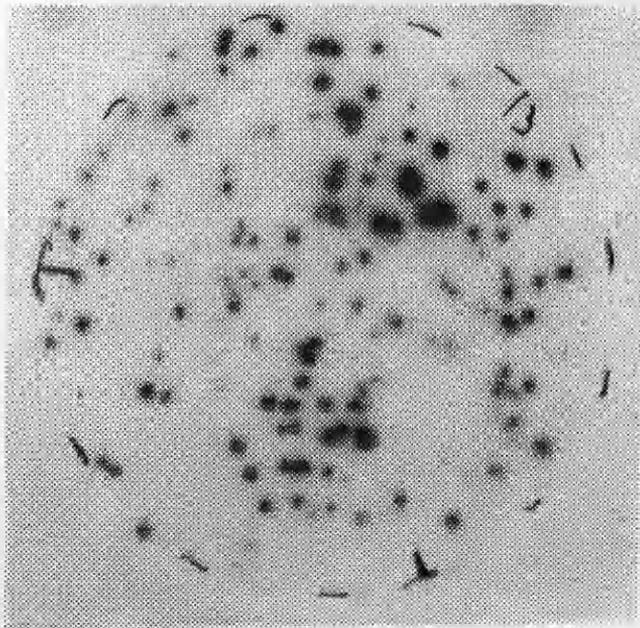
**Figure 7.11** Measurement of the DNA concentration by wavelength scanning . Arrows show the absorbance at 260 and 280 nm. A) Expression vector pBtrp/2; B) P57 gene



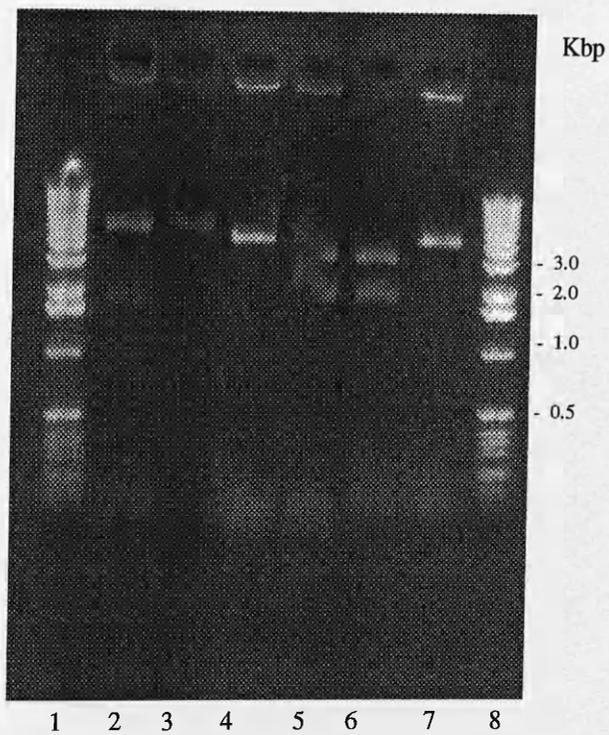
**Figure 7.12** Double digestion of PCR products (p57 gene) by Sal I and Nco I.  
Lines 1, 4 and 7: 1Kbp DNA marker; Lines 2, 3, 5 and 6: PCR products, samples 1-4 respectively.



**Figure 7.13** *E. coli* (DH10B) clones after transformation with recombinant DNA pBT57  
(14 hours after incubation at 37°C)

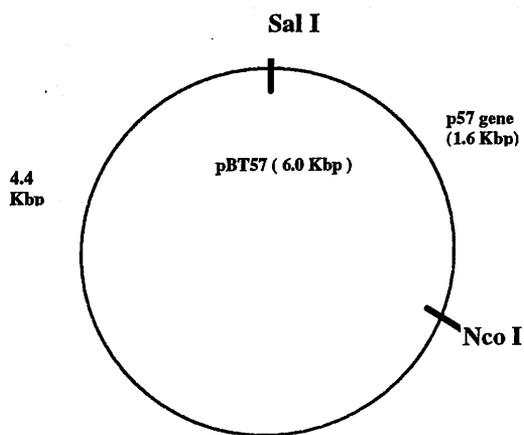


**Figure 7.14 Identification of clones containing the p57 gene by colony hybridization.**



**Figure 7.15** Triple digestion of pB57 plasmid (clones 2 and 6) by Sal I, Nco I and Pst I

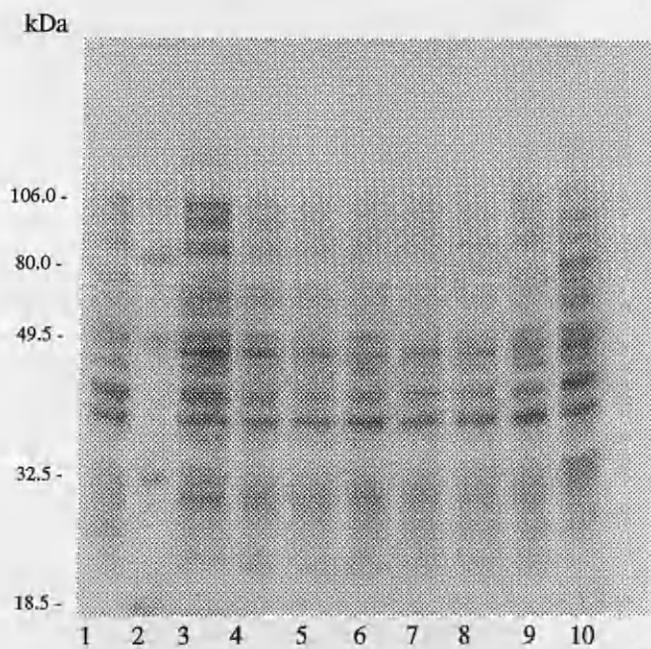
**Lines 1 and 8:** 1 Kbp DNA marker. **Line 2:** clone 2 + Sal I + Nco I. **Line 3:** clone 6 + Sal I + Nco I. **Line 4:** negative clone + Sal I + Nco I. **Line 5:** clone 2 + Sal I + Pst I. **Line 6:** clone 6 + Sal I + Pst I. **Line 7:** clone negative + Sal I + Pst I



**Figure 7.16** Diagram showing the digestion of pBT57 plasmid by Sal I and Nco I

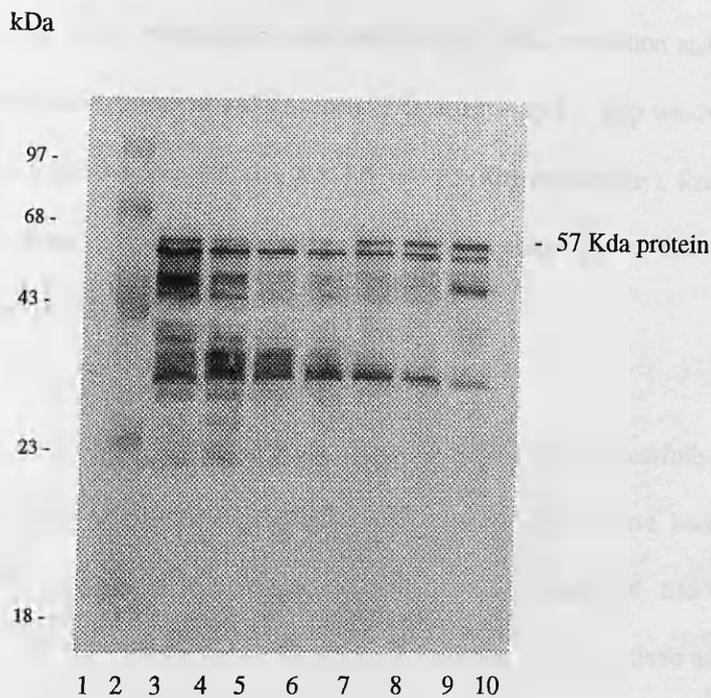
**Table 7.3 Absorbance of bacterial culture used for expression of p57 gene**

<b>OD<sub>600 nm</sub></b>	<b>Positive Clones (containing plasmid pBT57)</b>	<b>Negative Clones (No plasmid)</b>
<b>Overnight preinduction</b>	2.9	3.8
<b>T<sub>0</sub> preinduction</b>	0.7	0.8
<b>T<sub>1</sub> (1 hour after induction)</b>	1.0	1.2
<b>T<sub>2</sub> (2 hours post induction)</b>	1.6	1.8
<b>T<sub>3</sub> (3 hours post induction)</b>	1.7	1.6
<b>T<sub>4</sub> (4 hours post induction)</b>	2.5	2.5
<b>T<sub>5</sub> (5 hours post induction)</b>	2.0	2.5
<b>Overnight post induction</b>	2.2	3.4



**Figure 7.17** SDS-PAGE analysis of *E. coli* cells containing the plasmid pBT57.

- |  |                                    |
|--|------------------------------------|
| 1) Negative clone (Non induced)        | 2) Molecular weight marker.        |
| 3) Two hours after subculture, T0 .    | 4) One hour after induction T1.    |
| 5) Two hours after induction T2        | 6) Three hours after induction T3. |
| 7) Four hours after induction T4.      | 8) Five hours after induction, T5. |
| 9) Fifteen hours after induction, TON. | 10) Negative clone (induced, T0)   |



**Figure 7.18** Western blot analysis of *E. coli* cells containing the plasmid pBT57.

- |  |                                    |
|--|------------------------------------|
| 1) Negative clone (Non induced)        | 2) Molecular weight marker.        |
| 3) Two hours after subculture, T0 .    | 4) One hour after induction T1.    |
| 5) Two hours after induction T2        | 6) Three hours after induction T3. |
| 7) Four hours after induction T4.      | 8) Five hours after induction, T5. |
| 9) Fifteen hours after induction, TON. | 10) Negative clone (induced, T0)   |

## 7.4 DISCUSSION

The immunoscreening of a *R. salmoninarum* gene bank allowed the detection of bacterial clones containing DNA fragments probably coding for antigens which reacted with anti cell wall MAbs. Although these clones showed a strong background. It appears that these positive clones contained a DNA insert coding for a yet unknown cell wall antigen. The DNA restriction analysis of a selected positive clone showed that it contained a DNA insert of approximately 8.7 Kbp which could be split into six different digested fragments (0.7, 0.8, 0.9, 1.3, 1.5, and 3.5 Kbp respectively). Recently, Dheur *et al*, (1994), using the same approach, identified the gene encoding for a low molecular weight *R. salmoninarum* protein (LMWp).

The gene encoding for the 57 Kda protein from *R. salmoninarum* was successfully amplified from genomic renibacterial DNA (isolate ATCC 33209) by PCR. Similar primers have been use by Brown *et al*, (1994) to amplify a selected oligonucleotide sequence in the p57 gene of this bacterium which allowed the detection of the pathogen in infected tissue. Using this technique, these authors detected as few as two *R. salmoninarum* cells per egg. These results suggested the feasibility of PCR as a specific and sensitive diagnostic tool for this pathogen.

The blotting screening of the *R. salmoninarum* gene bank, performed in the present study, also showed positive clones to anti-induced ECP PAb (S7.1). This antisera has also been used successfully for the isolation of the gene encoding for the LMWp (Dheur *et al*, 1994), so it is possible that, at least, some of the positive clones detected may contain the LMWp gene.

A recombinant DNA molecule (termed pBT57), containing the p57 gene and the expression vector pBTrp/2, was produced and cloned into *E. coli* (DH10B). Clones containing the p57 gene were detected by colony hybridisation and DNA restriction analysis. Western blot analysis of these clones showed that p57 protein was produced even before the induction of the tryptophan promoter.

Recently, Grayson, Evenden, Gilpin and Munn (1995) reported the expression of the p57 gene in the plasmid vector vector pUC18 and subsequently a soluble fusion protein protein was produced using the pMAL expression vector system. Although the cloning and expression techniques described by these authors were different from those used in the present study, both systems showed similar results.

57 Kda protein showed degradation products with approximate MW of 54, 50, 48, 30, 25 and 20 Kda. This results are in agreement with previous reports describing the unstable nature of this protein . The study of Griffiths and Lynch (1991) demonstrated that the degradation of p57 protein increased with time and temperature. These authors suggested that degradation could be due to the presence of a serine proteinase enzyme in the ECP fraction. The study of Rockey *et al.*, (1991) demonstrated that this autologous serine proteinase cleaves p57 antigen (*even in situ*) resulting in fragments with molecular masses of 45, 36, 34, 25 and 20 Kda. P57 protein obtained from *E. coli* (XL1-blue) containing the recombinant plasmid pMC57HS (Grayson *et al.*, 1995) also showed several strongly immunoreactive bands of molecular masses from 20 to 58 kDa.

P57 is the predominant protein antigen found on the bacterial cell surface and is the major component of the extracellular protein (Getchell *et al.*, 1985; Wiens and Kaattari, 1989). It has been found in infected tissue and sera at approximate concentrations of 1 mg ml<sup>-1</sup> (Kaattari *et al.*, 1989). The same authors found a direct correlation between the amount of soluble protein (of which the 57 Kda protein is a major component) produced and significant decrease in the haematocrit of infected fish. Their studies demonstrated that soluble factors can, at concentrations normally found in infected fish, suppress the *in*

*in vitro* response to trinitrophenylated lipopolysaccharide (an antigen which is unrelated to Rs.). It was also observed that this treatment caused a decrease in the number of adherent cells *in vitro*. This observation is important because adherent cells have been found to be an essential in the generation of antibody responses in fish while also serving as a target for *R salmoninarum* cells. (Young and Chapman, 1978). Moreover, infected fish demonstrated a reduced ability to generate antibody producing cells as compared to normal fish.

Analysis of the amino acid composition (Dubreuil *et al.*, 1990) reveals that this protein is rich in glycine (18%), Asx (asparagine and aspartic acid), valine, and alanine, whereas methionine is absent. The molecule, comprising approximately 572 residues, is acidic, with Asx and Glx (glutamine and glutamic acid) representing 20.8% of the residues, and 101 % basic residues (lysine, histidine, arginine). It contains 33.4% hydrophobic residues. The isoelectric point is 4.8 under non denaturing conditions. This protein appears to require L-cysteine as a source of sulphur for synthesis (Bandin *et al.*, 1993). Listeriolysin O, from *Listeria monocytogenes* is another sulphur-dependant protein which enable the bacterium to escape from the phagosome to the cytoplasm (Portnoy *et al.*, 1992). Although the molecular weight of listeriolysin and p57 is identical, preliminary ELISA analysis using microplates coated with both purified proteins and a set of anti-renibacterium monoclonal antibodies showed no antigenic relationship (data not shown).

Wiens and Kaatari, (1989) developed eight anti-p57 MAbs classified on the basis of their differential recognition of these proteolytic breakdown products. Group I MAbs recognise p34 and p20 which have an identical amino-terminal sequence to p57. So, group I MAbs bind proximal to the amino-terminus of the protein. In addition, since agglutination activity was inhibited by these group of MAbs, the agglutinating domain may be near the amino-terminus. Group II MAbs. recognised p36 and p34 fragments. Antigenic binding sites recognised by group I and II MAbs are exposed on the surface of the bacterial cell. Group III MAbs bound proteolytic fragment p45 and two fragments p36 and p25. This group recognises an

antigenic binding site which is sterically unavailable when p57 is attached to the bacterial cell surface. The p36 and p25 fragments recognised by this group are also present in cell extracts from washed bacterial cells. These observations suggest that p36 and p25 may contain a determinant of p57 which is important for the attachment of the protein to the bacterial cell surface.

The function of p57 protein *in vivo* is unknown. However, a diverse number of *in vitro* activities have been attributed to it, including haemagglutination of rabbit, pigeon, horse, and rat erythrocytes (Daly and Stevenson, 1987, 1990), agglutination of salmonid spermatozoa (Daly and Stevenson, 1989), restoration of cell surface hydrophobicity (Daly and Stevenson, 1990), immunosuppression of salmonid lymphocytes (Turaga *et al.*, 1987) and agglutination of salmonid leukocytes (Wiens and Kaattari, 1991). P57 possesses characteristics resembling a number of proteinaceous adhesins. These include acidic pI, haemagglutinating activity, contribution to bacterial cell surface hydrophobicity and subsequently to the attachment of the bacterium to the host cells (Wiens and Kaattari 1991; Daly and Stevenson 1987, 1990).

The availability of the cloned p57 gene, and therefore the antigenic p57 protein, in a host more easy to culture than *R. salmoninarum* will allowed further biological, molecular, biochemical and immunological studies on this major soluble antigen aimed at better understand its role in the pathogenesis and immunity.

## CHAPTER 8

### GENERAL DISCUSSION

Bacterial kidney disease (BKD) represents a major problem in the farming of salmonid fish world-wide. The facts that there is as yet no effective vaccine, antibiotics are often ineffective, and the bacterium is transmitted vertically within the salmonid egg, make BKD one of the most difficult bacterial fish diseases to control. A combination of approaches, including microbiology, immunology and molecular biology, was employed in the present study to investigate the causative agent of BKD.

A library of *R. salmoninarum* isolates (18) were obtained from four different countries and analysed enzymatically, electrophoretically and immunologically using API ZYM, MLEE and antibody probes (both polyclonal and monoclonal), respectively. The effect of four different culture media on the growth, enzymatic and antigenic characteristics of the bacterium was also evaluated. Efforts were also made in the identification and purification of renibacterial antigens using chemical extraction, immune affinity chromatography and genetic engineering. The immunogenic and protective nature of some of these antigens was determined by immunising rainbow trout and measuring the antibody levels in the sera or by injecting antibodies into fish followed by challenge with live bacteria (passive immunisation).

The results from the API ZYM and MLEE analysis showed complete homogeneity in all the isolates tested. Based on these findings it is possible that, at least, all the *R.s* isolates tested are genetically closely related and that the distribution of this pathogen may have a clonal nature. Furthermore, these results support the antigenic and biochemical homogeneity of the *R. salmoninarum* isolates reported by Bullock *et al.*, (1974); Paterson *et al.*, (1981); Getchel *et al.*, (1985); Bruno and Munro, (1986); Austin and Austin, (1987) and Hsu *et al.*, (1991).

API ZYM and MLEE showed limited value in differentiating between *R. salmoninarum* isolates. The usefulness of these techniques could be improved by analysing a larger sample collection which should represent geographic and time spread of the bacteria in a statistically valid way, and also by detecting a wider range of enzymes. It seems probable, however, that immunological and molecular biology techniques such as the use of monoclonal antibodies, RAPDs and ribotyping analysis will provide a better discrimination between isolates.

The immunological analysis using PAb failed to differentiate between *R.s* isolates and showed cross-reaction with other closely related bacteria, particularly *Arthrobacter sp* and *Micrococcus luteus*. MAb, however, allowed the detection of a *R.s* strain which appears to lack p57 antigen (B88151), and to detect differences in the expression of antigens depending on the culture media used. Anti-PG MAb (12B7, and 11D11) showed no cross-reaction with any of the 31 bacterial species tested, except a very low level (< 14 %) observed with *M. luteus*. These MAbs provide useful diagnosis tools as they allow the identification of the bacterium itself rather than soluble antigens as occurs using MAb anti-ECP proteins such as anti-p57 MAb. The specificity of anti-PG MAb could be due to the nature of the peptide bridge of the renibacterial peptidoglycan which has been reported to be unique among the Gram positive bacteria (Kusser and Fiedler, 1983).

Highly specific antisera against the interpeptide bridges of PG were also raised by Seidl and (1978) and allowed to differentiate antigenically between *Staphylococcus* and *Micrococcus*. The same authors have concluded that the difference of even one amino acid (alanine or glycine) in the interpeptide bridge could be serologically detected.

Since PG reveals at least five main antigenic determinants there is a great heterogeneity of antibodies to PG. Antibodies of the various immunoglobulins classes and to the various antigenic determinants of PG may occur simultaneously and independently in the same antisera (Heymer *et al.*, 1985). Specific

antibodies to PG of the immunoglobulin IgG class (Wilhem *et al.*, 1982); of the IgA class (Franken *et al.*, 1984), of the IgM class (Verbrugh *et al.*, 1981; Wilhem *et al.*, 1982) and of the IgE class (Schopfer *et al.*, 1980) have been reported. In the present study, three out of four MAb against PG were of the Class IgM and only one belonged to the class IgG1.

The fact that no significant antigenic variations were observed when 18 renibacterial isolates were tested using eight monoclonal and four polyclonal antibodies are in agreement with previous reports of Bullock *et al.*, (1974); Paterson *et al.*, (1981); Getchell *et al.*, (1985); Wiens and Kaattari, (1989); Hsu *et al.*, (1991) and Austin and Austin, (1993) demonstrating the antigenic homogeneity of the pathogen. This may be of practical benefit in the preparation of a vaccine, in that one preparation may be able to cross-protect fish infected by other isolates. Renibacterial isolate B88151, however, showed a notorious loss of ECP antigens, especially p57. This finding is in agreement with the reports of Bruno, (1988) who described the presence of avirulent isolates of R.s which lack p57. The same author also reported that old cultures tend to lose ECP antigens and become avirulent.

The production of an effective *R. salmoninarum* vaccine requires a greater understanding of the pathogenesis of BKB. This appears to depend on the ability of *R. salmoninarum* to invade, survive and multiply within macrophage cells. Unfortunately, little is known about the virulence or pathogenicity of the bacterium. Enhancing the *in vitro* growth conditions of R.s using fermentors and attempting to mimic pathogen's natural environment may lead to the expression of important antigens which have been previously latent. For example, it is well recognised that iron-restriction *in vivo* affects the expression of classical virulence determinants such as bacterial toxins.

Recently, two genes encoding for *R. salmoninarum* iron dependant haemolysin have been cloned (Evdenden *et al.*, 1990; Grayson *et al.*, 1995). Another iron-dependant gene encoding for a low molecular weight protein has also been cloned and expressed at Pharos S.A. Belgium. Its biological properties are currently

being studied (Thiry, personal communication). All these findings revealed that the expression of genes, antigens, virulence factors and enzymes of *R.s* appears to vary with culture conditions. In addition, many bacterial pathogens, including *R. salmoninarum* attenuate rapidly on passage *in vitro* with the subsequent loss or inhibition in the production of important antigens or virulence factors. Therefore, the use of fresh isolates in antigenic and virulence studies is essential. Moreover, new cultures techniques, especially those that mimic the natural environment of the pathogen are required. Such studies may include bacterial growth inside macrophages cell lines or implantation of live bacteria contained within a dialysis bag, into the abdominal cavity of fish.

Other possible pathogenic mechanisms of BKD involve the formation of immune-complexes, peptidoglycans, and attachment to host's cells. Immune complexes have been detected by Kaattari *et al.*, (1989). One of their main biological properties is activation of complement with the subsequent increase in macrophage chemotactic factor, opsonisation of free bacterial cells and phagocytosis. A far more serious consequence of the immune complex generation is the possibility of a type III reaction which has been suggested to occur in chronic cases of BKD (Young and Chapman, 1978, Bruno, 1986, Raverty, 1992). Peptidoglycan appears to stimulate the release of numerous macrophage products including endogenous pyrogen (Oken *et al.*, 1979), interleukin 1 and prostaglandins (Gold and Mishell, 1981) which are important mediators of inflammation (Lewis, 1975; Morley, 1981). Based on these properties, it is not surprising that the injection of PG from various bacterial species into animals induced an intense inflammatory reaction (Otha, 1981; Fox *et al.*, 1982). In Atlantic and coho salmon, for example, the inoculation of purified renibacterial PG resulted in the formation of mild and multifocal pyogranulomata in the renal interstitium (Raverty, 1992). Activation of macrophages also plays an important role in the immunoadjuvanticity of PG, especially in the process called 'presentation' where macrophage process and present the antigen to the B cells for the production of antibodies.

The process of attachment of *R. salmoninarum* to the host cells is not clearly understood. However, there is information suggesting that the hydrophobic and hemagglutinating cell surface properties of the bacterium may be important (Bruno, 1988). In addition, the main extracellular and cell surface protein of this pathogen (p57) possesses certain characteristics resembling the adhesin of other bacterial pathogens (Daly and Stevenson, 1987). The importance of p57 in attachment of R.s to host cells is supported by the MAb study of Wiens and Kaattari, (1989). They showed that anti-p57 MAbs, specific to the supposedly cell surface exposed amino terminus of the protein, inhibited either or both of the hemagglutinating or leukagglutinating activities *in vitro*.

The work of Rose and Levine (1992) has shown that *R. salmoninarum* is able to bind to the C3b component of the complement system *in vitro*. This binding, followed by ligation to salmonid phagocytes bearing C3b receptors, could provide a possible mechanism for entry of this pathogen to the cells. Binding to C3b receptor may also be beneficial to an invading pathogen as entry to the phagocytic cells via this route does not result in the release and consequently exposure of the bacterium to hostile superoxide, hydrogen peroxide and arachidonic acid metabolites (Kaufmann, 1993).

Once inside the macrophage cell, R.s appears to survive and even multiply. Phagocytic cells possess a highly hostile environment to microorganisms and structural damage and function impairment results from the exposure to microbiocidal mechanisms such as reactive oxygen intermediates, reactive nitrogen intermediates, acid hydrolases, and cationic proteins, as well as conditions of low iron and pH. Intracellular micro-organisms can avoid death within the phagocytes by producing resistant cell surface molecules such as lipids and glycolipids, prevention of phagolysosomal fusion, inhibition of vacuolar acidification, and the inhibition of the release of reactive oxygen intermediates (Finlay and Falkow, 1989). The existence of such avoidance mechanisms has so far not been demonstrated in *R. salmoninarum* apart from apparent resistance to reactive oxygen intermediates (Kaattari *et al*, 1987; Bandin *et al.*, 1993).

Electron microscopic analysis of naturally infected rainbow trout kidney tissue performed in this study revealed *R. salmoninarum* cells inside phagosomes and also in the cytoplasm. The same finding has been recently observed by Gutenberger, (1993). These results suggest that *R. salmoninarum* possesses an active membrane damaging factor which allows the bacterium to escape from the phagolysosome to lie free in the cytoplasm. A similar membrane damaging factor has been identified in *Listeria monocytogenes*, another gram positive intracellular pathogen. This sulphur-dependant protein termed listeriolysin O is responsible for the escape of this pathogen from the phagosome (Portnoy *et al.*, 1992). It has been reported that p57 and listeriolysin O possess the same molecular mass and apparently the same sulphur dependant nature (Bandin *et al.*, 1992). Thus, future experiments to determine the relationship between these two proteins should be pursued.

The presence of high amounts of polysaccharide in the renibacterial cell wall could play an important role in the resistance to lysosomal enzymes. Other avoidance mechanisms such as catalase and iron reductase activity and phospholipase have also been suggested (Bruno and Munro, 1986c; Evenden *et al.*, 1993).

The studies of Gutenberger (1993) also showed that the bacterium does replicate slowly within the macrophage, but that there was concurrent killing by the cell. In addition, the studies of Bandin *et al.*, (1993) showed that both virulent and avirulent isolates of *R. salmoninarum* survived contact with trout macrophages for 3-4 days but declined thereafter.

Mycobacterium is another group of intracellular pathogens which shares many characteristics with *R. salmoninarum*. Two cell wall carbohydrates polymers (arabinomannan and lipoarabinomannan) have been demonstrated to induce a strong immunogenicity in these bacteria (Hunter *et al.*, 1986). The carbohydrates are known to suppress T- cell proliferation (Kaplan *et al.*, 1987; Moreno *et al.*, 1988; Molloy *et al.*, 1990), to inhibit the  $\gamma$ -interferon-mediated activation of macrophages (Sibley

*et al.*, 1988, 1990), to inhibit the synthesis of mRNA encoding IL-2 and IL-5 in human T- cells (Chujor *et al.*, 1992) and to enhance the production of tumour necrosis factor (TNF) by mononuclear cells (Moreno *et al.*, 1989). These combined results suggested that these carbohydrate compounds may have broad immunosuppressive effects in mycobacteriosis and contribute to the disease state (Levis *et al.*, 1987; Moreno *et al.*, 1988, 1989). It seems likely that renibacterial lipooligosaccharide (lipooligomanann) described by Kusser, (personal communication) could have some of these properties. This may explain, at least in part, some of the characteristic pathogenesis and tissue lesions of BKD.

In this study monoclonal antibodies have been used as tools towards the development of a recombinant vaccine by employing them in passive immunisation experiments to determine potential protective antigens and also as probes to “pull out” potential antigens expressed by a *R. salmoninarum* gene library. These studies have investigated the role of humoral immunity, however, it should be noted that induction of cell mediated immunity (CMI) may be even more important. The CMI responses including T lymphocytes and activated macrophages, are important in host resistance to intracellular bacteria. Development of the cell mediated immune response to facultative intracellular organisms involves the co-operative action of T lymphocytes as specific inducers and macrophages as non-specific effector cells. T lymphocytes recruit and assemble mononuclear phagocytes and release cytokines that activates macrophages for enhanced bactericidal activity (Denis 1990). Cytotoxic T cells may kill inactivated macrophages containing the bacterium for subsequent phagocytosis (Orne *et al.* 1992).

The results reported here from immunisation of rainbow trout with different antigens or bacterial preparations showed high levels of anti-cell wall antibodies were raised by fish. However when the fish sera were tested using purified peptidoglycan, only low levels of immune response were detected. This

could suggest that the chemical extraction destroyed important antigens in the peptidoglycan molecule. In addition, the results from chapter 5 suggested that purified polysaccharides are not immunogenic enough to induce an immune response in mice but once linked with peptidoglycan could become potent immunogen.

Fish naturally infected with *R. salmoninarum* showed a low level immune response to soluble antigens (normal ECP, induced ECP and LMWp) compared with that obtained using rabbit antisera. The studies of Hastings and Ellis, (1988) indicate that fish may be much more restricted in their ability to produce antibodies with a wide variety of specificities compared with mammals. Moreover, there may be intrinsic differences between Pacific salmon, Atlantic salmon and rainbow trout in their capacity to develop a protective immune response to the pathogen as suggested in the reports of Paterson *et al.*, (1981), Evelyn, (1984) and McCarthy *et al.*, (1984). Among the fish species examined to date, all appear to produce a single dominant class of immunoglobulin (Ig), a tetrameric molecule similar to IgM of mammals (Lamers, 1985). Though fish respond to immunisation by the production of specific antibodies, they do not have as broad an ability to respond to certain types of antigens as do mammals (Lamer, 1985; Stolen *et al.*, 1986).

Although none of the polyclonal or monoclonal antibodies used in the passive immunisation experiment showed any evidence of protection against experimental BKD infection, there was a delay in the onset of mortality in some of the groups. The same results were obtained by Kaattari *et al.* (1988), who challenged coho salmon with a mixture of live *R. salmoninarum* cells and high titre antiserum to homologous *R. salmoninarum* soluble proteins. They found that fish induced a specific but not protective immune response to ECP proteins, although fish took 25 % longer to die compared with control fish.

The poor results obtained in general in the vaccination of fish against *R. salmoninarum* may be due to the use of inadequate immunising preparations perhaps because they contain the wrong antigen, insufficient

of those necessary to confer protection, or perhaps the important antigen is too weakly immunogenic or response to them suppressed by other antigens (Munro and Bruno, 1988). ECP antigens, especially p57 have been associated with “agressins” which are basically toxins which act to destroy or impair the host immune system. The elaboration of such molecules by *R. salmoninarum* would not only limit the efficacy of any BKD vaccine based in ECP products or whole bacterial preparations, but would also result in the increased susceptibility to any infected fish to resist infection by other pathogens (Kaattari *et al.*,1989).

The extraction of important antigens, in relatively large quantities requires the improvement of the current culture system in order to imitate the natural environment of the pathogen. The combination of genetic engineering with immunological techniques is an invaluable approach to identify genes encoding for antigens with potential for vaccine formulation . This approach has been extensively used with other pathogens difficult to culture such as *Treponema pallidum* (Van Emden *et al.*, 1983), *Mycobacterium leprae* ( Ivany *et al.*, 1983), *M. tuberculosis* (Young *et al.*, 1985), *Plasmodium knowlesi* (Godson *et al.*,1983).

Recombinant DNA technology (or genetic engineering) was used in this study to try to identify the genes encoding for novel renibacterial antigens. The immunoscreening of the *R. salmoninarum* gene bank suggested the presence of a gene encoding for an unknown cell wall antigen, however due to the limited period of time available, no further characterisation was carried out. The cloning and expression of the gene encoding for p57 antigen is an indication of the value of this approach. The p57 gene has recently been expressed in another expression system (pMal vector) by Grayson *et al*, (1995) which supports the present results.

The poor results obtained in the vaccination of fish against *R. salmoninarum* by numerous researchers suggest that attempts at prophylaxis should centre upon the elicitation of cellular immunity rather than humoral immunity (Kaattari *et al*, 1989). Nevertheless, the promising results obtained by passive

immunisation with fish serum should be pursued. Most important among the cell mediated responses are those which give rise to enhanced phagocytic activity since the function of macrophage is not only essential in the phagocytosis of bacterial pathogens, but also in the processing and presentation of the antigen to the other cells of the immune system.

Future research should consider the enhancement of the cellular-mediated and the non-specific immune responses. Studies to improve the *in vivo* and *in vitro* growth of the bacterium using macrophages cell line, fermentors and implantation experiments should also be pursued. The investigation of the ecological relationship of *R.s* with other genetically and antigenically closely related bacteria such as *Arthrobacter* and *Micrococcus* is also an important avenue for research. In addition, the integration of immunological and molecular biology techniques is required to identify potential vaccine candidates, virulence factors and to understand the complex mechanisms of entry and survival of *R. salmoninarum* in phagocytic cells.

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# APPENDIX I

## STANDARD SOLUTIONS AND BUFFERS

### 1- Phosphate buffered saline ( PBS )

Disodium hydrogen orthophosphate-2-hydrate (2.56 g /l., BDH ), Sodium dihydrogen orthophosphate (0.876 g / l , BDH), Sodium chloride ( 8.77 g / l , Sigma ) and were dissolved in distilled water and pH was adjusted to 7.2 with 6 M Sodium hydroxide and autoclaved at 121 °C for 15 minutes.

### 2- Gram staining

#### 2.1. Crystal violet solution

Crystal Violet (2g, C.I.No.42555) was dissolved in 95% ethanol ( 20 ml ) and mixed with ammonium oxalate (0.5g) dissolved in distilled water (80 ml). The solution was allowed to stand 24 hr before filtration ( Whatman ).

#### 2.2. Iodine solution

Potassium iodine ( 2g ) was dissolved in as small as a volume of water. After addition of iodine ( 1g ) the solution was brought to 300 ml with distilled water and allowed to stand for 25 hr before filtration.

#### 2.3. Safranin solution

Safranin ( 0.25g, C.I.No 50240 ) was dissolved in 95% ethanol (10 ml). After addition of distilled water ( 90 ml ) the solution was allowed to stand for 24 hr and followed by filtration.

#### 2.4. Acetone

Acetone solution was prepared by mixing 50 ml acetone with 950 ml ethanol.

### 3. SDS-PAGE

#### 3.1. Separating gel buffer

Separating gel buffer was prepared using 1.5 M Tris ( 91 g / 500 ml, Sigma ) 0.4% SDS (2 g / 500 ml, Sigma ) and adjusting pH to 8.7 with HCl. The buffer solution was stored at

4 °C before use.

### **3.2. Stacking gel buffer**

Stacking gel buffer was made-up using 0.5 M Tris ( 6.05 g / 100 ml ), 0.4 % SDS ( 0.4 g/ 100 ml ) and adjusting pH of the solution to 6.8 with HCl. The buffer was stored at 4 °C before use.

### **3.3. Reservoir buffer**

Reservoir buffer was prepared by adding 0.125 M Tris (15.1 g / l ), 0.960 M Glycine (172.0 g/l) and 0.5% SDS ( 5.09 g / l ) to distilled water at a pH of 8.3.

### **3.3. Acrylamide stock solution**

Acrylamide ( 30.0 g /100 ml, Sigma ) and bisacrylamide ( 8.0 g/100 ml, Sigma) were mixed in distilled water then filtered through filter paper (Whatman No.1). The bottle was covered with aluminium foil and stored at 4 °C before using.

### **3.4. Sample buffer**

30 ml 10 % SDS, 12.5 ml stacking gel buffer and 10 ml glycerol were mixed and the pH adjusted to 6.8 with Hcl.

### **3.5. Staining solution**

0.1% Coomassie Blue R250 ( 2.4g )was dissolved in 1000 ml distilled water. After addition of 400 ml Glacial acetic acid (BDH chemicals Ltd. ) and 1000 ml methanol (BDH) the solution was filtered ( Whatman ).

### **3.6. Destaining solution**

Acetic acid (100 ml, Glacial) and methanol (400 ml ) were mixed with distilled water (500 ml).

## **4. ELISA**

### **4.1. Carbonate-bicarbonate buffer**

Sodium carbonate (  $\text{Na}_2\text{CO}_3$ , 1.59g ) and sodium bicarbonate (  $\text{NaHCO}_3$  , 2.93 ) were dissolved in 1 l. distilled water at pH 9.6.

### **4.2. Low salt wash (10x)**

Trisma base (24.2g , Sigma ), sodium chloride (222,2g, Sigma ), Merthiolate (1g) and

Tween 20 (5 ml) were dissolved in one liter distilled water and pH was adjusted to 7.3 with concentrated HCl.

#### **4.3. High salt wash (10x)**

Trisma base ( 24.2g ) , NaCl ( 292.2g ) , merthiolate ( 1g ) and Tween 20 ( 10 ml ) were dissolved in one liter distilled water and pH was adjusted to 7.7 with concentrated HCl.

#### **4.3. Substrate buffer**

Substrate buffer was prepared by mixing citric acid ( 21g, Sigma ), sodium acetate ( 8.2 g, Sigma) and adjusting pH to 5.4 with 1M NaOH. 33.3  $\mu$ l of H<sub>2</sub>O<sub>2</sub> was mixed with 100 ml substrate buffer.

#### **4.4. Chromogen**

Chromogen was made up by adding 42 mM 3'3'5'5'- Tetra methylbenzidine dihydrochloride (TMB) to 12 acetic acid distilled water. 120  $\mu$ l of this solution was added to 12 ml substrate buffer to prepare the substrate solution.

#### **4.5. Stop solution**

Stop solution was made up of 2M H<sub>2</sub>SO<sub>4</sub> in distilled water.

### **5. Western blot**

#### **5.1. Transfer buffer**

Transfer buffer was prepared at pH 8.3 by dissolving Glycine ( 13.5g, Sigma ) and Trisma base (3.03g, Sigma ) in 200 ml methanol ( BDH ) and bringing to one liter with nanopure water

#### **5.2. Tris buffered saline ( TBS )**

20mM Tris ( 2.42 g, Sigma), 0.5 mM NaCl ( 29.4g, Sigma) were dissolved in one liter nanopure water and pH was adjusted to 7.5.

#### **5.3. Tween tris buffer saline ( T-TBS )**

0.5 ml Tween 20 ( Sigma ) was added to one liter of TBS.

#### **5.4 Substrate solution**

Substrate solution was composed of 6mg 3,3'- diaminobenzidine ( DAB ) in 10 ml TBS and 30  $\mu$ l H<sub>2</sub>O<sub>2</sub> at pH 7.6.

### **6 Histology and immunohistochemistry**

#### **6.1 10% neutral buffered formalin**

Mix (4.01 g) sodium dihydrogen phosphate (monohydrate), 6.5 g sodium hydrogen phosphate (anhydrous), formaldehyde (100 ml) and distilled water (900 ml).

### **7 Electron microscopy**

#### **7.1 Sodium cacodylate buffer (0.1 M)**

Sodium cacodylate (10.7 g) in 500 ml distilled water. allow plenty of time to for the solution to dissolve before testing the pH and adjusting it to pH 7.2 - 7.4 with HCl.

#### **7.2 Karnovsky fixative (1.3% paraformaldehyde, 1.6% glutaraldehyde, Karnovsky, 1965)**

Mix paraformaldehyde (2g) in 25 ml distilled water in a 100 ml conical flask. Heat the mixture and shake constantly whilst maintaining the temperature at 60 - 70 °C for several minutes. before the mixture is cool add a few drops of 1N Na OH and shake the flask well to dissolve the precipitate. Leave the flask to cool before adding 0.025g calcium chloride (anhydrous) and 10 ml 25% glutaraldehyde ( or 5 ml of 50% glutaraldehyde). Make up to a total volume of 150 ml with the cacodylate buffer and re-adjust the pH to 7.2 - 7.4. Label with date and pH. store at 4 °C and discard if not used after 1 month.

#### **7.3 Rinsing solution (Sucrose 0.1 M in cacodylate buffer)**

Mix 6.84 g sucrose (MW 342) in 200 ml cacodylate buffer. sucrose is added to the cacodylate buffer solution in order to prevent osmotic shock to the tissues. Label with date and pH. store at 4 °C and discard if not used after 1 month.

#### **7.4 Osmium solution (1 %)**

Label a small brown bottle specifically kept for osmium and add a strip of hazard tape marked "Toxic" . Remove the label from one 0.25g ampoule of Osmium tetroxide. wrap the ampoule in the black paper and break the ampoule. Place both halves of the ampoule, together with any small crystals which may have dropped out onto the paper, into the bottle. Immediately add 25 ml cacodylate buffer, stopper the bottle and shake bottle carefully.

Leave to dissolve at least overnight and shake again carefully before use. do not attempt to pH this solution. This solution is toxic and must be stored in a fume cupboard. Discard if discoloured, by adding an equal volume of vegetable oil to the osmium and leaving for 24 hrs before disposing of down the sink with copious amounts of water.

#### **7.5 Uranyl acetate (watson, 1958)**

20% solution solution in absolute ethanol, stored at 4 °C.

#### **7.6 Lead citrate (Reynolds, 1963)**

Mix lead nitrate (1.33 g) and sodium citrate (1.76 g) in 15 ml distilled water, then mix together in a 50 ml volumetric flask. Stopper and shake for 1 min. Leave to stand for 30 minutes, shake at intervals, to ensure complete conversion of lead nitrate to lead citrate. Then add 8 ml of fresh 1N NaOH to dissolve the precipitate and make up to 50 ml with distilled water. store at 4°C. Centrifuge a small volume before use.

### **8 Molecular biology**

#### **8.1 Ethidium bromide solution (1000x, 0.5 mg/ml)**

50 mg ethidium bromide in 100 ml distilled water. Dilute stock 1:1000 for gels or stain solution. Working solution, 0.5 µg/ml. protect solution from light. Caution, ethidium bromide is a mutagen and must be handled carefully.

#### **8.2 SOC medium**

Mix yeast extract (0.5%), tryptone (2%), NaCl (10 mM), KCl (2.5 mM), MgCl<sub>2</sub> (10 mM), MgSO<sub>4</sub> (20mM), Glucose (20mM). Autoclave at 121 °C for 20 minutes.

#### **8.3 M9 medium**

Mix Na<sub>2</sub>HPO<sub>4</sub> x H<sub>2</sub>O (6g/l), KH<sub>2</sub>PO<sub>4</sub> (3g/l), NaCl (0.5g/l), NH<sub>4</sub>Cl (10g/l), casamino acid (5g), MgSO<sub>4</sub> 1M (1ml), CaCl<sub>2</sub> 0.5 M (0.2 ml), glucose 40 % (5 ml), thiamine B<sub>1</sub> 1mg/ml (10 ml), tryptophan 10 mg/ml (4ml).

#### **Mg<sup>(2++)</sup> stock solution**

Mix MgCl<sub>2</sub> x 6 H<sub>2</sub>O (203 g/l) with MgSO<sub>4</sub> x 7 H<sub>2</sub>O (247 g/l). Filter syterilised.

**Luria Berthini medium**

Mix Bacto tryptone (20 g/l), yeast extract (5g/l), NaCl (0.58 g/l), KCl (0.19 g/l), agar (20 g/l). Autoclave and add ampicillin (100 µg/l) filter sterilised. LB broth is made up as described previously but without agar.

**SOB medium**

Mix tryptone (20 g/l), yeast extract (5 g/l), NaCl (0.58 g/l), KCl (0.19 g/l), agar (20 g/l). Autoclave and then add Mg<sup>(2+)</sup> stock solution (10 ml/l) filter sterilised.

## APPENDIX II

### STANDARD TECHNIQUES

#### 2.1 TSA contamination test for *Renibacterium salmoninarum*

Suspected renibacterial colonies were subcultured into TSA plates and incubated up to 48 hours at 22°C. The absence of bacterial colonies in TSA after 48 h suggested the absence of contamination in the renibacterial culture.

#### 2.2 API ZYM

The biochemical characterisation was performed by using API ZYM System (La Balme les Grottes, 38390 Montalieu, France) according to Bruno and Munro, (1986). Five ml of tap water was added to the incubation tray and the API ZYM gallery was removed from the sealed container and placed in the incubation tray.

The gallery was inoculated with 100 ml well<sup>-1</sup> of freshly harvested bacterial suspension at OD<sub>610</sub> 1.0 (1x10<sup>9</sup> cells ml<sup>-1</sup>). After placing the lid on the tray, it was incubated for 28 h at 22°C. One drop of reagent ZYM A (250 g Tris, 110 ml of 37% HCl, and 100 g SDS made up to a final volume of 1 litre of distilled water) was added to each well followed by one drop of reagent ZYM B (3.5 g of Fast Blue BB per litre of 2-methoxyethanol). The colour was allowed to develop for five minutes with the gallery placed under a powerful light source for approximately 10 seconds with the bulb placed 4 inches above the gallery. This procedure eliminated any yellow colour which could have appeared in the wells due to any excess of fast blue which had not reacted. After light exposure the negative reactions became colourless. The results were read from the gallery by using the table provided.

#### 2.3 Measuring the bacterial concentration by direct count

Heat killed bacterial suspension, prepared as described in section 3.7.1, was diluted in sterile saline solution (0.09 % NaCl w/v) containing 0.01% (w/v) Thiomersal (Sigma Chemical Company) to OD<sub>610nm</sub> 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, under sterile conditions. Immediately before counting, each sample was diluted with sterile saline solution so that when the counter chamber was filled there was about 5-10 cells

per small square.

The diluted sample (25 ml) was placed in the Nebaur chamber and a clean cover glass placed on top. The bacteria was allowed to settle for five minutes and examined in phase contrast microscope (Olympus BH-2, Japan) at 40x. The bacteria were counted in squares selected randomly so that the total count was about 500. The total bacterial concentration per ml was calculated applying the following formula:

$$\text{TOTAL N}^\circ \text{ BACTERIA PER ML} = \frac{\text{COUNT} \times 25 \times 10^4 \times \text{DILUTION FACTOR}}{\text{N}^\circ \text{ SQUARES COUNTED}}$$

The counting was repeated twice and the average counts were calculated. The chamber and the cover glass were scrupulously cleaned and microscopically examined after each count.

## 2.5 Gram staining

Gram stain was performed following the method described by Friederich, 1984. The bacterial smear was made by placing the bacteria in a drop of distilled water on the surface of a clean glass slide. Using sterile wire loop and conditions some bacteria was remove from the plate of culture medium and emulsified in a drop of distilled water. the smear was allowed to air dry and was then heat fixed by passing the slide quickly through the Bunsen's flame. The slide was allowed to cool and then flooded with the ammonium oxalate-crystal violet solution for 1 minute. The excess stain was washed off with a flush of distilled water. The slide was then flooded with iodine solution and left for 1 minute. The excess stain was removed again with a flush of distilled water. The smear was then decolorized by washing with acetone for 2-3 second or until no more stain were remove and then washed again in distilled water as described previously. The smear was counterstained with safranin solution for 2 minutes and then washed in running tap water. The slide was blotted dry and examined under oil immersion at 100 x magnification on a light microscope.

Gram positive bacteria appeared blue or black in colour while Gram negative bacteria were red or pink.

## 2.6 Catalase reaction

50 ml (1 drop) of 10% hydrogen peroxide (Sigma Chemical Company) was placed in a clean glass slide and then a sample of R.s. taken from solid media was added and mixed. The presence of effervescence within 5 second indicated a positive reaction while negative reaction remained unchanged.

## **2.7 Cytochromo oxidase reaction**

The oxidase test was performed following the method of Kovacs described by Cowan and Steel, 1991. Filter paper Whatman N°1 was impregnate with 1% tetramethyl-p-phenylenediamine dihydrochloride (Sigma Chemical Company). A sample of R.s. taken from solid media by using a platinum wire was smeared across the surface of the impregnated paper. Oxidase positive organism turned the paper dark purpura in colour while in negative ones it remains clear.

## **2.8 Reconstitution of lyophilised bacteria**

The vial of lyophilised bacteria (e.g. those purchased from the NCIMB) were cracked open using a file and the powdered Rs were carefully resuspended in fresh made KDM-2 broth by using a sterile Pasteur pipette, under sterile conditions. The bacterial suspension was then inoculated onto two sterile bottles containing 100 ml of KDM-2 broth and incubated at 15°C for 15 days with agitation.

An aliquot of each isolate was transferred to KDM-2 plates which were incubated at 15°C for 15 days. Plates were then tested for contamination as described in section 2.2.3.1. Non contaminated cultures became master cultures and were stored sealed at 4°C until use.

## **2.9 Storage of viable bacterial cells**

Rs. grown on KDM-2 broth and then solid media as described in section 2.2.2.2 were transferred to sterile tubes containing beads in cryopreservative, mixed well and allowed to stand for 10 minutes at room temperature. Then the cryopreservative was completely removed and discarded. Beads were then stored at -70°C.

## **2.10 TISSUE PROCESSING FOR HISTOPATHOLOGICAL EXAMINATION**

Tissue samples were trimmed to a maximum thickness of 4 mm, using standard procedures, and processed on a Tissue Tek II Tissue Processor (Miles Laboratories, Slough, Berks), such that samples were dehydrated through graded alcohols from 70-100%, cleared with chloroform, and impregnated with molten paraffin wax. Tissue were then embedded in wax. Sections 4-5mm thick were cut on a rotary microtome (No 1512 Leitz (Instruments Ltd., Luton, Beds.), floated out in a water bath at 50°C which incorporated 0.5% albumin for adhesion, and transferred to pre-cleaned glass slides which were subsequently dried in a hot air oven at 45°C for 12-24 hours before staining.

Sections for routine histological examination were stained with haematoxylin and eosin as followed. Xylene (5 min), alcohol I (2 min), alcohols II (1.5 min), was in running tap water, haematoxylin (5 min), wash in tap water, acid alcohol (3 quick dips), wash in tap water, scott's tap water (30 sec), wash well in tap water, eosin (5 mins), quick wash in tap water, methylated spirit (3 0 sec), alcohol I (2 min), alcohol II (1.5 min), xylene I (5 min), xylene II (5 mins). Sections were coverslipped after the last xylene in the staining series.

## **2.11 TISSUE PROCESSING FOR ULTRA-STRUCTURAL EXAMINATION**

Karnovsky fixed samples were washed and left in 0.1 M cacodylate buffer at 4°C for 12 hours, and then fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer at 4°C for one hour. After two further 15 min washes in buffer, tissues were dehydrated by passing through graded alcohols (60 %, 90 %, 100 %, 90%, 100%, 100% anhydrous alcohol, 10 min each wash). After being washed in a 50/50 mixture of propylene oxide and absolute anhydrous alcohol for 10 min, then in 100% propylene oxide for 10 min, samples were infiltrated with epoxide resin (EMix, EMScope Laboratories Ltd., Ashford, Kent) of medium hardness, firstly in a 50/50 mixture of propylene oxide and resin for 30 min at 37°C, then in 100% resin for one hour at 37°C. Samples were embedded in resin in polythene capsules and polymerised in a hot air oven for 48 h at 60°C.

Resin blocks were roughly trimmed with a razor blade. Final trimming, semi- and ultra-thin section cutting were performed on a ultramicrotome (OM U3, Reichter-Jung, Slough, Berks) fitted with a glass knife made on an LKB knife maker (Bromma, Sweden). Semi-thin sections were picked up on glass slides, ultra-thin sections were floated out onto uncoated copper grids.

Semi-thin sections of 1 µm thickness were stained with alkaline toluidine blue (Bancroft and Stevens, 1977). Ultra-thin resin sections were double stained in alcoholic uranyl acetate and lead citrate solution (Raynolds, 1963). Submerge grids face up in drops of uranyl acetate on parafilm (2 mins). Rinse in stream of distilled water. Blot grids on filter paper. Float grids face down on small blob of lead citrate on parafilm (2 mins). Rinse in a stream of distilled water. Slides were routinely viewed with a Reichter-Jung Polyvar wide field photomicroscope (Reichter-Jung, Slough, Berks). Photomicrographs were taken on Kodak Ektacrome (daylight) 200 film (Eastman Kodak Co., Rochester, N.Y.).

## **2.12 Lyophilisation procedure**

Sterile glass ampoules were loaded with 400 ml of the material to be freeze dried. Ampoules were placed in the loaded carrier plate on top of the chamber spinner. The desiccant was carefully loaded in the corresponding tray. Samples were lyophilised following the direction given by the manufacturers. After 12 to 24 hrs (depending on the water content of the samples), the ampoules were vacuum sealed overnight, freeze dried and stored at 4°C until use.

NUCLEOTIDE SEQUENCE OF THE GENE ENCODING FOR THE 57 kDa PROTEIN of  
*R. salmoninarum* (Chien *et al.*, 1992)

1	Met	Lys	Ile	Lys	Lys	Ile	Leu	Ala	Leu	Thr	Ala	Val	Thr	Ala	Val	Thr	16
1	ATG	AAA	ATA	AAA	AAA	ATT	TTA	GCG	CTG	ACT	GCA	GTC	ACT	GCA	GTC	ACT	48
17	Phe	Phe	Gly	Val	Ala	Pro	Leu	Ala	Gln	Ala	Ser	Gln	Gly	Glu	Gly	Asn	32
49	TTT	TTT	GGG	GTG	GCT	CCT	CTT	GCG	CAA	GCT	TCG	CAA	GGT	GAA	GGG	AAT	96
33	Ser	Ser	Thr	Ser	Thr	Val	Gln	Gly	Phe	Ser	Ser	Val	Asn	Ile	Phe	Gln	48
97	TCT	TCC	ACT	TCA	ACA	GTA	CAA	GGC	TTC	AGC	AGT	GTT	AAC	ATT	TTT	CAA	144
49	Gln	Gly	Gly	Tyr	Ser	Ala	Phe	Phe	His	Glu	Leu	Arg	Pro	Asp	Gly	Thr	64
145	CAG	GGT	GGT	TAT	TCT	GCT	TTC	TTT	CAC	GAG	TTA	AGG	CCT	GAC	GGG	ACC	192
65	Ser	Ser	Ala	Ala	Gly	Gly	Pro	Val	Ala	Ala	Gly	Gly	Ser	Tyr	Ser	Ser	80
193	TCC	AGC	GCC	GCA	GGA	GGA	CCA	GTT	GCA	GCT	GGT	GGC	TCT	TAT	AGT	TCT	240
81	Gly	Asp	Leu	Gly	Lys	Lys	Gly	Phe	Glu	Asp	Gly	Asp	Leu	Val	Val	Pro	96
241	GGT	GAT	TTA	GGT	AAA	AAG	GGC	TTC	GAA	GAC	GGA	GAT	CTT	GTT	GTT	CCC	288
97	Val	Val	Asn	Ala	Val	Phe	Gly	Asn	Glu	Gln	Lys	Gly	Ser	Ser	Phe	Val	112
289	GTA	GTA	AAC	GCG	GTT	TTT	GGA	AAT	GAG	CAA	AAA	GGA	AGC	TCC	TTT	GTT	336
113	Tyr	Asn	Lys	Asp	Gly	Pro	Ala	Lys	Glu	Leu	Lys	Val	Trp	Gly	Thr	Thr	128
337	TAT	AAC	AAA	GAT	GGT	CCT	GCC	AAG	GAA	CTG	AAA	GTC	TGG	GGG	ACG	ACT	384
129	Leu	Asn	Val	Gly	Phe	Gly	Cys	Asn	Asp	Asn	Ala	Pro	Ser	Pro	Met	Gly	144
385	CTA	AAC	GTA	GGT	TTC	GGG	TGT	AAC	GAT	AAT	GCG	CCA	TCC	CCA	ATG	GGA	432
145	Cys	Gly	Pro	Ala	Asn	Lys	Pro	Val	Ala	Ser	Gly	Leu	Gln	Pro	Pro	Ala	160
433	TGT	GGC	CCG	GCA	AAT	AAA	CCT	GTT	GCA	TCC	GGA	CTT	CAG	CCT	CCG	GCG	480
161	Gly	Pro	Leu	Gly	Gly	Gly	Thr	Val	Val	Lys	Val	Asp	Gly	Ser	Asn	Leu	176
481	GGT	CCT	CTT	GGC	GGC	GGT	ACT	GTG	GTG	AAG	GTG	GAT	GGC	AGC	AAC	CTA	528
177	Phe	Gly	Ala	Ser	Gln	Val	Ser	Phe	Gly	Asp	Lys	Pro	Gly	Thr	Asp	Ile	192
529	TTC	GGC	GCA	TCG	CAA	GTC	TCC	TTT	GGA	GAC	AAA	CCG	GGA	ACG	GAC	ATT	576
193	Ala	Val	Ala	Gln	Asp	Gly	Asn	Ser	Leu	Thr	Val	Lys	Thr	Pro	Ala	Val	208
577	GCC	GTC	GCT	CAG	GAT	GGT	AAC	TCG	TTG	ACT	GTA	AAA	ACA	CCA	GCA	GTA	624
209	Asp	Ala	Ala	Gly	Pro	Val	Lys	Val	Thr	Val	Thr	Asn	Pro	Gly	Gly	Glu	224
625	GAT	GCC	GCT	GGC	CCG	GTA	AAG	GTT	ACA	GTC	ACT	AAC	CCC	GGT	GGG	GAG	672
225	Thr	Val	Thr	Tyr	Glu	Ser	Phe	His	Tyr	Phe	Gly	Ser	Ala	Pro	Thr	Ala	240
673	ACG	GTA	ACG	TAC	GAG	AGT	TTC	CAC	TAC	TTC	GGA	AGC	GCC	CCG	ACA	GCA	720
241	Thr	Leu	Glu	Pro	Lys	Thr	Gly	Pro	Arg	Asp	Gly	Gly	Thr	Val	Val	Lys	256
721	ACT	CTC	GAG	CCT	AAG	ACT	GGG	CCT	CGT	GAC	GGC	GGT	ACT	GTG	GTG	AAG	768