THE USE OF GENE CLONING TECHNIQUES

IN THE STUDY OF THE FISH PATHOGEN

Renibacterium salmoninarum

by

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ABSTRACT

A programme of research was undertaken to assess the feasibility of the application of gene cloning techniques to *Renibacterium salmoninarum*, an important, but as yet poorly characterized pathogen of salmonid fish. Difficulties encountered during *in vitro* culture, have restricted our knowledge of the bacterium, especially with respect to its mechanisms and molecules of pathogenicity.

A collection of *R.salmoninarum* isolates was made, and an initial study conducted to determine target molecules for cloning. All strains tested were found to have uniform protein profiles when analysed with SDS-PAGE, and uniform antigenic profiles when subject to immunoblotting experiments utilizing rabbit antisera raised against both whole cell and ECP preparations of the bacterium. The presence of haemolytic and proteolytic activity, putative pathogenicity determinants of *R.salmoninarum*, could not be established.

Genomic DNA extracted from isolates of *R.salmoninarum* was used to construct gene libraries in a variety of *E. coli K12* host vector systems. Gene libraries were screened with antisera for the presence of *R.salmoninarum* antigens, and with erythrocytes and a range of natural and synthetic substrates for the presence of haemolytic and proteolytic components.

As a result of the screening process, a stable haemolytic *E. coli* clone was detected and isolated from a pHC79/*R.salmoninarum/Sau3A* gene library. This clone contained the recombinant cosmid molecule pRHLY1 bearing a gene for RHLY, a putative *R.salmoninarum* haemolysin on a DNA insert of approximately 32 kbp.

The RHLY encoding gene, designated *rsh*, was subcloned on a 1.65 kbp DNA fragment to form the recombinant plasmid pRHLY11, for which a preliminary restriction endonuclease cleavage map was constructed. Southern blot hybridization was performed, and established that the fragment containing the *rsh* gene was a true representative of a *R.salmoninarum* genomic DNA sequence, present in all isolates studied.

Minicell analysis of the cloned *rsh* gene and immunoblot of the pRHLY-containing *E. coli* clone with antiserum to *R.salmoninarum* whole cells, revealed that haemolytic activity is mediated by a protein of an approximate size of 48-50 kDa. Haemolytic activity could not be detected in cell-free assay and RHLY was not purified, but experiments conducted with RHLY-containing *E. coli* indicated that the protein is active aginst a wide spectrum of animal erythrocytes, across a wide temperature range. The specific mode of cytolytic activity was not established. Immunoblot analysis utilizing an antiserum raised to a β-galactosidase/RHLY fusion protein, revealed that a similar protein is found in whole cell preparations of *R.salmoninarum*.

The nucleotide sequence of the *rsh* gene was determined, and was shown to contain an ORF of 1248 bp. Putative transcriptional/translational control regions were identified for the *rsh* gene, and the primary amino acid sequence of the RHLY protein determined. A computer-aided search of the PIR protein sequence database, failed to detect any protein with significant homology to RHLY, therefore, conclusive evidence for structure/function of the molecule was not obtained. However, the RHLY protein was found to have a 30-35 amino acid motif, conserved and repeated twice, that shares significant homology with a repeated motif in the sequence of *dniR* protein,the product of a gene in *E. coli K12* that regulates the expression of the enzyme nitrite reductase.

Future studies of the RHLY protein, the *rsh* gene, and other components of *R.salmoninarum* are discussed.

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This thesis is dedicated to the memory of my father
Bryan Gordon Evenden
1932-1985

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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ABBREVIATIONS

A . . . . . adenine
mA . . . . . milliampere
ATP . . . . adenosine triphosphate
dATP . . . deoxyadenosine triphosphate
ddATP . . dideoxyadenosine triphosphate
BHI . . . . brain heart infusion medium
BKD . . . . bacterial kidney disease
bp . . . . . base pairs
C . . . . . cytosine
°C . . . . . degrees centigrade
CMI . . . . cell mediated immunity
dCTP . . . deoxycytosine triphosphate
ddCTP . . . dideoxycytosine triphosphate
DMF . . . . dimethylformamide
DMSO . . . dimethylsulphoxide
DNA . . . . deoxyribonucleic acid
DNase . . . deoxyribonuclease
ECP . . . . extracellular products
EDTA . . . ethylenediaminetetraacetic acid
ELISA . . . enzyme-linked immunosorbent assay
FAT . . . . fluorescent antibody test
FCA . . . . Freund's complete adjuvant
G . . . . . guanine
g . . . . . gram
fg . . . . . femtogram
kg . . . . . kilogram
mg . . . . . milligram
pg . . . . . picogram
μg . . . . . microgram
dGTP . . . deoxyguanosine triphosphate
ddGTP . . . dideoxyguanosine triphosphate
x g . . . . . acceleration due to gravity
h . . . . . hour
i.p. . . . . intraperitoneal
IFAT . . . . indirect fluorescent antibody test
IPTG . . . . isopropyl-β,D-thiogalactopyranoside
kbp . . . . . kilobase pairs
kDa . . . . kilodaltons
KDM . . . . kidney disease medium
KDM2 . . . kidney disease medium version 2
LB . . . . . Luria-Bertani medium
ml . . . . . millilitre
μl . . . . . microlitre
cm . . . . . centimetre
mm . . . . . millimetre
μm . . . . . micrometre
M . . . . . molar
mM . . . . millimolar
μM . . . . . micromolar
MHC . . . . Mueller-Hinton cysteine media
min . . . . . minute

dNTP . . . deoxynucleoside triphosphate
ddNTP . . . dideoxynucleoside triphosphate
p57 . . . . . *R.salmoninarum* major surface antigen
PAGE . . . polyacrylamide gel electrophoresis
PBSA . . . phosphate buffered saline pH 7.2 (Dulbecco A)
PBSM . . . PBS/skim milk powder suspension
PCR . . . . polymerase chain reaction
PGS . . . . phosphate gelatin saline
PIR . . . . . Protein Identification Resource
% . . . . . percent
RHLY . . . putative *R.salmoninarum* haemolysin.
RNA . . . . ribonucleic acid
mRNA . . . messenger RNA
rRNA . . . ribosomal RNA
RNase . . . ribonuclease
SDS . . . . sodium dodecyl sulphate
sec . . . . . second
T . . . . . thymine
TBE . . . . tris borate EDTA buffer
TE . . . . . tris EDTA buffer
Tris . . . . . tris(hydroxymethyl)aminomethane
dTTP . . . . deoxythymidine triphosphate
ddTTP . . . dideoxythymidine triphosphate
U . . . . . uracil
UV . . . . . ultraviolet
v . . . . . volt
v/v . . . . . volume/volume
w/v . . . . . weight/volume
Xgal . . . . 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

CHAPTER 1: INTRODUCTION.

The Gram-positive diplobacillus *Renibacterium salmoninarum* (Sanders and Fryer, 1980), is the aetiological agent of bacterial kidney disease (BKD), a chronic, debilitating and often fatal condition affecting fish of the family *salmonidae* (salmon, trout and char). The systemic granulomatous BKD infection is known to occur in at least 13 species of salmonid fish, throughout the world, in both their freshwater and marine habitats. Although BKD is detectable in wild salmonid populations, it has attained most significance amongst those fish reared by intensive aquacultural methods for use as food or in fisheries stock enhancement. BKD is a considerable obstacle to the successful hatchery rearing of salmonids and is recognised as one of the most difficult bacterial infections to control. The disease is largely unresponsive to chemotherapeutic intervention, and there is as yet, no effective vaccine preparation (Fryer and Sanders, 1981; Austin and Austin, 1987; Elliot *et al.*, 1989).

Our knowledge of the pathogenesis of BKD is limited and even less is known about the mechanisms and molecules involved in *R. salmoninarum* pathogenicity.

It is known however, that susceptible fish can aquire the pathogen by being in close proximity to other infected individuals (horizontal transmission)(Munro and Bruno, 1988), or as a result of development from an infected egg (vertical transmission)(Evelyn *et al.*, 1984). Once a *R. salmoninarum* infection has been established, apparently initially in kidney tissue, it can rapidly disseminate throughout the host affecting many internal organs. This dissemination, and the inability of the host to overcome the spread of the infection is attributed to the ability of *R. salmoninarum* to invade, survive and multiply within professional phagocytic cells namely macrophages and polymorphonuclear leucocytes (Young and Chapman, 1978). Infected fish are seen to mount an immune response to *R. salmoninarum*, and high titres of circulating antibody to bacterial antigens can be detected (Bruno, 1987; Bruno and Munro, 1988), however these antibodies are

apparently non-protective, and may even exacerbate BKD pathology (Sakai *et al.*, 1989; Villena *et al.*, 1989).

It is therefore clear that *R. salmoninarum* is a complex intracellular pathogen, and future success in the prevention and control of BKD in the salmonid population lies in the aquisition of a greater knowledge of the disease process in general and the molecular basis of *R. salmoninarum* pathogenicity in particular.

Detailed bacteriological studies of *R. salmoninarum* particularly the isolation and characterization of its cellular components have always been hampered by the difficulties encountered in the *in vitro* cultivation of the organism (Austin and Austin, 1987). Initial isolation of the bacterium from infected fish, it is now known, may require up to 20 weeks incubation on solid culture medium (Benediktsdottir *et al.*, 1991), and even established laboratory cultures of *R. salmoninarum* are extremely slow-growing. As a result, cultures are often contaminated by the overgrowth of faster growing heterotrophs such as fungi and other species of bacteria (Bruno, 1984). Plus, the long incubation times required are likely to adversely affect labile cellular components produced. It is therefore clear, that for studies of *R. salmoninarum* to progress, a reliable and productive source of macromolecules for further analyses is required.

New impetus for studies of the molecular basis of bacterial pathogenicity has been provided over the past decade by the development of gene cloning techniques and their application to this field of biological research (Dougan, 1989; Finlay, 1992). Not only have these modern molecular biology methods been applied with great success to the investigation of well-characterized bacterial pathogens such as *Staphylococcus aureus* (Foster *et al.*, 1990) and *Vibrio cholerae* (Miller and Mekalanos, 1988), they have given a long-awaited new direction to studies of important bacterial species such as *Mycobacterium leprae* (Young and Cole, 1993) for which laboratory cultivation is either problematic or near impossible. This new direction results from the ability to isolate a

bacterial gene sequence and transfer it to an alternative host organism such as *Escherichia coli* where it can be made to constitutively express its encoded macromolecule(s). In this way it has become possible to produce relatively large amounts of bacterial proteins for use in further analyses.

It is this invaluable aspect of gene cloning technology that provides the stimulus for this research project. The aims of which are:

1. To utilize DNA from *R.salmoninarum* to construct a number of gene libraries in a number of *E. coli* host/vector systems.

2. To screen these gene libraries for recombinant viral and bacterial clones producing antigens, enzymes or other components from *R.salmoninarum*

3. To isolate and characterize any cloned *R.salmoninarum* molecules and their encoding gene sequences, both to assess their role in the pathogenicity of the bacterium and to provide impetus for future studies.

In attempting these aims, it is hoped that, above all, the feasibility of the application of cloning techniques to future studies of *R.salmoninarum* will be established.

CHAPTER 2 : LITERATURE REVIEW .

2.0 BACTERIAL KIDNEY DISEASE OF SALMONID FISH.

2.1 OVERVIEW.

Bacterial kidney disease (BKD), caused by the Gram-positive diplobacillus *Renibacterium salmoninarum* (Sanders and Fryer, 1980), is a chronic infection affecting fish of the family *salmonidae*, namely salmon, trout and char.

Historically, BKD was first described in the Atlantic salmon (*Salmo salar L.*) in the freshwaters of the rivers Dee and Spey in Scotland (Mackie *et al.* 1933 ; Smith, 1964) and was assigned for obvious reasons, the name 'Dee disease.' At approximately the same time, an infection of hatchery-reared brook trout (*Salvelinus fontinalis*), (now known to be BKD), was reported in the United States of America (Belding and Merrill, 1935). Since these early reports of the condition, BKD, (also known as Corynebacterial kidney disease or salmonid kidney disease) has been found to affect at least 13 species of salmonid fish, both in their freshwater and marine habitat (Fryer and Sanders, 1981 ; Hoffman *et al.*,1984).

Although primary reports and studies of BKD concentrated on the impact of the disease on wild fish populations, the bulk of scientific studies have been applied to BKD as it affects salmonids reared in aquaculture. Over the past three decades, intensive salmonid culture has become an important industry in many parts of the world. Fish are farmed intensively, and under conditions of high population density infectious disease can and does constitute a major obstacle to the successful rearing of salmon and trout, either for food or fishery stocks. BKD, although present in the wild fish population, at only seemingly low levels, has become one of the most important infective conditions of farmed salmonids. It is considered by many, to be amongst the most difficult of bacterial infections to control, being largely unresponsive to chemotherapy and as yet uncontrolled by immunoprophylaxis (Austin and Austin, 1987 ; Ellis, 1988 ; Elliot *et al.*, 1989).

2.2 DISEASE CHARACTERISTICS.

Fish with BKD can show a variety of symptoms. Sometimes, infection with *R. salmoninarum* may not be accompanied by visible changes in appearance and/or behaviour (asymptomatic infection). However clinically diseased salmonids can show any or a combination of the following. Fish may be lethargic and dark in appearance with an accompanying lack of balance (Bruno, 1988a), their abdomens distended and eyes protuding (exophthalmia). Lesions, ranging from small blood-filled blebs or blisters to abcesses may be present on the skin surface,and in severe cases, haemorrhaging can occur through the muscle blocks and the vent (Fryer and Sanders, 1981 ; Hoffman *et al.* 1984 ; Bruno 1986a ; Austin and Austin 1987).

Internal examination of infected fish reveals the presence of characteristic grey/white lesions disseminated throughout the tissues of the kidney, and in acute cases, also in the liver, spleen, heart, intestine and even the brain. Affected organs can be swollen and in the case of the kidney may become severely distended and ruptured. Internal haemorrhagic lesions may be visible especially in the lining of the peritoneum, and a fibrous pseudomembrane may be observed covering infected organs.

Histopathological studies of infected fish show that the BKD lesions are granulomatous in nature and consist of a purulent mixture of leucocytes and macrophages, bacteria and cellular debris. Areas of focal necrosis are present in all infected areas but predominate in the kidney tissues where the ultrastructural changes in the glomeruli and renal tubules are considered similar to those found in mammalian glomerulonephritis (Young and Chapman 1978). During infection *R. salmoninarum* is present both extracellularly and intracellularly. The bacteria are found regularly within macrophages and polymorphonuclear leucocytes where they can apparently survive and multiply to fill the cell to bursting point. This intracellular growth of *R. salmoninarum* is thought to facilitate the systemic spread of the pathogen, allowing dissemination of the infection to many

tissues and organs of the host.

Closer examination of the pseudomembrane (Wolke, 1975) reveals layers of fibroblasts and histiocytes along with degenerating macrophages and leucocytic cells, the main macromolecular component of the membrane appears to be fibrin.

Haematological analysis reveals many changes. There is a general reduction in haematocrit and haemoglobin levels in infected fish along with a loss of serum protein and serum cholesterol. Erythrocytes exhibit a reduction in mean cell diameter and the total population is diminished. Other cell populations appear to be enhanced, especially monocytes, neutrophils and thrombocytes (Bruno, 1986b). Physiochemical imbalances are also detectable, for example, an increase in blood urea nitrogen. Such imbalances are thought to reflect dysfunction of the organ systems precipitated by gross tissue damage.

The way in which BKD causes death is unknown but a number of possibilities exist (Fryer and Sanders 1981; Bruno 1986a). Notably, the proliferation of granulomatous necrotic lesions may impair or obliterate tissue and organ function, or death may result from direct damage to such tissues by products of *R. salmoninarum* or hydrolytic and oxidizing products liberated from damaged host cells such as macrophages. It may also be the case that death is the result of heart failure, caused by myocardial infection or intoxication, or a combination of any of the above events.

2.3 EPIZOOTIOLOGY.

All published evidence relating to *R. salmoninarum* suggests that this bacterium is an obligate pathogen of salmonids. No data has been found that points to the organism being a normal member of the aquatic environment, causing disease in an opportunistic manner.

The status of *R. salmoninarum* in the environment was studied by Austin and Rayment in 1985. Water and sediment samples were collected from 56 fish farms and when cultured, no viable *R. salmoninarum* were recovered. Survival experiments using the bacterium showed that it could survive in association with organic matter in the form of

sediment for up to three weeks, but could not be isolated from the overlying water. Using laboratory-grown cultures, the authors also demonstrated that *R. salmoninarum* could survive in river water for limited periods, after which the number of viable organisms dropped dramatically.

Experimental evidence indicates that *R. salmoninarum* can survive for a short time outside of its fish host, presumably long enough to ensure successful transmission, but cannot withstand lengthy exposure to the aquatic environment (Evelyn, 1988). In addition, the bacterium has to date, only routinely been isolated from asymptomatically infected ('carrier') or clinically diseased fish (Austin and Austin, 1987). These findings would tend to confirm the status of *R. salmoninarum* as a primary bacterial pathogen.

The source of infecting *R. salmoninarum* is still unclear, but is thought to be mainly through exposure to surface waters holding populations of infected fish (Wood and Wallis, 1955; Wolf, 1966; Bucke, 1978; Mitchum *et al*, 1979; Paterson *et al.*, 1979, 1981). It is also worth noting that this source may be either wild or farmed populations. Culture systems such as cage farms with high fish densities and low water flow, are particularly susceptible to BKD outbreaks, infections regularly occurring in juvenile salmonids such as smolts, where these fish are placed in close proximity to survivors of earlier outbreaks of the disease (Munro and Bruno, 1988).

R. salmoninarum is regularly detected in faeces of both infected wild and farmed fish (Austin and Rayment, 1985), and this may possibly indicate the spread of BKD via the faecal-oral route. Infection has been shown to arise from ingestion of viable bacteria, with the work of Wood and Wallis (1955) showing that *R. salmoninarum* could be transmitted by diets made from infected fish offal. This route, is today, thought less important due to the introduction of feed pasteurisation (Elliot *et al.*, 1989). Other routes of transmission suggested have been via injuries to skin and external organs (Hoffman *et al.*,1984; Lall *et al.*, 1985) or by a parasitic vector (Sniesko and Griffin, 1955; Smith, 1964; Wood &

Yasutake, 1956; Coulson, 1977; Putz, 1972). The possibilty of BKD being transmitted from infected fish species other than salmonids has also been suggested. Bell *et al.* (1990), found that the sablefish (*Anaplopoma fimbria*) is also susceptible to experimental BKD infection, and juvenile Pacific herring (Bruno 1988a) have also been identified harbouring *R. salmoninarum.*

The majority of *R. salmoninarum* infections, especially in farmed fish populations, would seem to originate via a horizontal route of transmission (Munro and Bruno, 1988), but much attention has also been focused on the role of infected salmonid eggs in the spread of the disease. This vertical transmission route appears to be unique amongst bacterial fish pathogens (Allison, 1958; Wolf, 1966; MacLean and Yoder, 1970; Mitchum *et al.*, 1979; Evelyn *et al.*, 1984). Egg involvement was indicated as early as 1958 by Allison, who observed that BKD occurred following transfer of ova from an infected site. Other authors have implicated eggs in the spread of the disease, even those disinfected before distribution. (Bullock *et al.*, 1978, Evelyn, 1984; Elliot *et al.*, 1989). *R. salmoninarum* has been shown to associate with both mature eggs and developing oogonia. Bacterial cells are thought to enter developing ova by pinocytosis and mature ova, possibly via the micropyle or the numerous pore canals (Bruno and Munro, 1986). Egg infection is thought to be due to exposure, in the female fish, to large numbers of bacteria in coelomic (ovarian) fluid. Evelyn (1984) demonstrated the presence of *R. salmoninarum* in 11-15% of eggs from an infected female coho salmon (*O. kisutch*). The coelomic fluid of this fish was seen to be cloudy due to large numbers of bacteria present. The ability of *R. salmoninarum* to exist in such large numbers in salmonid reproductive fluids has been attributed to the abilty of the host fish to tolerate a large number of bacterial cells, where such levels of other pathogens (such as *Aeromonas salmonicida*) would probably prove fatal (Lee and Evelyn, 1979).

2.3.1 FACTORS AFFECTING BKD INFECTION.

The manifestation of BKD seems to be affected by a number of factors such as water quality, temperature, salinity and diet. Coupled with this, the physiological and genetic status of the host fish may also play an important role.

The effect of temperature on BKD has been studied by many workers (Fryer and Sanders, 1981). Earp (1953), found that most BKD outbreaks occurred between the temperatures of 8 and 18°C, with the majority in the autumn-winter period when the water temperature was declining. However it was found that the highest number of BKD-associated mortalities occurred at higher temperatures, an observation confirmed by Austin (1985). In periods of low water temperature, BKD still occurs, apparently causing continual low mortality levels (Sniesko and Griffin, 1955). In studies made on sea-caged Atlantic salmon (*S. salar L.*), BKD was found to occur and cause mortalities in the spring-summer period, when temperatures were rising (Bruno, 1988a). It is therefore possible that a period of temperature change, rather than a critical specific ambient temperature, is important to the manifestation of BKD outbreaks. This may occur at different periods of the year, according to geographical location.

Salinity is also an important factor when studying the characteristics of BKD in salmonid fish. Being anadromous in nature, salmon and some species of trout exist in both freshwater and marine environments. It has been postulated that BKD infection is a major factor affecting the ability of salmonids to acclimatize to and survive the oceanic environment (Frantsi *et al.*, 1975 ; Fryer and Sanders, 1981),. The studies of Earp *et al.* (1953), Bell (1961) and Bruno (1988a) show that transfer of salmonids to sea water can induce BKD. Banner *et al.* (1986), investigated the occurrence of *R. salmoninarum* infection in salmonids captured in the Pacific ocean off the states of Oregon and Washington in N.W. United States. Infection was detected in chinook salmon (*Oncorhynchus tshawytscha*), chum salmon (*O. keta*), coho salmon (*O. kisutch*) pink

salmon (*O. gorbuscha*), sockeye salmon (*O. nerka*), cutthroat trout (*O. clarki*) and steelhead trout (*O. mykiss*). Levels of infection in these species, based on positive immunofluorescence tests, ranged from 1-11% of the captured population. More recent studies (Dr. D. Rockey, pers. comm.) show that oceanic survival of hatchery-reared salmonids is related to the level of *R. salmoninarum* infection in juvenile populations. So, salmonids infected in early life, presumably via infected eggs, are less likely to survive the transition to an oceanic existence without succumbing to BKD.

Another predisposing factor in BKD outbreaks, and of paramount importance to all bacterial diseases of fish, is the effect of environmental stress on the susceptible host. As previously mentioned, changes in water quality, population density and intervention by husbandry methods, confer upon aquaculture an innate susceptibility to attract fish mortality through infectious disease (Austin and Austin, 1987).

The effect of nutritional (dietary) variation on the development of BKD is only partially understood. Some diets are known to enhance the disease, whilst others may control its occurrence. Feed containing corn gluten (Wedemyer and Ross, 1973) or lipid (Austin, 1985) have been shown to increase the prevalence of *R. salmoninarum* infection within a population. Work by Paterson *et al.* (1981), indicated that the supplementation of salmonid diets with vitamins and trace elements, especially vitamin A, iron and zinc, reduced the incidence of BKD. Supplementation with iodine and fluorine also seems to be efficacious in BKD control (Bowser *et al.*, 1988), having an apparent effect on the levels of both clinical and asymptomatic infections.

Variations in susceptibility to BKD also exist amongst the different species of salmonid (Sanders *et al.*, 1978 ; Bell *et al.*, 1984). What is clear, is that the Pacific races of salmonid species appear to be more susceptible than their Atlantic counterparts, the most affected seems to be pink salmon (*O. gorbuscha*) then the sockeye (*O. nerka*), chinook (*O. tshawytscha*), chum (*O. keta*) and the least coho (*O. kisutch*). Information on the

Atlantic salmonids is limited, but european strains of Atlantic salmon (*S. salar L.*) and rainbow trout (*O. mykiss*) seem to show modest resistance to the disease. Genotypic variation between species may correlate with disease susceptibility, with one study (Suzomoto, 1977) appearing to indicate that limited resistance to BKD on coho salmon (*O. kisutch*) is related to a transferrin genotype.

It is obvious that information on the factors influencing BKD infection is still incomplete after over 3 decades of research. Our knowledge, especially of host, pathogen and environmental factors needs to be improved before more effective control strategies can be formulated.

2.4 THE ATTEMPTED CONTROL OF BKD.

Over the past forty years, a large portion of the scientific studies made on BKD have addressed the problem of disease control. Many workers have investigated ways in which the prevalence of *R. salmoninarum* infection, and the consequent associated mortalities can be reduced (Elliot *et al.*, 1989).

Historically, the first effective method for controlling BKD in hatchery-reared salmonids was adopted in the early 1960's. The process of pasteurising salmon carcasses and viscera used for fish diets, prevented the accidental transmission of *R. salmoninarum* to susceptible hosts (Fryer and Sanders, 1981). At around the same time, a growing interest in antimicrobial chemotherapy had resulted in the evaluation of a number of compounds with potential efficacy in combatting *R. salmoninarum* infection (Wolf & Dunbar, 1959). Since these early days, research has carried on apace, not only into more potent chemotherapeutic agents, but into methods of control such as vaccination and modified husbandry practices (Elliot *et al.* ,1989).

2.4.1 CHEMOTHERAPY AND CHEMOPROPHYLAXIS.

As early as 1951, research into the control of BKD using chemotherapy and chemoprophylaxis had begun. In that year, Rucker *et al.* (1951) reported that by

incorporating sulphadiazine into diets at 264 mg/kg body weight for 1 week, and at 132mg/kg body weight for a further 3 weeks, BKD associated mortalities in juvenile sockeye salmon (*O. nerka*) could be reduced. Other sulphonamide drugs were also found to be efficacious (Earp *et al.*,1953; Rucker *et al.*, 1954; Snieszko and Griffin, 1955; Wood and Wallis, 1955; Allison, 1958) but a reoccurrence of mortality was found on cessation of the chemotherapeutic regimen.

The most widely used compound for the chemical control of BKD, the macrolide antibiotic erythromycin, was first evaluated by Wolf and Dunbar (1959). These authors found that a daily dose in feed of 100 mg/kg body weight produced the biggest reduction in mortality. Oral administration of erythromycin for the chemoprophylaxis and chemotherapy of BKD has been carried out in many salmonid rearing facilities across North America and Europe (Groman and Klontz, 1983; Austin, 1985). Administration of the compound via this route rapidly results in high levels of the antibiotic (above the *in vitro* MIC for *R. salmoninarum*) in the plasma and tissues of fish (Groman, 1983). After drug withdrawal, the tissue and plasma concentration declines below detectable levels within 3 weeks, after which, reinfection with BKD can occur. Erythromycin has also been administered to salmonids via immersion and injection (Groman and Klontz, 1983) and both methods seem to produce promising results, but to date have yet to be properly evaluated for the successful control of BKD.

Other antibacterial compounds have been shown to have some efficacy in controlling BKD infection. In a study of 79 compounds, Austin (1985) revealed that in addition to erythromycin, other antibiotics such as Penicillin G, clindamycin, kitasamycin and spiramycin were chemotherapeutic, whilst Cephradine, lincamycin and rifampicin showed some efficacy in prophylaxis of the disease.

The inability of antimicrobial compounds to eliminate *R. salmoninarum* from all treated fish has been attributed both to the intracellular nature of the bacterium and its consequent

inaccessibility (Getchell *et al.*, 1985) and to feed rejection of orally administered compounds due to unpalatability (Schreck and Moffit, 1957). Whatever the case, it is evident that more studies are needed on this approach to BKD control.

2.4.2 VACCINATION.

A potent method of controlling infectious disease in salmonid fish, lies in vaccination (Gould *et al.*, 1979; Amend *et al.*, 1983; Adams *et al.*, 1988; Ellis, 1988). Vaccination against BKD was first suggested by Evelyn in 1971, who not only demonstrated the production of specific agglutinins in sockeye salmon (*O. nerka*) following injection of heat-killed cells of *R. salmoninarum*, but showed that a definite anamnestic response followed a second challenge with the bacterial preparation. Unfortunately the ability of vaccinated fish to resist BKD infection was not tested. Subsequent work by Paterson *et al.*, (1981; 1985) produced a set of similar results. Immunization consisted of a set of bacterins administered by intra-peritoneal injection or by hyperosmotic infiltration to juvenile Atlantic salmon (*S. salar L.*) As the fish were held in a facility annually affected by BKD, not only could the authors measure any specific antibody response, they could also assess the ability of each bacterin to confer resistance to a natural challenge by *R. salmoninarum*. The highest circulating antibody titres were found in fish injected with killed bacteria combined with Freunds complete adjuvant, as opposed to fish immunized with the preparation without adjuvant or by hyperosmotic infiltration. A detectable immune response was found in fish for over a year and there appeared to be a reduction in the prevalence of BKD, however, levels of *R. salmoninarum* as detected by an Indirect Fluorescent Antibody Technique (IFAT) did not vary between vaccinated and control fish.

Another investigation, by McCarthy *et al.*, (1984) consisted of the administration of four different bacterins to rainbow trout (*O. mykiss*) at least 34 days before injection challenge of live *R. salmoninarum*. Bacterins were unaltered *R. salmoninarum* from broth culture at double and single strength and pH-lysed preparations of double and single strength cell

preparations. All bacterins were administered without adjuvant either by intra-peritoneal injection, hyperosmotic infiltration or by direct immersion. A lack of protection against *R. salmoninarum* was found in those fish immunized by infiltration and immersion, whilst the authors suggested that fish immunized by injection were protected against infection. Comparison however, with the studies of Paterson (above) cannot be made, as McCarthy and his colleagues did not measure circulating antibody levels and did not use a comparable *R. salmoninarum* detection method (Gram stain smears instead of IFAT).

Another approach taken for the production of an anti-BKD vaccine has been to combine a *R. salmoninarum* antigen with a preparation shown to induce protective immunity to another bacterial pathogen of salmonids. This was attempted by Kaattari *et al.* (1987), when they combined a soluble antigen fraction from *R. salmoninarum* with killed cells of *Vibrio anguillarum*. The authors also reversed the formulation, combining a soluble antigen fraction with killed cells of *R. salmoninarum*. Groups of coho salmon were vaccinated by intra-peritoneal injection and the antibody and cellular responses to *R. salmoninarum* tested after 21 days, just prior to a challenge with the bacterium. A variable response was found to all of the preparations, and a lack of correlation with protection observed. However, comparison of mortalities within the vaccinated groups did suggest that at least three of the antigen preparations may have had some potential in immunoprophylaxis. A subsequent vaccine trial however, (Kaattari *et al.*, 1988) failed to demonstrate any protective effect.

Another study made by Amend and Johnson (1984) involved the combination of antigens from *V. anguillarum, A. salmonicida* and *R. salmoninarum* in vaccine preparations. It was the aim of the authors to study multivalent fish vaccines and the possible reduction in efficacy by antigen competition. Encouragingly, results showed no antigenic competition, but no conclusion could be drawn as to the benefits of a multivalent anti-BKD vaccine, as fish were only challenged after vaccination with *A. salmonicida* and *V. anguillarum*.

Although these various studies suggest that a protective anti-BKD vaccine is a possibility, there are other factors to be considered. Salmonids are obviously able to produce specific antibodies to experimental *R. salmoninarum* bacterins, but there is to date no conclusive proof that such antibodies form part of a protective immune response. Other workers have demonstrated elevated antibody titres in both wild and farmed salmonids exposed naturally to *R. salmoninarum* (Weber and Zwicker, 1979; Bruno, 1987), but again no definite indication of protective immunity has been found. Although in his study, Bruno (1987) found that titres of antibody in sera of non-infected, sub-clinically infected rainbow trout, generally correlated with the level of BKD infection, no comparative correlation could be found in Atlantic salmon.

It is also possible that the nature of any antigen preparation selected for a BKD vaccine may be important for its ability to confer consequent protective immunity. In the work of Turaga *et al.* (1987), preparations of soluble antigens were shown to suppress antibody production by both normal coho salmon lymphocytes and those lymphocytes isolated from BKD infected individuals.

With reference to the work of Bruno (1987) described above, it is also the belief of some workers that the particular species of salmonid may influence the success of an anti-BKD vaccine preparation. An extensive study carried out by Evelyn *et al.* (1988) resulted in the failure of a number of bacterin preparations to induce antibody responses in coho and sockeye salmon to both natural and experimental challenge with *R. salmoninarum*. These results led the authors to postulate that members of the salmonid genus *Oncorhynchus* are unable to mount a protective immune response compared to that reported for fish of the genus *Salmo*.

The general lack of success in controlling BKD using antimicrobial agents and/or vaccine immunoprophylaxis, has stimulated research into control methods based on the improvement of salmonid husbandry (Munro and Bruno, 1988). By changing fish rearing

practices, it is hoped that levels of BKD infection in both farmed and wild populations may be influenced in a beneficial manner.

2.4.3. DIETARY MODIFICATION.

In the previous section of this chapter dedicated to studies of BKD epizootiology, a number of studies were mentioned where it was suggested that the dietary/nutritional status of salmonid fish could influence the prevalence of *R. salmoninarum* infection and associated mortality (Woodall and La Roche, 1964; Wedemeyer and Ross, 1973; Wood, 1979; Bell *et al.*, 1984; Lall *et al.*, 1985; Paterson *et al.*, 1985; Sakai *et al.*, 1986; Bowser *et al.*, 1988). The variation of fish diets with respect to the levels and nature of protein, lipid, carbohydrate, vitamins, minerals and trace elements has been utilised with a number of salmonid species. Results have, in general, been inconsistent, and studies have shown no demonstratable effect, whilst others have shown encouraging influences on BKD infection. Dietary modification may prove a useful contribution to disease control strategies in the future, but it seems unlikely that this approach could be singularly and universally effective.

2.4.4 PREVENTION OF DISEASE TRANSMISSION.

The interruption of the horizontal and vertical transmission routes of *R. salmoninarum* would obviously facilitate a reduction in the prevalence of BKD in farmed fish populations. Austin and Rayment (1985) along with Evelyn (1988) have demonstrated that *R. salmoninarum* can survive for limited periods in both freshwater and marine fish culture systems, and therefore susceptible host fish may become infected by viable bacteria shed from diseased fish kept in close proximity. One way in which such a transmission pattern could be avoided would be the treatment of the water supply to remove infective *R. salmoninarum* cells. Although impractical for large freshwater farms and marine facilities, some benefit may result from the disinfection or sterilisation of freshwater supplies for salmonid hatcheries. In one study, Austin (1983) tested ozonisation

for disinfecting laboratory effluent heavily contaminated with *R. salmoninarum,* and after preparing cultures from samples collected at monthly intervals, demonstrated that the method was effective in killing the organism. Ultra-violet irradiation for use in aquacultural water sterilisation was studied by Flatow (1981), and although the method does seem to be efficacious in reducing bacterial loads in water supplies, no mention is made of the effect of U.V. irradiation on *R. salmoninarum.* A review of published literature finds no more studies on this aspect of BKD control.

The bulk of studies made on the control of *R. salmoninarum* infections via the interruption of transmission routes have concentrated on the vertical spread of the disease. Workers have investigated the treatment of salmonid eggs, antibiotic therapy for prespawning adults and the segregation and culling of infected broodstock.

Evidence that suggested egg contamination was a major source of BKD infections (Allison, 1958: Evelyn, 1984), intiated several experimental studies in which salmonid eggs thought to be exposed to *R. salmoninarum* were treated by hardening in the presence of antibacterial compounds. It has been found that treatment with iodophor (Evelyn *et al.*, 1984; Bullock *et al.*, 1986; Evelyn *et al.*, 1986b) and erythromycin (Groman, 1983; Groman and Klontz, 1983; Bullock and Leek, 1986; Evelyn *et al*, 1986c) is largely ineffective for preventing vertical transmission of *R. salmoninarum.* This is probably due to the short contact time with the compounds used and the failure of such treatments to deliver the antibacterial agent to the egg yolk, where *R. salmoninarum* can occur (Evelyn *et al.*, 1986a).

The injection of prespawning adult salmonids with erythromycin phosphate has also been performed to assess its utility in controlling BKD. This form of treatment seems to reduce prespawning mortalities (Groman and Klontz, 1983) and also may reduce vertical transmission of BKD (Paterson, 1982; Groman and Klontz, 1983; Bullock and Leek, 1986; Evelyn *et al.*, 1986a). Infections caused by *R. salmoninarum* seem to be reduced

in severity by this treatment, and the antibiotic appears to persist in the egg yolk for extended periods (Sakai, 1986). Possible drawbacks to this treatment have however been mentioned. Firstly, the timing of injection for prespawning adults seems critical, and secondly, the large scale use of the antibiotic could result in the selection of erythromycin resistant *R. salmoninarum* strains (Evelyn *et al.*, 1986a).

At the time of salmonid spawning, there may also be an opportunity of reducing the number of infected progeny that will later form a part of a larger population. The removal of *R. salmoninarum* infected adult fish from the spawning population would reduce the levels of egg infection and could prove to be a very effective way of controlling BKD. Infected broodstock fish could then be culled and their reproductive material withdrawn from fertilization. Available evidence leads to the conclusion that it is eggs and not sperm that are responsible for the vertical transmission of *R. salmoninarum*, and successful segregation depends on the sensitive and selective detection of the organism in spawning female fish and their eggs (Klontz, 1983; Evelyn *et al*, 1986 a,c). Such detection and diagnosis of BKD in salmonid population forms the next section of this review.

2.5 THE DETECTION AND DIAGNOSIS OF BKD.

By studying the published information on the detection and diagnosis of BKD, it is clear that a great deal of confusion surrounds which of the available methods is most suitable. It is also clear that no single method has yet been developed that ensures the sensitive and selective detection of *R. salmoninarum*.

The first methods for the diagnosis of BKD consisted of the histological examination of kidney tissue for the presence of Gram-positive coccobacilli (Smith, 1964). The reliability of this method was somewhat impaired by the presence of morphologically similar bacteria and by melanin granules found in the tissue samples (Chen, 1974). A more recent histochemical diagnosis technique was described by Bruno and Munro (1982), who developed a technique for staining glycogen in *R. salmoninarum* cells using Lillie's

allochrome. However, this improved technique is again, not ideal (Austin and Austin, 1987), as interference by morphologically similar glycogen-containing bacteria would still exist.

The development of culture techniques for *R. salmoninarum* did initially lead to an improvement in the diagnosis of BKD. However the apparent slow growth of the organism significantly influences the effectiveness of this method, and the recent study by Benediktsdottir *et al.* (1991) suggests that up to 20 weeks incubation may be needed for growth resulting from an initial isolation. Although many workers gave up their reliance on culture methods in favour of the more rapid immunological-based detection systems developed in the 1970s and 1980s, they remain in use to this date in many fish disease laboratories (Dr. D.W. Bruno, pers. comm.) This is due to the fact that culturing is still regarded as the most reliable method for verifying the presence of viable *R. salmoninarum* in both environmental and pathological samples and since the selectivity of some immunological methods has been found to be dubious (see below). It was Evelyn (1978) who first contradicted the utopian opinion of serology, by reporting that culturing using KDM medium was more sensitive than the up to date method of fluorescent antibody detection by a factor of 10:1. In a later study (Evelyn *et al.*, 1981), this view was enforced when experiments were undertaken to determine whether or not there was correlation between culturing and fluorescent antibody (FA) based diagnosis of the BKD carrier state. Again culturing was reported as more sensitive than FA techniques. The proponents of culture-based diagnosis systems for BKD continued to develope improved formulations of growth media and selective kidney disease medium (SKDM; Austin *et al.*,1983), charcoal agar (Daly and Stevenson, 1985) and SKDM-C (Benediktsdottir *et al.*, 1991) have all apparently increased the possibility of the primary isolation of *R. salmoninarum.*

Serological diagnostic tests for *R. salmoninarum* and BKD became a focus for attention

in the mid 1970s, with an immunodiffusion test, based on the detection of soluble *R. salmoninarum* antigen in infected fish tissue being the first to appear (Chen *et al.*, (1974)). In the same year, Bullock *et al.* (1974) coupled the method with the more traditional agglutination reaction. Immunodiffusion was of course much quicker than bacterial cultivation, and the diagnosis of BKD (based on the development of precipitin lines) took no more than 24 hours.

A considerable improvement, in terms of speed, resulted from the development of indirect fluorescent antibody techniques (IFAT; Bullock and Stuckey, 1975; Mitchum *et al.*, 1979; Paterson *et al.*, 1979; Laidler, 1980) and direct fluorescent antibody techniques (FAT; Bullock *et al.*, 1980). The IFAT has found use for detecting asymptomatic or overt cases of BKD (Bullock and Stuckey, 1975) and indeed, in the work by Paterson *et al.* (1979) pointed to the possible enzootic nature of BKD in one canadian river. This work also found that IFAT was seemingly more sensitive than the examination of Gram-stained kidney tissue and bacterial cultivation on Mueller-Hinton Agar supplemented with 10% (v/v) Foetal calf serum and 0.1% L-cysteine hydrochloride.

Elliot and Barila (1986) modified the fluorescent antibody staining procedure for the detection of *R. salmoninarum* in the coelomic fluid of chinook salmon. It was found that the detection of the bacterium could be made more sensitive by membrane filter concentration.

The co-agglutination test of Kimura and Yoshimizu (1977) also showed promise for the rapid diagnosis of BKD. This method utilizes specific anti-*R. salmoninarum* antibody coated staphylococcal cells, which are reacted with the supernatant from heated (100°C for 30 min.) kidney tissue.

Solid phase enzyme-linked antibody/antigen assays have also been developed for the detection of *R. salmoninarum* and the diagnosis of BKD. A great deal of interest has been shown in the development of an enzyme-linked immunosorbent assay (ELISA) (Voller *et*

al. 1979) for BKD. The technique offers a combination of speed and sensitivity for the detection of bacterial antigens in a large number of samples (Ruitenberg *et al.*, 1977). Dixon (1987), reported the development of an ELISA technique for the detection of *R. salmoninarum*. The antibody used in the assay was preabsorbed with two species of cross-reacting bacteria (*Bacillus sphaericus* and *Rothia denticariosa*) to make a specific test system. *R. salmoninarum* could be detected in clinically diseased fish within 30 min. of preparing a kidney sample. A double-sandwich ELISA (Voller *et al.*, 1979) has been developed (Pascho and Mulcahy, 1987) and more recently the technique has been enhanced with the use of anti-*R. salmoninarum* monoclonal antibodies (Hsu *et al.*,1991; Rockey *et al.*, 1991).

Membrane blotting procedures have also found a role in the detection of *R. salmoninarum* antigens in infected fish tissues and body fluids. Coupling specific anti-*R. salmoninarum* antibodies to peroxidase (Sakai *et al.*,1987a) and using an avidin-biotin complex (Sakai *et al.*, 1987b) appear to enhance the sensitivity and speed of BKD diagnosis. A 'western' blotting technique for the detection of *R. salmoninarum* antigens in salmonid serum and reproductive fluids was developed by Griffiths and Lynch (1991), although this method appeared sensitive for the detection of the major soluble antigen of the bacterium, possible confusion in diagnosis may arise from the variable patterns of immunoreactive bands caused by differences in sample handling and processing.

It must be noted that a number of the serological methods of BKD diagnosis and *R. salmoninarum* detection should be examined sceptically, especially those developed before any detailed taxonomic study of the bacterium (Austin and Austin, 1987). These authors stated correctly, that it is unclear how workers knew that the aetiological agent of BKD possessed a unique antigenic profile, distinguishing it from other Gram-positive bacteria. Unfortunately, the reliability of early serological methods may now be questioned insofar as cross-reactions with apparently unrelated organisms have been recorded. Bullock *et al.*

(1980), observed large bacteria in faecal samples of brook trout, which fluoresced with antiserum to *R. salmoninarum.* Cross-reactions were also shown by Austin and Rayment (1985) who reported false positive reactions with coryneform bacteria obtained from fish, a fish pathogenic *Mycobacterium spp.* and *Rothia denticariosa.* It is therefore clear that polyclonal anti-*R.salmoninarum* antibodies should be cross-absorbed before use in serological detection proceedures, and where possible specific monoclonal antibodies employed (Arakawa *et al.*, 1987).

The rapid development of nucleic acid probe technology over the last 10 years has greatly improved detection methods for pathogenic microorganisms, and properly developed gene probes can be highly specific and show incredible sensitivity when coupled to an amplification method such as the polymerase chain reaction (Sakai *et al.*, 1988) . A number of research groups are at present applying this technology to the detection of *R. salmoninarum* in both environmental and clinical samples, and there have been a small number of publications. Friojonsson *et al.* (1991) report the development of a radioactively labelled DNA probe based on a gene sequence encoding a 57 kDa surface protein of *R. salmoninarum.* The probe was found to be specific, and sensitive to a detection limit of less than 1 ng of genomic DNA. The same authors also report a probe specific to *R. salmoninarum* 16s rRNA which was used in a PCR-based assay with a 1-2 day processing time. The DNA probe described by Mattson *et al.* (1991) is also complementary to *R. salmoninarum* 16s rRNA, but was non-specific cross reacting with nucleic acid isolated from two *Arthrobacter* species. The probe was seen to hybridize to DNA from colonies classified by culture to be *R. salmoninarum*, and the detection level was determined to be 2.5 x 10^4 cells.

2.6 RENIBACTERIUM SALMONINARUM; THE AETIOLOGICAL AGENT OF BKD.

The true nature of the aetiological agent of BKD was unknown for many years. Early descriptions of the condition included the presence of small coccobacilli in the lesions and tissues of affected fish (Belding and Merrill, 1935; Smith, 1964), but the inability to culture this organism in the laboratory meant that a full characterization of the bacterium was impossible. Only in the last twenty years has this task been undertaken with any success, but it is evident that much work has yet to be done.

2.7 CULTURE OF *R. salmoninarum*.

The cultivation *in vitro* of the aetiological agent of BKD was not achieved until a nutrient-rich medium containing fish extract, glucose, yeast extract and bovine serum/meat infusion at incubation temperatures of 15 and 20 °C was used to isolate the organism from chinook salmon (Earp, 1950). The culture method was improved by using Dorset egg medium or minced chick embryos in 1% (w/v) agar (Earp *et al.*, 1953). The growth obtained using this medium, was nevertheless poor, even after incubation periods lasting over 2 weeks. To prove that this growth was of the aetiological agent of BKD, Earp inoculated the bacterial culture into healthy chinook salmon and eventually recovered it again from a kidney lesion. This work was continued by Ordal and Earp (1956), who supplemented Dorset egg medium with 0.05-1.0% (w/v) L-cysteine, tryptone and yeast extract to successfully isolate the pathogen in 21-28 days following incubation at 17°C This work led to the formulation of cysteine blood agar, used by Ordal and Earp (1956) in the experiments that finally fulfilled Kochs' postulates for *R.salmoninarum*. A modification of this medium was proposed by Evelyn *et al.* (1973) where foetal calf serum was substituted for human blood. Four years later this was developed further by removing sodium chloride and substituting peptone for tryptone and beef extract to produce KDM2 medium (Evelyn, 1977), a medium now used by many laboratories for the routine culture

of *R. salmoninarum.*

In a parallel development of growth medium, Wolf and Dunbar (1959) used Mueller-Hinton agar supplemented with 0.1% (w/v) L-cysteine hydrochloride (MHC) to culture the pathogen. The value of this medium has been confirmed by Bullock *et al.* (1979) and Bruno (1984). The medium was further developed by Paterson *et al.* (1979), when it was supplemented with 10% (v/v) foetal calf serum.

A replacement for serum in media for *R. salmoninarum* was proposed by Daly and Stevenson (1985), who obtained good growth of the bacterium using charcoal.

A problem associated with the use of these rich media for the culture of *R. salmoninarum* is their suitability for the growth of many fast-growing microorganisms that may rapidly out compete and overgrow the slower-growing BKD organism. One solution to this problem was proposed by Evelyn (1977), and consisted of a drop plating technique (analagous to dilution plates) which could dilute out potential interference by fast-growing heterotrophs.

The problem of contamination in *R. salmoninarum* cultures was to some extent alleviated by the advent of selective isolation techniques, which made the initial isolation of the pathogen from fish tissues less of an uncertain affair. The selective isolation medium, known as SKDM (Austin *et al.*, 1983) proved to be effective for the isolation of *R. salmoninarum* from even dilute samples.

A rich semi-defined medium for *R. salmoninarum* was formulated by Embley *et al.* (1982) as part of a major study into biological aspects of the bacterium (Embley, 1983). Devoid of serum, this medium was not suitable for the isolation of the pathogen from infected fish tissues. However, the development of this medium must be seen as an important advance in the characterization of the kidney disease bacterium, as it was used to provide both inocula and biomass for the major taxonomic, nutritional and physiological study of the organism (Embley *et al.*, 1983).

2.8 CHARACTERIZATION AND TAXONOMY.

At various times during its' study, the aetiological agent of BKD has been linked with *Corynebacterium* (Ordal and Earp, 1956; Smith, 1964; Sanders and Fryer, 1978; Austin and Rodgers, 1980), *Brevibacterium* (Smith, 1964), *Listeria* (Bullock *et al., 1975), Lactobacillus* (Vladik *et al., 1974)* and *Rickettsia* (Snieszko and Griffin, 1955). As it was subsequently appreciated that the organism is sufficiently unique to warrant separate species status, it was described by Sanders and Fryer (1978) as *Corynebacterium salmoninus*. However, with further information, these authors realised that the pathogen belonged in a new, as yet undescribed genus and, therefore, proposed *Renibacterium*. Thus the aetiological agent of BKD became classified as *Renibacterium salmoninarum* (Sanders and Fryer, 1980).

The difficulties encountered whilst culturing *R. salmoninarum* have contributed significantly to the uncertainty over its precise taxonomic status. Early workers only emphasized a small number of morphological features, namely the presence of small (0.3-1.5 x 0.1-1.0 μm), Gram-positive, asporogenous, non-motile and non-acid-fast rods, frequently occurring in pairs. Evidence of pleomorphism, metachromatic granules and a 'coryneform' appearance (Ordal and Earp, 1956; Smith, 1964) led to an initial tenuous association with *Corynebacterium*. Later studies however, failed to substantiate the 'coryneform' appearance of *R. salmoninarum* cells (Young and Chapman 1978), and such morphological studies should always be treated cautiously, as should all *a priori* characters in taxonomic studies.

Proteolytic and catalase activities were demonstrated along with the growth requirement for cysteine by Earp (1950) and Ordal and Earp (1956). From then on, other attributes of the organism were slowly discovered. In particular Smith (1964) indicated the temperature range of growth, i.e. most rapid at 15°C, slow at 5 and 22°C, and not at all at 37°C. Following this study, during the period of the late 1970s to the present day, more

knowledge on *R. salmoninarum* has accumulated.

Characteristically, *R. salmoninarum* produces cream (non-pigmented), shiny, smooth growth on solid media, whilst older cultures may exhibit a granular or crystalline appearance due to the precipitation of cystine from the growth medium. In broth culture, growth can vary from uniform turbidity in some cultures to a clumping sedimentary appearance in others (Austin and Austin, 1987).

The biochemical properties of *R. salmoninarum* have been investigated (Embley, 1983; Goodfellow *et al.* 1985), the amalgamated findings of these studies are shown in Table 2.1.

The cell wall peptidoglycan of *R. salmoninarum* contains D-alanine, D-glutamic acid, glycine and lysine as the diamino acids. The principal cell wall sugar is glucose, but arabinose, mannose and rhamnose are also present (Sanders and Fryer, 1980). However, this disagrees with the more recent work of Kusser and Fiedler (1983), who reported the principal cell wall sugar being galactose, with lesser amounts of N-acetyl-glucosamine, rhamnose and N-acetyl-fucosamine.

Lipid studies reveal that mycolic acids are absent, with methyl-branched fatty acids forming over 92% of the total fatty acid component of the bacterium, with 12-methyltetradecanoic (anteiso-C_{15}), 13-methyldecanoic (iso-C_{15}) and 14-methylhexadecanoic (anteiso-C_{17}) as the major components. Straight chain fatty acids generally accounted for 1% of the total fatty acids, and unsaturated fatty acids were not detected at all. Over 81% of the total fatty acids were composed of the lower melting point anteiso acids, possibly contributing to membrane fluidity at low temperatues. All strains studied also contain diphosphotidylglycerol, two major and six or seven minor glycolipids and two unidentified minor phospholipids.

The guanine plus cytosine content of the DNA, was originally calculated as 53 % (Sanders and Fryer, 1980), but more contemporary studies have revealed a slightly higher

Character	Response	Character	Response
Production of:		RNA	-
Acid phosphatase	+	Starch	-
Alkaline phosphatase	+	Testosterone	-
Butyrate esterase	-	Tributyrin	+
Caprylate esterase	+	Tween 40	+
Catalase	+	Tween 60	+
Chymotrypsinase	-	Tween 80	-
Cystine arylamidase	-	Tyrosine	-
α-fucosidase	-	Xanthine	-
α-galactosidase	-	Acid production from sugars	
β-galactosidase	-	Growth on/at:	
β-glucosaminidase	-	pH 7.8	+
α-glucosidase	+	Bile salts (0.025%)	-
β-glucosidase	-	Crystal violet (0.0001%)	+
β-glucuronidase	-	Methylene blue (0.001%)	-
Leucine arylamidase	+	Nile blue (0.00001%)	+
α-mannosidase	+	Phenol (0.005%)	-
Myristate esterase	-	Potassium thiocyanate (1%)	+
Oxidase	-	Sodium chloride (1%)	+
Trypsinase	+	Sodium selenite (0.01%)	-
Valine arylamidase	-	Thallous acetate (0.001%)	-
Nitrate reduction	-	Utilization of :	
Degradation of:		4-umbelliferyl (4MU) acetate	+
Adenine	-	4MU-butyrate	+
Aesculin	-	4MU-βD-cellobiopyranoside	-
Arbutin	-	4MU-elaidate	-
Casein	+	4MU-αL-arabinopyranoside	-
Chitin	-	4MU-2 acetamido-2-deoxy-βD	
Chondroitin	-	galactopyranoside	-
DNA	-	4MU-βL-fucopyranoside	-
Elastin	-	4MU-heptanoate	+
Gelatin	-	4MU-laurate	+
Guanine	-	4MU-nonanoate	+
Hyaluronic acid	-	4MU-oleate	+
Hypoxanthine	-	4MU-palmitate	-
Lecithin	-	4MU-propionate	+

Table 2.1 Biochemical characteristics of *Renibacterium salmoninarum*. The table shows an amalgamation of the results of biochemical characterization of *R.salmoninarum* in the studies of Embley (1983) and Goodfellow *et al* (1985). The character + indicates a positive response to the given test for 100% of the strains tested. The character - indicates a negative response.

figure of 55% (Banner, 1992).

The exact taxonomic position of *R. salmoninarum* remains uncertain. However, the numerical phenetic study of Goodfellow *et al.* (1985) confirmed the homogeneity of the taxon whilst demonstrating its dissimilarity to Lactobacillus and Listeria (*Listeria denitrificans*). This study would also seem to confirm the unique position of *R. salmoninarum* as a genospecies.

2.9 *R.salmoninarum* PATHOGENICITY: MECHANISMS AND MOLECULES.

Bacterial pathogens are highly adapted microorganisms with a survival strategy that requires multiplication on or within another living organism. The particular strategies used for this purpose are complex and microbial pathogenicity should be seen as a multifactorial process of interaction between host and infecting organism (Mims, 1987; Dougan, 1989). Whilst different pathogens have evolved separate and distinct mechanisms for overcoming host or environmental barriers to infection, several common themes are repeatedly demonstrated during the disease process (Finlay and Falkow, 1989), and these may be considered with reference to *R. salmoninarum.*

The first interaction between a pathogenetic bacterium and the host invariably involves attachment to a cell surface. There are several distinct and alternative means of cell attachment, none of which have been conclusively shown to occur in *R. salmoninarum* infection. However there is a body of information that suggests that hydrophobic and haemagglutinating cell surface properties of the bacterium may be important (Bruno, 1988; Bandin *et al.*, 1989; Daly and Stevenson, 1987,1989,1990). The predominant cell surface protein of *R. salmoninarum* is the 57 kDa molecule, variously referred to as Antigen F, Haemagglutinin, and more recently, p57 or MSA (major soluble antigen). Certain characteristics of p57 such as the acidic isoelectric point (pI, 4.5-4.8), *in vitro* reassembly and the hydrophobic and haemagglutinating properties, bear resemblance to other molecules of bacterial adherence (Daly and Stevenson, 1990). However, although

agglutination of mammalian erythrocytes has been shown (Daly and Stevenson, 1987), p57 binds to, but does not usually agglutinate, salmonid fish erythrocytes (Bandin *et al.*, 1989; Kaattari et al., 1985) A further study by Wiens and Kaattari (1991) indicates that it is the supposedly cell surface exposed amino terminus of the p57 protein that is responsible for the binding and haemagglutinating/leucagglutinating properties.

Another possible attachment mechanism, suggested by the work of Dubreil *et al.* (1990) is via peritrichous fimbrial structures found at the surface of the *R. salmoninarum* cell. Further analysis revealed that these fimbriae to be composed of an immunodominant, hydrophobic, 57 kDa protein, and it seems likely that this fimbrial adhesin and p57 are the same surface component.

Bacterial pathogens often attach to host cells via eucaryotic carbohydrate receptors (Mims, 1987) and the utilisation of this mechanism by *R. salmoninarum* formed part of the study performed by Daly and Stevenson (1987). Using a number of carbohydrates, these workers attempted to block the haemagglutinating activity of the bacterium *in vitro* and establish the role of sugar receptor binding. However, no reduction in haemagglutinating properties was observed and therefore attachment to host cells by this method seems unlikely.

For some time, *R. salmoninarum* has been regarded as an intracellular pathogen with the ability to enter, survive and multiply within the phagocytic cells of the salmonid host (Young and Chapman, 1978; Bruno, 1986). The means by which the infective bacterium enters these cell types and the nature of the host/pathogen interaction is as yet unknown. However, recent work by Rose and Levine (1992) suggests a possible mechanism for entry. *R. salmoninarum* has been shown to bind to the C3b component of the complement pathway *in vitro*. This binding, followed by ligation to salmonid phagocytes bearing C3b receptors, could provide an easy means of access via internalization of these surface-borne receptors. This complement receptor entry pathway has been suggested as the preferred

route of entry for another intracellular pathogen *Legionella pneumophila* (Horwitz, 1988). This means of entry into a phagocyte has benefits for the invading pathogen as internalization by this route does not result in the release and consequent exposure of the bacterium to hostile superoxide, hydrogen peroxide and arachidonic acid metabolites.

A successful invading bacterium must multiply sufficiently to establish itself in the host or to reach sufficient numbers to ensure efficient transmission. Evidence from histological studies suggest that the successful establishment of *R. salmoninarum* infection seems to rely on the ability of the pathogen to survive and multiply within the phagocytic cells of the host (Austin and Austin, 1987; Young and Chapman, 1978; Bruno, 1986). These studies made on *R. salmoninarum* infected tissue indicate that the bacterium can resist the hostile environment created by phagolysosomal fusion as well as escape the phagocytic vacuole to lie free in the cytoplasm. A rapid escape to the cytoplasm has recently been shown by the electron microscopic studies of Gutenberger *et al.* (1991), although these authors failed to show cytoplasmic bacterial multiplication. Studies made on intracellular parasites other than *R. salmoninarum* such as *Listeria monocytogenes, Mycobacterium tuberculosis, Salmonella typhi, Leishmania donovani* and *Legionella pneumophila*, have shown the many ways in which the invading pathogen can avoid destruction within phagocytes (Finlay and Falkow, 1989). These avoidance mechanisms involve resistant cell surface molecules such as lipids and glycolipids, prevention of phagolysosomal fusion, inhibition of vacuolar acidification and the release of reactive oxygen intermediates. Apart from apparent resistance to reactive oxygen intermediates (Kaatari *et al.*, 1987; Bandin *et al.*, 1992), the existence of such avoidance mechanisms has not been demonstrated in *R. salmoninarum*. However it has been inferred by other workers that other mechanisms such catalase and iron reductase activity, along with phospholipase and membrane-active cytolysin may play a role in infection (Bruno and Munro, 1986; Grayson et al., 1991a,1991b). Another possible determinant of *R. salmoninarum* pathogenicity and the

progression of BKD within the susceptible host, is a proteolytic enzyme produced by the bacterium. The true nature of this enzyme is unknown, but it may be the serine protease described by Griffiths and Lynch (1991) and Rockey *et al.* (1991) or the strong proteolytic component found in BKD lesions (Sakai *et al.*,1989).

The mechanisms by which *R. salmoninarum* avoids immune clearance are still largely unknown, but research in this area appears well established.Some of the first steps taken toward an understanding the pathogenesis of BKD at a molecular level were taken by Kaatari *et al.* (1985,1986,1987,1988), who undertook a series of studies designed to isolate and identify antigenic components of *R. salmoninarum* and enhance their ability to activate host defences, in particular cell-mediated immunity. These authors noted that aspects of the pathology of *R. salmoninarum* infections bore some resemblance to corynebacterial, mycobacterial and listerial infections of mammals; i.e. chronic granulomatous, inflammatory reactions with extensive tissue necrosis caused by the ability of the pathogen to survive and multiply intracellularly. They reasoned that the intracellular location of *R. salmoninarum* provided a protected environment for the pathogen and a constant stimulus for the immune sytem of the host which was capable of responding, but in an inappropriate manner (Young and Chapman, 1978). Like Bruno and Munro (1988) it was noted that the lack of protection conferred by agglutinating antibody and the importance of temperature in the resistance of fish to the disease appeared to provide evidence for the crucial role of cell-mediated immunity acting to eliminate infection. It was also noted that immunological tolerance may be important in the apparent inability of some fish to overcome *R. salmoninarum* infection. So-called 'vertical' transmission of the bacterium via salmonid eggs may mean that the developing fry will be exposed to and thus be tolerant of any antigens produced by *R. salmoninarum* during this phase of the life cycle, thereby disrupting an effective immune response when the animal is older. Recent studies suggest an important role for the p57 protein in the interaction with the salmonid

immune system. Work has shown that the proteolytic degradation of this molecule generates most of the immunoreactive components of *R. salmoninarum* extracellular products (Griffiths and Lynch, 1991; Rockey et al., 1991) and that the breakdown products are present in high concentration amongst infected tissue (Kaattari *et al.*, 1987; Turaga *et al.*, 1987). In addition to these findings p57 has been shown to have a potent effect on the mitogenic, plaque-forming cells and chemiluminescence responses of leucocytes *in vitro*. (Kaattari *et al.*, 1988, Turaga *et al.*, 1987).

This brings to a close the review of the published literature on BKD and *R.salmoninarum.* This review is now available as part of a recent publication, discussing both our present knowledge on the subject and future routes to a better understanding of the pathogen and its role in the disease process (Evenden *et al.*, 1993).

2.10 GENE CLONING: APPLICATIONS IN THE STUDY OF BACTERIAL PATHOGENS

The past twenty years have seen great advances in experimental biology, advances brought about by our rapidly growing knowledge of the molecular basis of life. This great leap in our understanding, has for the main part, been brought about by the development of genetic manipulation techniques, the so-called recombinant DNA technology (Weinberg, 1991). At the heart of this technology is gene-cloning, which enables the isolation, characterization and manipulation of genetic sequences, the ultimate control mechanism for all living organisms. It is cloning, more than any other factor that has changed the face of biology. Cloning has not merely expanded the repertoire of laboratory techniques, but has precipitated a revolution in all biological disciplines, including the development of new approaches to the study of human disease and the rapid establishment of a modern biotechnology industry. The theory and practice of gene cloning is too large and complex to be covered by this review,but the reader can gain suitable knowledge of the subject from the excellent

publications on the subject, for example, Old and Primrose (1989), Singer and Berg (1991), Winnacker (1987) and Watson *et al.* (1987).

Recombinant DNA technology was born out of the painstaking research of microbial geneticists, and it seems only fair that microbiology in general, and bacteriology in particular should have benefitted from its invention. For one community of research workers, those whose interest lies in the molecular basis of bacterial pathogenesis, gene cloning has become a powerful and enabling tool (Dougan, 1989; Finlay and Falkow, 1989). The complex molecular interactions made between a bacterial pathogen and its host organism (the majority under strict genetic control), constitute an area of investigation particularly suited for the application of gene cloning (Miller *et al.*, 1989; Dorman and Ni' Bhrain, 1992). The advantages of a gene cloning approach to this area are strikingly evident, and the various genetic manipulation and characterization techniques have been applied with some success.

2.10.1. MOVING GENES AROUND.

The major advantage of cloning genetic material from bacterial pathogens comes from the ability to move genes encoding putative pathogenicity and virulence factors into a so-called 'clean' host cell in which their structure, function and regulation may be readily studied (Singer and Berg, 1991; Finlay, 1992). The host cell used in the majority of experiments is *Escherichia coli* strain K12. This strain is an attractive choice because it has been the standard tool of microbial genetics for many years, it not only supports the replication of a large number of bacteriophage and plasmids that are potentially useful vectors for foreign (target) DNA, but its genetic and physiological behaviour is known in considerable detail (Neidhart *et al.*, 1987). A number of other prokaryotic and eukaryotic cell types have also been used as host/vector systems, but all fulfill the same function, namely the propagation and possible expression of the gene(s) of interest away from the often confusing and interfering molecular mechanisms of the organism from which the

gene was isolated. In this sense, gene cloning may be seen as a technique of biochemical dissection, the selective removal of a genetic sequence and its encoded molecule(s) from a complex biochemical/ physiochemical environment for further study (Dougan, 1989).

Once successfully introduced into its new host, the DNA of interest can be investigated in many ways. Firstly, if the DNA contains a gene encoding a particular molecule thought to be involved in bacterial pathogenicity, and the means exist to detect this molecule, it may be possible to express this molecule *in vitro*, isolate and purify it for further characterization. The value of this approach was brilliantly illustrated by Stanley Falkow and his colleagues at Stanford University in the late 1970's. These workers, probably the first people to apply molecular approaches and gene cloning to the study of bacterial virulence, succeeded in cloning the heat-stable and heat-labile toxins (Smith and Linggood, 1971) of enterotoxigenic *Escherichia coli* strains. This work, (So *et al.*, 1976, 1978) played a significant role in the subsequent molecular characterization of these important bacterial toxins. The cloning and expression of bacterial pathogenicity and virulence factors is now commonplace, even a cursory examination of the published literature reveals a wealth of information obtained using these techniques.

2.10.2 THE STUDY OF GENETIC CONTROL.

The expression of genes involved in bacterial pathogenicity and virulence is often governed by complex control mechanisms (Finlay and Falkow, 1989), involving coordinate alterations in the expression of sets of genes and operons in response to environmental stimuli of both a biochemical and physiochemical nature (Miller *et al.*, 1989). Gene cloning and manipulation has enabled detailed studies into the control of various bacterial virulence genes (Stock, 1989), and a particularly illustrative example of this approach is the research carried out on *Bordetella pertussis*, the aetiological agent of whooping cough. The virulence determinants of *B. pertussis* are all regulated by a single genetic locus *vir*, encoding at least 20 gene products including filamentous haemagglutinin

(Cowell *et al.*, 1982), pertussis toxin (Katada and Ui, 1982) adenylate cyclase (Wolff *et al.*, 1980), haemolysin, fimbrial subunits and dermonecrotic factor (Cowell *et al.*, 1979). By using gene cloning techniques, Alison Weiss and her co-workers were able to study the *vir* locus in detail, establishing its control by factors such as temperature fluctuation and varying levels of chemical compounds such as magnesium sulphate and nicotinic acid (Weiss *et al.*, 1983; Weiss and Hewlett, 1986). To date, a wide range of bacterial virulence genes have been studied utilising gene cloning techniques, other notable examples being the *vir* genes of *Shigella* (Maurelli and Sansonetti, 1988), the *yop* genes of *Yersinia pestis* (Pollack, 1984) and the *tox* genes of *Vibrio cholerae* (Miller *et al.*, 1987).

2.10.3.THE STUDY OF GENE STRUCTURE

Genetic manipulation techniques also allow the study of the fine structure of cloned DNA fragments. By using methods developed not long after the introduction of recombinant DNA techniques (Sanger *et al.* 1977), the nucleotide base sequence of the gene under study can be determined. DNA sequencing has generated much information about many pathogenicity and virulence factors. Besides allowing the determination of the predicted primary structure of the factor under study, it can often yield clues about the function of the gene product if amino acid homology is found with previously characterised proteins. Amino acid homology searches often define groups of related proteins, whilst discrete areas of amino acid sequence homology may provide clues to the functions of various domains (Finlay, 1992). A good example of the application of nucleotide base sequencing and subsequent amino acid sequence homology analysis is the research undertaken by workers studying the 15 functionally and immunologically related bacterial toxins, known as the thiol-activated cytolysins (Geoffroy and Alouf, 1984). These toxins are produced by Gram-positive bacterial species, namely by the 4 genera *Streptococcus, Bacillus, Clostridium* and *Listeria*, and the initial characterization of these

molecules suggested a similar mechanism of action and possible common evolutionary origin (Kehoe and Timmis, 1984). However, in this work, the authors, in hybridization experiments using cloned toxin genes, failed to detect homology with DNA from species producing thiol-activated toxins. Kehoe's research group then suggested that these toxin genes may have undergone a considerable degree of divergence and that functionally important structures might therefore only be identifiable as conserved regions in the primary amino acid sequences of the proteins. This was confirmed by Kehoe *et al.* (1987) by comparing the amino acid sequences of streptolysin O and pneumolysin predicted from the DNA sequence of the coding genes. It was found that when the amino acid sequences were aligned, 42% of the residues matched exactly and that alignment of the sequences using similar residues showed even higher homology. This work resulted in valuable clues to the structure and function of the thiol-activated toxins, which are now recognised as important determinants of bacterial pathogenicity (Portnoy *et al.*, 1992).

2.10.4. PRODUCTION OF DNA PROBES.

Cloned genes from bacterial pathogens, may also be used as sensitive DNA probes to be utilized in a number of different studies. Using cloned DNA in hybridization experiments, allows the research worker to search for the organism from which it was isolated in both the environment and in infected tissues and cells (Viscidi and Yolken, 1987). These epizootic studies allow investigation of possibly undetected habitats of a given pathogen, plus offer new approaches to disease detection and pathogenesis (Walker and Dougan, 1989; Hopkin and Wakefield, 1990; Macario and Macario, 1990). DNA probes may also used in taxonomic studies, discovering other genera or species containing the same or similar genes. The studies made by Neill *et al.*,(1990) on *Staphylococcus aureus*, and by Datta *et al.*(1990) on *Listeria monocytogenes* indicate the utility of gene probes in the detection of virulence factors and the subsequent detection and characterization of pathogenic species of bacteria. In the former research project (Neill *et al.*, 1990), a

number of DNA sequences were evaluated as gene probes for the specific detection and differentiation of *S. aureus* strains containing genes encoding enterotoxins A (SEA), B (SEB) and C (SEC) (Spero *et al.*, 1988) and toxic shock syndrome toxin 1 (TSST-1) (Blomster-Hautamaa *et al.*, 1988). Identification of sequences unique to each toxin gene, based on knowledge of their nucleotide sequences, led to the preparation of specific 18-base oligonucleotide probes for use in colony hybridization experiments. These probes were found to hybridize specifically with DNA from toxin-producing strains and there was over 90% correlation between the hybridization results and toxin protein production as detected by immunoassay. The authors concluded that the synthetic oligonucleotide probes provided an effective alternative approach for the study of *S. aureus* toxigenicity *in vitro.* The second gene probe study (Datta *et al.*, 1990), utilized a portion of cloned DNA containing the listeriolysin O toxin gene to produce 2 specific gene probes for *Listeria monocytogenes.* Hybridization experiments confirmed the specificity of each probe and it was found that the probes could be used to differentiate between pathogenic *L. monocytogenes* and other non-pathogenic *Listeria* species and it was concluded that the DNA probes could be readily utilized in further studies of the epidemiology of listeriosis and the detection of virulent *L. monocytogenes.*

2.10.5. CONSTRUCTION OF MUTATED GENES.

Mutant bacterial strains can be powerful tools for the analysis of bacterial pathogenicity and virulence, and strains differing in the expresssion of a putative virulence factor can be used in both *in vitro* and *in vivo* studies of disease progression (Smith, 1990). Recombinant DNA technology and gene cloning has provided the means for both controlled and defined mutation of DNA sequences, resulting in great advances in our knowledge of the molecular basis of bacterial infection.

A study of the published literature concerned with the mutation of putative virulence genes via genetic manipulation reveals that this has been acheived utilizing a number of

different methods. However, for purposes of classification and review these methods can be seen to fall in to two groups; Transposon mutagenesis and site-directed mutagenesis.

Transposons are mobile genetic elements which can insert at random into plasmids or the bacterial chromosome independently of the host cell recombination system. The insertion of a transposon into the DNA disrupts the nucleotide sequence and this produces a mutation. Apart from the genes involved in transposition, transposons also carry genes conferring new phenotypes on the host cell, and these phenotypes (usually antibiotic resistance) are used to identify possible mutant bacteria and those containing mutated plasmids. By producing mutations in this way, genes coding for specific virulence factors can be identified and aspects of their structure and function studied.

An example of the use of transposon mutagenesis in the study of a putative virulence factor is shown by the work of Gaillard *et al* (1986). These authors used the transposon Tn1545 to mutate the gene encoding the haemolytic toxin protein from the Gram-positive pathogen *Listeria monocytogenes* in an attempt to elucidate its role in the virulence of the bacteria. Virulent, haemolytic strains of *L. monocytogenes* were exposed to Tn 1545 and resultant mutated organisms deficient in the production of haemolytic toxin selected. The DNA of the mutant bacteria was isolated and purified, and then used in Southern hybridization experiments to identify those mutants carrying a single inserted transposon. The virulence of the defined mutants was assayed using a mouse model and found to be considerably reduced compared to wild type *L. monocytogenes*. confirmation of the contribution of haemolytic toxin to virulence was obtained by the restoration of a virulent phenotype observed in a toxin producing revertant obtained by the spontaneous loss of transposon Tn 1545. A large number of transposons are available for use in the mutagenesis of both Gram-positive and Gram-negative bacterial species (Foster 1991). These transposons can be delivered to the DNA of interest using bacteriophage or plasmid

vectors (Foster 1984), as conjugative vectors (Gaillard *et al.*, 1986) or by so-called 'suicide' plasmids (Miller and Mekalanos, 1988).

An advantage of transposon mutagenesis is that it can be performed without knowledge of the structure of the gene coding for the putative virulence factor being inactivated. This is not the case with the second type of mutagenesis methodology, whose utility is dependent on a detailed knowledge of the gene sequence to be mutated. The site-directed mutagenesis methods depend on the use of *E. coli* host/ vector systems to mutate cloned gene sequences by the insertion, deletion or substitution of nucleotide bases. These precise controlled mutations can then be used to study both structural and functional aspects of the gene under study. The simplest method of site-directed mutagenesis is the single primer method (Zoller and Smith, 1983) involving the priming of DNA synthesis with a synthetic oligonucleotide carrying a base mismatch within its complementary sequence. After synthesis, the newly synthesized DNA will bear the directed deletion, insertion or substitution mutation (Old and Primrose, 1989). Oligonucleotide-mediated mutagenesis was used with some success in the study of the structure/ function relationships of the thiol-activated toxins of the Gram-positive pathogens *Streptococcus pyogenes, Streptococcus pneumoniae* and *Listeria monocytogenes.* (Boulnois *et al.*,1989; Saunders *et al.*, 1989; Pinkney *et al.*, 1989; Michel *et al.*, 1990; Pinkney and Kehoe, 1990). These authors produced mutations in the genes of these toxins to facilitate the substitution of amino acid residues thought to be vital to toxic activity, and compared the *in vitro* haemolytic activities of the altered protein produced with that encoded by the wild type gene. Amino acid substitutions of the single cysteine residue, and alternate substitutions in three tryptophan residues aided the decision that the toxicity of the thiol-activated cytolysins was dependent on the presence of a tryptophan residue and not a cysteine in the conserved 11 residue sequence of amino acids (Geoffroy and Alouf, 1991).

Another valuable directed mutagenesis method, used for gene inactivation

in virulence studies is recombinational allelic replacement. As the name suggests, this method is dependent on a homologous recombination event in which wild type bacterial DNA is replaced by a mutated homologous region of DNA isolated and characterized by gene cloning techniques (Foster, 1991). As is the case with other site-directed mutagenesis methods, the design of an allelic replacement experiment depends on the prior cloning and sequencing of the gene in question. Similar controls to those described above for transposon induced mutants should also be performed in order to demonstrate that the *in vitro* mutation has displaced the wild type allele (Finlay, 1992; Foster, 1992). The use of allelic replacement is shown by the work of Szeto and Shuman (1990) on the major secretory protease (Msp) of *Legionella pneumophila* (Dreyfus and Iglewski, 1986). Several lines of evidence suggest that Msp may be an important virulence determinant in the pathogenesis of Legionnaires disease (Horwitz, 1992). A mutation in a cloned Msp gene was induced using the transposon Tn9, resulting in the loss of Msp expression. This mutated gene was isolated and then cloned into a plasmid incapable of replication in *L. pneumophila*, when the recombinant plasmid was introduced into wild type *L. pneumophila* the resulting allelic exchange resulted in a mutant organism with less than 0.1% of the protease activity of the wild type cell. The mutant bacterium however, was able to multiply within and kill human macrophages. From these experiments, it was concluded that intracellular existence and Msp production are not linked and therefore the proteolytic activity of *L. pneumophila* is not a significant influence on this phase of Legionnaires disease.

2.10.6. THE STUDY OF FASTIDIOUS PATHOGENS.

By combining the ever expanding repertoire of gene cloning techniques, it is become possible for the research scientist to gain a valuable insight into the role of specific genes and gene products in the complex multifactorial process of bacterial pathogenicity. Genetic manipulation has both supported and enhanced our knowledge of previously well

characterized pathogens, it has also, more importantly allowed essential investigation into those bacterial species for which, to a larger degree, the range of molecules and mechanisms involved in the disease process is unknown.

For two species of fastidious bacteria in particular, gene cloning techniques have become invaluable in providing a long awaited impetus for pathogenicity studies. Both cause significant worldwide infections of humans and are largely resistant to even modern *in vitro* culture, these organisms are the leprosy bacillus *Mycobacterium leprae* and the aetiological agent of syphilis , *Treponema pallidum.*

The limited multiplication of *T.pallidum* in *in vitro* culture has long restricted the isolation and characterization of macromolecules important in the pathogenesis of syphilis (Norris, 1982). The most widely used cultivation method, utilizing the testis of rabbits (Baseman, 1990) is seen as far from ideal as *T. pallidum* components can only be isolated in small amounts, and are prone to contamination by host proteins (Hsu *et al.*, 1988).

The last decade of syphilis investigation has been enriched with the developments of recombinant DNA technology, a particular benefit being the ability of gene cloning to provide relatively large quantities of purified and well characterized proteins for studies of *T. pallidum* pathogenicity (Norris *et al.*, 1987; Strugnell *et al.*, 1990; Schouls, 1993). The first successful gene cloning studies were performed in the early 1980s (Fehniger *et al.*, 1984), and have continued apace to this date, resulting in the identification of 26 different recombinant antigens (Schouls, 1993). Cloning has been particularly useful in the isolation of membrane proteins (Fehniger *et al.*, 1986; Norgard *et al.*, 1986; Hindersson *et al.*, 1987; Peterson *et al.*, 1987) as well as endoflagellar subunits (Issacs *et al.*, 1989; Palleson and Hindersson, 1989; Champion *et al.*, 1990).

Genetic engineering as applied to the leprosy bacillus *Mycobacterium leprae*, has followed a somewhat similar path to that of *T. pallidum*. Again, the failure to cultivate *M. leprae in vitro* has proved problematic, culture methods using the footpads of mice,

and more recently heavily infected armadillos are far from suitable (Wolinsky, 1990). The potential of cloning in studies of *M. leprae* was first discussed in print by Young *et al.* (1985) after which a great deal of effort was made to utilize the technology. The first genes to be cloned from mycobacteria were those encoding protein recognised by monoclonal antibodies and by T-lymphocytes (Young *et al.*, 1985; Mustafa *et al.*, 1986; Ottenhoff *et al.*, 1986). Over 20 *M.leprae* antigens have been isolated using gene cloning, and in several cases, nucleotide sequence analysis of these genes has allowed identification of the functional role of the encoded protein (Young *et al.*, 1990). Notably, a number of prominent antigens have been identified as members of conserved heat-shock protein families (Young *et al.*, 1988; Mehra *et al.*, 1992). A fruitful approach in the detection of recombinant *M. leprae* antigens has involved the use of serum from leprosy affected patients (Sela, 1991). Gene cloning has also stimulated other ambitious studies of mycobacteria, one project already in progress is dedicated to the characterization of the *M. leprae* genome (Young and Cole, 1993), whilst other genetic manipulation studies into interspecies gene transfer in mycobacteria and the expression of foreign gene sequences (Jacobs *et al.*, 1987; Husson *et al.*, 1990).

The contribution of gene cloning techniques to the detailed molecular analysis of bacterial pathogens should not be underestimated. There have been great advances in our knowledge of a huge number of different species, the techniques have been used to both accentuate bacteriological experimentation and to provide an ever expanding repertoire of methods for new research direction.

CHAPTER 3: MATERIALS AND METHODS.

3.1 LABORATORY REAGENTS & GROWTH MEDIA.

Details of formulation and preparation of the laboratory reagents and growth media, can be found in Appendix I at the end of this thesis.

3.2 BACTERIOLOGY.

3.2.1 BACTERIAL STRAINS.

The strains of *Renibacterium salmoninarum* utilised in this thesis are show in chapter 4 (Table 4.1), whilst the various strains of *Escherichia coli* K12 used in gene cloning experiments of this project are shown in Table 3.1.

3.2.2 LABORATORY CULTURE AND STORAGE OF BACTERIA.

Unless stated otherwise, the routine culture of *R.salmoninarum* was performed using Mueller-Hinton medium supplemented with 0.1% (w/v) cysteine hydrochloride (MHC). MHC agar was produced by the addition of 1.5% w/v agar No.2 (Oxoid) which was dissolved by steaming prior to autoclaving. Cultures were incubated aerobically at 15°C. in a cooled incubator. Petri dishes containing agar cultures of *R.salmoninarum* were sealed with paraffin film to prevent contamination during extended incubation periods. Visible microbial contamination was removed by careful excision of the affected area of agar using a sterile scalpel.

Formulations of other media utilised in the culture of *R.salmoninarum* during this project, can be found in Appendix I.

Strains of *E.coli* K12 were routinely cultured using Luria-Bertani (LB) medium. LB agar was produced by the addition of 1.5% w/v agar No.2 (Oxoid) which was dissolved by steaming prior to autoclaving. Those strains bearing plasmids were cultured in LB medium supplemented with the appropriate selective antibiotic(s). Cultures were incubated aerobically at 37°C.

Both *R.salmoninarum* and *E.coli* K12 strains were stored frozen as follows; cells

Q359	*supE hsdR φ80 P2*	A *supE* host used to select Spi⁻ λ recombinants
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)*	A recombinant-deficient *lac*⁻ strain for use with vectors carrying the β-galactosidase gene.
DH1	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	A recombinant deficient strain used for routine plating and growth of plasmids and cosmids.
C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	A suppressor strain often used for making phage lysates
XL1-BLUE	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻ F'[proAB lacI^q lacZΔM15 Tn10(tet^r)]*	Improved blue/white screening strain, used in this project for selection of plasmids for DNA sequencing
Y1089	*araD139 ΔlacU169 proA+ Δlon rpsL hflA150 [chr::Tn10(tet^r)] pMC9*	A strain used for gene cloning in λgt11, deficient in the *lon* protease ,which may allow increased stability of foreign proteins.
Y1090	*supF hsdR araD139 Δlon ΔlacU169 rpsL trpC22::Tn10 (tet^r) pMC9*	A strain used for screening of expression libraries and propagation of λgt11
DS410	*F' rpsL minA minB*	A mini-cell producing strain for translation analysis of recombinant plasmids.

Table 3.1 Strains of *E. coli K12* used in this research project

harvested from routine culture were resuspended in the appropriate growth medium containing 20% (w/v) sterile glycerol as a cryoprotectant, and distributed in 1ml aliquots in polypropylene microcentrifuge tubes. Tubes were flash frozen in liquid nitrogen and stored at -70°C. Additionally, plate cultures of *E.coli* K12 strains for routine use were stored at 4°C, subcultures were made every 4 to 6 weeks.

3.2.3 CHARACTERIZATION OF BACTERIAL CULTURES.

Bacterial cultures were routinely characterized to establish culture purity. Cultures of *R.salmoninarum* were checked by Gram stain (Burke, 1922) and microscopic examination, followed by enzyme testing. The criteria for purity was a monoculture of Gram positive small diplobacilli, giving negative oxidase reaction and positive catalase reaction (Cruickshank *et al.* 1975).

Cultures of *E.coli* K12 were confirmed by Gram stain and microscopic examination, and by the inhibition of growth on glucose minimal medium by valine.

3.3 BACTERIOPHAGE λ METHODS.

3.3.1 PLATING BACTERIOPHAGE λ.

The routine method for plating bacteriophage λ was as follows:

A 10 ml culture of the appropriate *E.coli* host strain was grown overnight in LB broth supplemented with 0.2% (w/v) maltose (to increase number of phage receptors (Sambrook *et al.*,1989)) at 37°C.

A set of tenfold serial dilutions (10^{-2} to 10^{-8}) were made of the phage suspension using SM buffer. A 0.1 ml volume of the phage dilution to be assayed was placed into a capped 5 ml test tube along with 0.2 ml of an overnight culture of the host *E.coli* culture, mixed gently and left at 37°C for 30 min. to allow the phage to adsorb. Next, a 3 ml volume of molten soft agar was added to each tube, the contents were mixed gently, and poured to cover the surface of an LB agar plate. Each agar overlay was allowed to set for approximately 5 min., and unless stated otherwise, the plates were incubated overnight

at 37°C.

3.3.2 PREPARATION OF BACTERIOPHAGE λ STOCKS.

3.3.2.1 STOCK SUSPENSIONS FROM SINGLE PHAGE PLAQUES.

Distinct, well separated plaques were removed as plugs from agar overlays using a sterile Pasteur pipette, and then placed into 1 ml of SM buffer containing 10 μl of chloroform to kill any bacteria present. A period of 1 h. was allowed to facilitate the diffusion of phage particles from the agar, after which the suspension was briefly vortexed. This stock suspension was stored at 4°C. these suspensions remain viable for long periods (in some cases; years).

3.3.2.2 STOCK SUSPENSIONS FROM CONFLUENT LYSIS PLATES.

Larger stocks of phage were prepared from whole plate lysates. Dilutions of the appropriate phage were plated out as in the above method, and those plates exhibiting near-confluent lysis chosen for phage preparations. The soft agar overlay containing phage was removed by gently scraping with a sterile glass spreader. The agar was macerated using the spreader, placed into a microcentrifuge tube and centrifuged at 13,000 x g for 10 min.. The resultant supernatant was added to an equal volume of SM buffer containing a small amount of chloroform. And stored at 4°C until use .

3.4 PROTEIN ANALYSIS TECHNIQUES.

3.4.1 CONCENTRATION OF BACTERIAL PROTEIN SOLUTIONS.

Bacterial protein solutions, either broth culture supernatant or cell lysate were routinely concentrated using ammonium sulphate (Scopes, 1982). Solid ammonium sulphate was added slowly to precooled (4°C) protein solution and allowed to dissolve, giving a final concentration of 662 mg/ml (90% saturation). This solution was placed at 4°C for at least 1 h. to facilitate protein precipitation. Protein was then pelletted by centrifugation at 15,000 x g for 30 min. at 4°C, and redissolved in a small volume of PBSA. Ammonium sulphate was removed by dialysis against several changes of PBSA at 4°C The protein

content was determined, and the concentrate stored frozen at -70°C .

3.4.2 DETERMINATION OF PROTEIN CONCENTRATION.

The concentration of proteins in solution was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Herts., U.K.) utilising the protein-dye binding assay described by Bradford (1976). Standard protein solutions were made using bovine serum albumin (BSA) dissolved in PBSA.

3.4.3 SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was performed using a discontinuous buffer system as described by Hames and Rickwood (1986). The electrophoretic apparatus used in this project was either the Mini-Protean II system (Bio-rad, Herts.,U.K.) or the LKB 2001 vertical electrophoresis system (LKB, Bromma, Sweden). Electrophoresis gels consisted of a main, separating/resolving gel, with an upper stacking gel formed between two glass plates. Polymerization of gels was performed using the ammonium persulphate(APS)/TEMED system. Details of gel formulation and reagents used can be found in Appendix I.

3.4.3.1 FORMATION OF SDS/PAGE GELS.

Separating gel mix of the appropriate concentration were prepared without APS and de-gassed under vacuum for 15 min. APS was added and the gel mix was poured into the gel former. The preparation was overlayed with a small amount of water saturated isobutanol to exclude air and thus promote gel polymerization. A period of 45 min. was allowed for polymerization to occur. After which, the isobutanol was removed and the top of the gel washed with distilled water to remove traces of isobutanol and unpolymerized gel mix. The upper stacking gel was formed in the same way as the lower gel by adding APS to a de-gassed gel mix and pouring into the gel former. Sample wells were constructed in the stacking gel by inserting the desired well-forming comb prior to polymerization. Another period of 45 min. was allowed for the stacking gel to form. The comb was

removed and the preformed sample wells washed out with both distilled water and electrode buffer before use.

3.4.3.2 ELECTROPHORESIS.

Complete gel sandwiches were placed into the electrophoresis apparatus and the reservoirs filled with electrode buffer. Samples were electrophoresed at 200 V/45 min. (Mini-Protean) or 350 V/4 h. (LKB/2001).

3.4.4 STAINING OF SDS/PAGE GELS.

Gels from SDS/PAGE were stained for protein utilising the coomassie brilliant blue-R stain as described by Hames and Rickwood (1986). The method was as follows. Electrophoresed gels were removed carefully from the glass sandwich support and placed in coomassie blue fixing and staining solution. The gels were allowed to fix/stain for a period of 18 h. at ambient temperature with gentle agitation. After this time the gels were removed and destained by gently agitating in several changes of destaining solution over a period of 4-5 h.

3.4.5 IMMUNOBLOTTING (WESTERN BLOTTING).

3.4.5.1 PROTEIN TRANSFER.

Proteins separated by SDS/PAGE were transferred from polyacrylamide gels to nitrocellulose membranes according to the electrophoretic transfer method of Towbin *et al* (1979). The apparatus utilised was either the Bio-Rad Transblot system (Bio-Rad, Herts.,U.K.) or the semi-dry Milliblot system (Millipore, Watford, U.K.). Electrophoretic transfer was carried out in the manafacturers recommended buffer at 30 mA/18h. (Transblot) or 275 mA/ 45 min. (Milliblot).

3.4.5.2 IMMUNOSTAINING.

Blots were initially equilibrated in PBSA for 10 min, after which, the free protein binding sites on the nitrocellulose membrane were blocked using a solution of 2% w/v skimmed-milk in PBSA (PBSM). Blocking was carried out at ambient temperature with gentle

agitation for 60 min. After blocking, the membrane was washed for 2 x 5 min. in PBSA. Primary antibody exposure was performed by incubating the membrane for 90 min. at ambient temperature in PBSM containing the optimal concentration of serum or antibody. After this time period, the membrane was rinsed in PBSA using 4 x 5 min. washes, to remove unbound antibody. The bound antibody was then labelled with an peroxidase/secondary antibody conjugate at the appropriate concentration. Unless otherwise stated, this antibody was peroxidase-conjugated swine anti-rabbit immunoglobulins (P217, DAKO). Again, exposure was performed by incubating the membrane in PBSM/antibody solution for 90 min. at ambient temperature and the membrane washed for 4 x 5 min. in PBSA. Bound, labelled antibody was visualized by development using the 3'3' diaminobenzidine tetrahydrochloride (DAB) system with Nickel enhancement.(Harlow & Lane, 1988). A positive reaction product giving a purple/brown colour.

3.4.6 PRODUCTION OF ANTISERA.

3.4.6.1 ANIMALS.

New Zealand white rabbits, were kept at the Animal House, University of Plymouth. All procedures were carried out under Home Office licence PPL 30/00176.

3.4.6.2 IMMUNIZATION.

Unless otherwise stated, Immunizations were made using antigen preparations with a protein concentration of 0.5-1.0 mg/ml Initial immunization consisted of a sub-cutaneous injection of 1 ml of antigen preparation combined with an equal volume of Freunds complete adjuvant (FCA) whilst subsequent 'boost' challenges, made at 4 week intervals, were made using the same volume of antigen combined with Freunds incomplete adjuvant (FIA).

3.4.6.3 PREPARATION OF ANTISERA.

Test bleeds to ascertain the level of circulating specific antibody were performed utilising

the marginal ear vein. Qualitative estimation of antibody levels were made by immunoblotting using dilutions of the antigen preparation. Blood from immunized animals exhibiting suitable specific antibody levels was taken via the marginal ear vein or by total exsanguination using cardiac puncture under anaesthesia. The serum from these blood samples was harvested by allowing the whole blood to clot at 37°C for 1 h. and by contracting the clot at 4°C for 18 h. Serum was then removed by a sterile Pasteur pipette and aliqotted into 1.5 ml microcentrifuge tubes. Serum samples were stored at -70°C. Once thawed, antiserum samples were stored at 4°C and protected from microbial contamination by the addition of sodium azide to a final concentration of 0.02% (w/v).

3.5 DNA ANALYSIS METHODS.

3.5.1 ISOLATION AND PURIFICATION OF BACTERIAL GENOMIC DNA.

Genomic DNA was isolated from the bacterial species used in this project by either a modification of the method of Marmur (1961), or by ultracentrifugation.

3.5.1.1 LYSIS OF BACTERIA.

Bacteria were lysed by one of the two methods described below. Gram-negative species by method 1. and Gram positive species utilising method 2.

METHOD 1.

Bacteria from a 2 l. broth culture grown to stationary phase were harvested by centrifugation at 5,000 x g for 20 min. at 4°C These cells were then lysed according to the method of Borck *et al* (1976) using lysozyme and Triton X100. The harvested cells were resuspended in 50 ml. of an ice-cold sucrose/Tris solution, to which a 7 ml. volume of lysozyme solution was added and the suspension shaken gently at 37°C for 1 min. and placed on ice for 10 min. To this cooled suspension, a 25 ml volume of ice-cold EDTA solution was added followed after a period of 5 min. by 50 ml of Triton lysis solution. Lysis of cells was usually complete after a further 30 min. on ice.

METHOD 2.

Bacterial cells were harvested by centrifugation at 5,000 x g for 20 min. at 4°C and resuspended in 30 ml of TE buffer containing 0.5% SDS and 25 μg/μl Protease K. After thorough mixing, the suspension was placed at 60°C for 1h. or until lysis had occurred. The bacterial lysate was centrifuged at 15,000 x g for 30 min. at 20°C, after which the nucleic acids were precipitated using isopropanol (see 3.5.3 below). The pelletted precipitate was then subjected to purification by eqilibrium centrifugation in caesium chloride-ethidium bromide gradients (3.5.4.1 below).

3.5.1.1 DNA ISOLATION BY THE MARMUR METHOD.

Perchlorate was added to the bacterial lysate to a final concentration of 1 M and the mixture shaken with an equal volume of chloroform/isoamyl alcohol mixture (24:1) for 30 min. in a conical flask with a ground glass stopper. The resulting emulsion was separated into 3 layers by centrifugation at 5,000 x g for 5 min. at ambient temperature. The upper aqueous phase containing the nucleic acids was carefully removed using a Pasteur pipette in to a beaker. The nucleic acids were precipitated by gently layering 2 volumes of 95% ethanol on the aqueous phase and mixing the layers gently with a glass rod, at the same time 'spooling' the threads of precipitate. The precipitate was drained free of excess ethanol and dissolved in 15 ml of dilute saline-citrate solution, the concentration of this solution was then adjusted to that of standard saline citrate by the addition of an amount of concentrated saline-citrate.

The nucleic acid solution was then shaken as before with an equal volume of chloroform-isoamyl alcohol mixture for 15 min., centrifuged and treated as above. This deproteinisation proceedure was repeated several times until no protein was observed at the interface of the organic and aqueous layer. The final supernatant was precipitated with 95% ethanol and the precipitate dissolved in saline citrate (approx. half of supernatant volume). RNase was added to a final concentration of 50μg/ml. and the mixture incubated

for 30 min. at 37°C. Following RNA digestion, the solution was again subjected to the deproteinisation proceedure as described above. After deproteinisation, the nucleic acid was again precipitated with 95% ethanol, drained and dissolved in 9 ml of dilute saline-citrate. To this solution, 1 ml. of acetate-EDTA solution was added. The solution was rapidly stirred with a glass rod attached to a motorised stirrer and a 0.54 volume of isopropanol added dropwise into the vortex. The DNA precipitated as fibrous threads which wound around the glass rod. The DNA was redissolved and precipitated as before and the precipitate, adhering to the glass rod, was washed free of acetate by stirring the rod in progressively more concentrated solutions of ethanol. The precipitate, finally drained of ethanol, was dissolved in a 1 ml volume of TE buffer.

3.5.2 ISOLATION OF PLASMID DNA FROM *Escherichia coli* .

3.5.2.1 MINIPREP METHOD.

This rapid method, based on the alkaline extraction technique of Birnboim & Doly (1979), produces plasmid DNA of sufficient purity for use as a substrate for restriction endonuclease cleavage and transformation.

A 1.5 ml volume of an overnight culture of the appropriate plasmid bearing strain of *E. coli* was pipetted into a plastic snap-top microcentrifuge tube. The bacteria were then pelletted by centrifugation in a microcentrifuge for 30 s. at 13,000 x g. The supernatant was discarded and the cells resuspended in 100 μl of B & D solution I followed by 10 min. incubation at ambient temperature. After this time, a 200 μl volume of freshly prepared B & D solution II was added and mixing was effected by gentle inversion of the tube. The mixture was placed on ice for 10 min. after which a 150 μl volume of ice cold B & D solution III was added followed by gentle mixing and the return of the tube to ice for a further 10 min.

The resultant white floculant precipitate formed at this stage of the proceedure was pelletted by 10 min. centrifugation at 13,000 x g and the supernatant (approx. 400 μl)

transferred to a fresh microcentrifuge tube. The plasmid DNA was precipitated by adding a 1 ml volume of analytical grade ethanol, mixing, and incubating on ice for 5-10 min. Precipitated DNA was pelletted by centrifugation at 13,000 x g for 5 min., dried of ethanol under vacuum, and resuspended in 50 μl of RNase solution. The solution was incubated at 37°C for 1 h., after which, the RNase and residual bacterial proteins werwe removed by phenol/chloroform extraction. The plasmid DNA was precipitated, pelletted and dried as before and finally resuspended in 10 μl of TE buffer.

3.5.2.2 MAXIPREP METHOD.

This method, again a variation of Birnboim & Doly (1979), was used to isolate plasmid DNA from larger volumes of bacterial cells. Up to 2 litres of broth culture could be processed using this method, providing a large amount of plasmid for use as a convenient laboratory stock.

Bacterial cells were harvested by centrifugation at 5,000 x g for 15 min. at 4°C and resuspended in 10 ml of B & D I solution. The suspension was left at ambient temperature for 10 min. Lysis of the bacteria was facilitated by the addition of 20 ml of B & D II solution, mixing gently, and incubating on ice for 15-20 min. Bacterial cell debris and genomic DNA were complexed by adding 15 ml of ice-cold B & D III solution, mixing, and returning the tube to ice for a further 10 min. The complexed material, present as a floculant white precipitate was pelletted by centrifugation. Supernatant liquid from this centrifugation was removed and placed in a 250 ml centrifuge tube. Plasmid DNA was then precipitated by the addition of 0.6 volumes of isopropanol, incubation at ambient temperature for 10-15 min. and centrifugation at 5,000 xg. for 20 min. at 4°C. The resultant pellet was drained, dried and dissolved in a suitable volume of TE buffer prior to purification by caesium chloride gradient equilibrium centrifugation.

3.5.3 CONCENTRATION OF DNA SOLUTIONS.

DNA solutions were routinely concentrated in the laboratory by precipitation with ethanol or isopropanol. A 1/10 volume of 3M sodium acetate was added to the solution to facilitate precipitation, followed by either 2.5 volumes of ice cold analytical grade ethanol or 0.6 volumes of isopropanol. Precipitation was allowed to occur in ethanol for 10 min. - 2h. at < 0°C or in isopropanol at ambient temperature for 15-30 min. Unless otherwise stated, the precipitate was pelletted by centrifugation at 13,000 xg for 10 min. in a microcentrifuge. After which, the supernatant solution was removed and the pellet washed in 70% v/v ice-cold ethanol prior to another centrifugation step. The final pellet was then dried under vaccuum and resuspended in an appropriate volume of TE buffer.

3.5.4 DNA PURIFICATION.

3.5.4.1 EQUILIBRIUM ULTRACENTRIFUGATION IN CAESIUM CHLORIDE-ETHIDIUM BROMIDE GRADIENTS.

High purity solutions of both genomic and plasmid DNA were prepared by eqilibrium centrifugation in caesium chloride-ethidium bromide gradients (Sambrook *et al.* 1989). DNA molecules separate out in these gradients according to their density into discrete bands and can be readily harvested for use in a wide range of genetic manipulation techniques.

The method described here is designed for use in the Beckman L7 Ultracentrifuge (Beckman,Bucks.,UK) utilising 38 ml 'Quick-Seal' tubes in the Ti 70i rotor.

The nucleic acid pellet to be purified was dissolved in 28 ml of TEB. To this, 31 g of caesium chloride was added slowly until solution was achieved. A 3 ml volume of ethidium bromide solution was added, gentle mixing was performed and the solution placed in the dark at ambient temperature for 18 h. During this time period a complex of caesium chloride, ethidium bromide and bacterial protein is formed and is visible as a floculant pink 'scum' on the surface of the solution. This unwanted material was then

removed by centrifugation at 5,000 x g for 15 min. at 20°C.

The supernatant containing the DNA was the dispensed using a Pasteur pipette into the ultracentrifuge tube. The tubes were then balanced to within 50 mg of each other, sealed, and centrifuged at 40,000 rpm. for 48 h. at 20°C.

Smaller, quicker preparations were often made using the Beckman L100 Micro-ultracentrifuge. For this method, volumes of TEB, caesium chloride and ethidium bromide were scaled down to fit 3.5 ml 'Quick-Seal' tubes. Gradients were formed by centrifugation at 80,000 rpm. for 24 h. at 20°C.

After centrifugation, DNA bands were visualized using U.V. light and removed using a hypodermic syringe fitted with a large gauge needle.

3.5.4.1.1 REMOVAL OF ETHIDIUM BROMIDE AND CAESIUM CHLORIDE FROM DNA PURIFIED BY ULTRACENTRIFUGATION.

Ethidium bromide was removed from purified DNA solutions by organic phase extraction using isopropanol saturated with both water and caesium chloride. An equal volume of isopropanol was added to the DNA solution and the two phases mixed by vortexing. The phases were allowed to separate for 5 min. and the upper phase, consisting of ethidium bromide and isopropanol removed. This extraction procedure was repeated until all the pink colour had dissapeared from both phases. The clear aqueous phase was then dialysed against several changes of TEB for 24 h. at 4°C. The DNA was precipitated using analytical grade ethanol, pelletted by centrifugation, dried and finally dissolved in a suitable volume of TE buffer for long-term storage at 4°C.

3.5.4.2 PHENOL/CHLOROFORM EXTRACTION PURIFICATION.

This method was used regularly to remove protein and other contaminating macromolecules from small volumes of DNA solutions in microcentrifuge tubes.

The DNA solution, plus an equal volume of phenol/chloroform mixture (1:1) was mixed by vortexing for 20 s.. This mixture was then centrifuged for 2 min. at 13,000 x g to

separate into 2 layers. The upper aqueous layer was removed carefully and placed in to a fresh container. To this solution was added an equal volume of chloroform. The mixture was vortexed, centrifuged as above and the aqueous layer removed. More chloroform was added and the proceedure repeated. DNA in the resulting aqueous solution was precipitated using ethanol and pelletted by centrifugation. The DNA pellet was then dried and dissolved in the appropriate buffer.

3.5.5 DETERMINATION OF DNA CONCENTRATION

The amount of DNA in a given solution was determined using ethidium bromide fluorescent quantitation. This method utilises the ultraviolet induced fluorescence emitted by ethidium bromide molecules intercalated into the DNA (Sambrook *et al.*, 1989).

DNA solutions to be analysed were electrophoresed in agarose gels containing 0.5 μg/ml ethidium bromide using a standard DNA solution of known concentration (bacteriophage λ DNA, 0.05 μg/μl). After electrophoresis, the agarose gel was exposed to U.V. light on a U.V. Transilluminator (U.V. Products inc.,California, USA). The quantity of DNA in the sample was estimated by comparing the fluorescent yield with that of the λ DNA standard.

3.5.6 AGAROSE GEL ELECTROPHORESIS OF DNA.

Agarose gel electrophoresis of DNA was carried out using gel electroporesis apparatus supplied by Pharmacia (Milton Keynes, UK). Agarose gels were prepared by adding the desired percentage of agarose (Molecular biology grade, Sigma) to 50 ml. of TRIS-borate-EDTA buffer (TBE) and heating this mixture for 4 min. in a microwave oven to ensure solution. Molten agarose was placed in a water bath at 45°C for 20 min. after which a 2.5 μl volume of ethidium bromide solution (10 mg/ml) was added and the mixture swirled gently. Gels were cast by pouring the agarose-ethidium bromide into the gel former supplied with the electrophoresis apparatus and inserting the sample well comb. The gels were allowed to set for 30 min. before use. Unless otherwise stated, DNA samples were

loaded on to gels in Ficoll DNA gel loading buffer (Sambrook *et al.*, 1989) and electrophoresis carried out in TBE buffer at a constant 120 V. DNA electrophoresed by this method was visualised by a U.V. Transilluminator and photographed using a Polaroid CU 5 camera system utilising type 665 film.

3.6 DNA MANIPULATION METHODS

3.6.1 CLEAVAGE OF DNA MOLECULES USING RESTRICTION ENDONUCLEASES.

Restriction endonuclease digestion was carried out using excess enzyme in the suppliers recommended buffer system. Typically 1 μg of DNA was digested in the following reaction mixture:

1. DNA solution 1 μl.
2. x10 restriction endonuclease buffer 1 μl.
3. Analytical grade H_2O 7 μl.
4. Restriction endonuclease 1 μl.

The individual components were added in the above order, the solution mixed and incubated at 37°C for 3-18 h.

The products of endonuclease digestion were analysed by agarose gel electrophoresis in comparison with DNA molecular size markers.

Larger amounts of DNA were digested by scaling up the reaction mixture, ensuring that the endonuclease buffer was present at 1/10 concentration.

3.6.2 ALKALINE PHOSPHATASE TREATMENT OF CLEAVED VECTOR DNA MOLECULES

In order to suppress self-ligation and recircularisation of cleaved vector DNA molecules, these were treated prior to ligation experiments, with calf intestinal alkaline phosphatase (CIAP). Digested vector DNA was ethanol precipitated and the resultant pellet dried by vacuum desiccation for 20 min. The dried pellet was resuspended in analytical grade water

containing a 10% volume of x10 strength CIAP buffer (Northumbria biological, Framlington, UK). Approximately 1 unit of CIAP (Northumbria biological) was added per 0.1 μg of vector DNA and the reaction allowed to proceed at 37°C for 30 min. The reaction was halted by the addition of EGTA solution pH 8.0 to give a final concentration of 10mM, and by heating at 65°C for 45 min. Dephosphorylated vector DNA was recovered by phenol/chloroform extraction and ethanol precipitation prior to use in ligation reactions.

3.6.3 INTERMOLECULAR LIGATION OF DNA MOLECULES.

The ligation of target (insert) DNA to vector DNA was performed using T4 DNA ligase in the suppliers recommended buffer system. In addition, the reaction mixture contained the condensing agent Hexamine cobalt chloride at a concentation of 1.0 μM to increase molecular crowding (Rusche & Howard-Flanders, 1985).

Unless otherwise stated, DNA ligations were typically performed with a target DNA to vector DNA ratio of 2:1 and at 15°C for 18 h. Successful ligation was confirmed by agarose electrophoresis in comparison with unligated DNA solutions.

3.6.4 TRANSFORMATION OF *E.coli* K12 STRAINS.

Cells of *E.coli* K12 strains were routinely transformed by the method of Hanahan (1985) using frozen competent cells.

3.6.4.1 PREPARATION OF TRANSFORMATION COMPETENT *E.coli* K12 CELLS.

Several 2-3 mm colonies were picked off a 24h. streak culture on SOB agar. These colonies were dispersed into 1 ml of SOB medium by vortexing, and this suspension used to inoculate 1 litre of SOB medium contained in a 5 litre conical flask.

The culture was incubated shaking at 37°C until A_{600} = 0.6. The incubated culture was distributed into 500ml polythene centrifuge tubes and chilled on ice for 15min., after ice incubation cells were pelletted by centrifugation at 1000 x g for 15 min. at 4°C. The

Supernatant fluid was removed and the cells resuspended in 300 ml of RF1 medium. This suspension was again placed on ice for 15min.. Once more the cells were pelletted as described above and resuspended, this time in 80ml of RF2 medium, and then placed on ice for a further 15min. Aliquots of 200 μl were distributed into 1.5 ml microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -70°C.

3.6.4.2 TRANSFORMATION USING FROZEN COMPETENT *E.coli* CELLS.

Tubes containing competent cells of the appropriate strain were removed from frozen storage and allowed to thaw at ambient temperature until just liquid. Thawed cells were then kept on ice. The DNA solution was added in a volume of < 20 μl, with DNA at a concentration of 0.2-0.5 μg and the tube gently swirled to mix the DNA evenly with the cells. The tube was returned to ice for 10-60 min. before 'heat shocking' at 42°C for 2 min. in a water bath. After heat shock, tubes were returned immediately to ice. A 0.8 ml volume of SOC medium was added and the transformation mixture incubated at 37°C. for 90 min.

After this incubation period, a series of dilutions were made of the transformed cell suspension. The diluted cell suspensions were then spread plated on to dried LB agar plates containing the appropriate selective antibiotic. Plates were incubated at 37°C. for 24 h.

3.6.4.3 TRANSFORMATION OF $CaCl_2$ TREATED *E. coli K12*.

Transfomations were also performed on *E. coli K12*, made competent by treatment with $CaCl_2$. Overnight cultures of the appropriate host strain of *E. coli K12* were grown in LB medium at 37°C. A 1.5 ml aliquot of the bacterial culture was placed in a microcentrifuge tube, and the bacteria pelletted by centrifugation at 13,000 x g for 30 seconds. The culture supernatant was discarded and the bacterial cells resuspended in 1 ml of ice-cold 50 mM $CaCl_2$. This suspension was briefly vortexed to wash the bacteria, and placed on ice for 20 min. The bacteria were then pelletted by another centrifugation at 13,000 x g for 30

seconds and the supernatant discarded. Bacterial cells were then resuspended in 100 μl of ice-cold $CaCl_2$ to which was added the plasmid DNA to be transformed (0.2-0.5μg).

After the addition of plasmid DNA, the transformation protocol followed was that described in 3.6.4.2 (above).

3.6.5 DETECTION OF RECOMBINANT PLASMIDS IN *E. coli K12* HOSTS UTILISING *lac* GENE COMPLEMENTATION (BLUE/WHITE SCREENING).

Host *E. coli K12* strains for blue/white screening were maintained on media that selected for the presence of the F' episome. The loss of the F' episome, results in the loss of the *lac* genes required for complementation with the *lac* genes present on the cloning vector and can lead to false positive reactions. The *E. coli K12*

strain JM109 was maintained on minimal medium (M9) plates supplemented with thiamine HCl, whilst *E. coli K12* XL1-blue was maintained in LB medium containing tetracycline (15 μg/ml).

Screening was performed using LB agar plates containing the appropriate antibiotic for the selection of the cloning vector utilised. These plates were dried at 45°C for 15 min. prior to overlaying with a further LB agar plus antibiotic(s) to which had been added 40μl of X-Gal solution and 40μl of IPTG solution. This overlay was allowed to set prior to the spread plating of the appropriate transformation mixture.

After incubation (usually 37°C for 24 h.) plates were examined for the presence of blue (*lac*$^+$, non recombinant) colonies, and white (*lac*$^-$, recombinant) colonies.

CHAPTER 4: INITIAL CHARACTERIZATION OF *R. salmoninarum* ISOLATES.

4.1 INTRODUCTION.

In comparison with many pathogens, there is a paucity of knowledge concerning *R.salmoninarum* and its constituent macromolecules, which to date has hampered the successful characterization of the bacterium (see chapter 2). Although a novel molecular biological approach may eventually aid this process, at the outset of this study of *R.salmoninarum,* it was clear that a number of problems may arise from this general lack of information that could ultimately affect the success of any gene cloning work undertaken.

Firstly, there occurs the problem of possible strain variation. Although all published work to date indicates that the bacterium comprises a homogeneous genospecies with no biochemical or serological differences between isolates (Austin and Rodgers, 1980; Getchell *et al.*, 1985; Goodfellow *et al.*, 1985), variation may occur in yet uncharacterized antigens or enzymes. It was therefore necessary that the *R.salmoninarum* isolates used during this research project should reflect any possible variation in the pathogen due to factors such as geographical origin, host species affected or number of laboratory passages.

A second major problem was also foreseen as a direct result of the reported difficulties encountered in the *in vitro* culture of *R.salmoninarum.* The slow growth of the bacterium in even semi-defined bacteriological media requires long incubation periods and cultures are often subject to contamination by faster growing microorganisms (Embley, 1983; Austin and Austin, 1987). As the success of any molecular microbiological study is ultimately dependent on the purity of the source material, a particularly critical factor being the DNA used for gene-cloning experiments, it was important that a reliable method for the routine laboratory culture of the organism should be established, and precautions taken to ensure an uncontaminated source of *R.salmoninarum* macromolecules was

available for further study.

Among the stated aims of this project was the investigation of putative pathogenicity and virulence factors of *R.salmoninarum* by the application of gene cloning technology, a major decision therefore had to be made concerning the specific *R.salmoninarum* molecules to be targeted using these techniques. Possible molecules of interest fall into two groupings; a) the cell-associated protein antigens of the bacterium, and b) aggressive and possibly toxic factors which may contribute to the pathology of BKD.

Of the many recorded antigenic components of *R.salmoninarum* the 57 kilodalton major surface antigen (Haemagglutinin, p57 protein or MSA) of the bacterium has been the subject of most studies. As a result of these studies, not only has this molecule been implicated as a major contributor to the pathogenicity and virulence of *R.salmoninarum* (Bruno, 1988b; Wiens and Kaattari, 1991) it has also been found to play a major role in the immunological interactions that occur between the pathogen and its salmonid host (Kaattari *et al.*, 1985, 1986, 1987, 1988). This molecule would make an attractive target for gene cloning, not only because of its apparent importance in the disease process, but because it is seemingly produced in relatively large quantities *in vitro* (Turaga *et al.*, 1987), and is readily detected by immunochemical methods (Wiens and Kaattari, 1989).

In contrast, little is known about the possible aggressive and toxic components of *R.salmoninarum*. The existence of these molecules and their relevance to the pathogenic processes of the bacterium, is disputed by a number of workers and has yet to be conclusively established. However, there are two reported properties of the pathogen that warranted further investigation, these being proteolytic and haemolytic activity.

Biochemical profiles of *R. salmoninarum* reveal the presence of a proteolytic activity capable of the breakdown of substrates such as casein and gelatin (Austin and Rodgers, 1980; Goodfellow *et al.*, 1985). The true nature of this proteolytic activity remains unknown, but may however be present in the form of a trypsin-like enzyme, the detection

of which forms part of the API-ZYM enzymic profile routinely used in the identification of *R. salmoninarum* isolates (Austin and Austin, 1987). This proteolysis may play an important part in the pathogenesis of BKD, possibly being responsible for the destruction of host tissues observed in the necrotic foci of *R.salmoninarum* infection (Bruno, 1986; Young and Chapman, 1978).

Another property of *R. salmoninarum* reported by workers studying BKD pathogenesis is haemolytic activity. Although this property is not described in the initial taxonomic studies of the bacterium by Sanders and Fryer (1980), nor in the description given to the genospecies in Bergey's Manual of Systematic Bacteriology, a number of studies do however report β-haemolysis as a property for *R.salmoninarum* isolates. Experiments carried out by Bruno (1984) as part of his Ph.D. study of *R. salmoninarum* and BKD indicate that the haemolytic agent is present in the extracellular products of the bacterium. No haemolytic molecule has to date been isolated from *R. salmoninarum* and there are some that dispute its existence (Bandin *et al.*, 1991). Again the isolation of such a molecule, if it exists, could form part of a gene cloning based study of *R. salmoninarum.*

The experimental work described in this chapter therefore, was intended to represent the preliminary study needed before gene-cloning techniques can be applied to isolates of *R. salmoninarum.* This study involved the establishment of a collection of *R. salmoninarum* isolates, followed by the assessment of methods for their routine laboratory culture. Finally the detection of *R. salmoninarum* components as target molecules for gene cloning experiments was attempted.

4.2 COLLECTION OF *R. salmoninarum* ISOLATES.

A number of *R. salmoninarum* isolates were obtained from either fish disease research establishments or culture collections (Table 4.1). As well as the type strain of *R. salmoninarum,* isolates were collected so as to detect any possible variation in the bacterium arising from differences in geographical location, species of host fish infected,

Strain	Host salmonid	Origin	Source	Remarks
MT419	Salmo salar L.	Scotland	D.W. Bruno	low passage strain
MT444	Salmo salar L.	Scotland	D.W. Bruno	low passage strain
MT452	Oncorhynchus mykiss	Scotland	D.W. Bruno	low passage strain
Round Butte	Oncorhynchus tshawytscha	Oregon, USA	S.K. Gutenberger	from freshwater hatchery
Marion Forks	Oncorhynchus tshawytscha	Oregon, USA	S.K. Gutenberger	from freshwater hatchery
84-019 OC	Oncorhynchus tshawytscha	Pacific ocean	S.K. Gutenberger	isolated from ocean caught salmonid
DR-128	Oncorhynchus mykiss	Canada	T. Evelyn	high passage strain
DR-143	Salvelinus fontinalis	Canada	T. Evelyn	high passage strain
DR-384	Oncorhynchus kisutch	Canada	T. Evelyn	high passage strain
Lea	Oncorhynchus tshawytscha	USA	C.J. Rodgers	ATCC 33209 type strain
PPD	Oncorhynchus mykiss	England	C.J. Rodgers	-
NCIMB 1111	Salvelinus fontinalis	USA	NCIMB	-
NCIMB 1116	Salmo salar L.	Scotland	NCIMB	'Dee disease' strain

Table 4.1 : Initial collection of *Renibacterium salmoninarum* strains.

aquatic environment (freshwater or marine) and the number of laboratory passages. Cultures of *R. salmoninarum* from laboratories outside of the U.K. or from the NCIMB culture collection were received in sealed ampoules as freeze dried cells whilst the remainder were received as agar plate cultures.

4.3 INITIAL RECOVERY OF *R. salmoninarum* ISOLATES.

On arrival in the laboratory, freeze-dried cultures of *R. salmoninarum* were resuscitated by the addition of 500μl. of distilled water, whilst inocula from agar plate cultures were prepared by using a loopful of bacterial growth suspended in 1 ml PBSA. All cultures were plated on to selective kidney disease medium (SKDM; Austin *et al.*, 1983) and grown aerobically for 6 weeks at 15°C.

4.4 CONFIRMATORY TESTS FOR *R. salmoninarum*.

All putative cultures of *R. salmoninarum* recovered on SKDM and from all subsequent laboratory cultures set up during this project were subject to the following confirmatory tests; a) Gram stain and microscopic examination, b) Oxidase test, c) Catalase test, and d) A slide agglutination test using rabbit antiserum raised to the *R. salmoninarum* ATCC type strain (kindly donated by Dr. C.J. Rodgers, M.A.F.F. fish disease laboratory, Weymouth. U.K.). Gram positive short rods in pairs, with an oxidase negative, catalase positive phenotype that agglutinated with antiserum, were confirmed as *R. salmoninarum*.

4.5 LABORATORY CULTURE OF *R. salmoninarum*.

4.5.1 CULTURE USING KDM2 MEDIUM.

Initially, the routine laboratory culture of *R. salmoninarum* was performed using the second modification of kidney disease medium (KDM2; Evelyn, 1977) as a broth, or as a solid medium formed by the addition of 1.5% agar. Cultures were incubated aerobically at 15°C. Growth appeared in these cultures after a varying time period, ranging between 1 and 6 wks. Cultures were regularly found to be overtly contaminated with other microorganisms such as fungi and other unidentified bacterial species. Complete

overgrowth on solid medium could be alleviated by daily examination of the plates coupled with careful excision of contaminating microbial colonies, however, contamination of broth cultures could not be dealt with in this manner and consequently, a large number of such cultures had to be discarded.

4.5.2 CULTURE USING MHC MEDIUM.

After the problems encountered during the initial attempts to culture *R. salmoninarum*, advice was sought from Dr D.W. Bruno of the S.O.A.F.D. Marine Laboratory, who has considerable experience in the culture of *R. salmoninarum* for bacteriological studies.

As a result of this consultation, it was decided that the routine laboratory culture of *R. salmoninarum* should be performed using Mueller-Hinton medium supplemented with 0.1% w/v cysteine hydrochloride (MHC). MHC agar was formed by the addition of 1.5% w/v agar. Cultures were incubated at 15°C and all agar plates were sealed using parafilm. Although cultures were still found to be susceptible to contamination, the use of MHC medium and the sealing of plate cultures considerably reduced the incidence of overgrowth by other microorganisms, and this method was adopted for the routine culture of *R. salmoninarum* isolates during this research project.

4.6 RATIONALIZATION OF THE NUMBER OF *R. salmoninarum* ISOLATES FOR USE IN THIS RESEARCH PROJECT.

Despite a significant reduction in the incidence of contamination facilitated by the culture of *R. salmoninarum* using MHC media, difficulty was experienced in the maintenance of 26 different cultures (13 plate, 13 broth) of the bacterium for use in preliminary bacteriological studies. It was therefore decided to reduce the number of *R.salmoninarum* isolates used in these experiments and as the source of macromolecules for the remainder of this research project. The 5 isolates, chosen as before to circumvent any possible phenotypic variation were ATCC33209, DR143, MT444, NCIMB1116, and PPD

4.7 STUDY OF THE COMPONENT PROTEINS OF *R. salmoninarum*.

4.7.1 SDS-PAGE ANALYSIS.

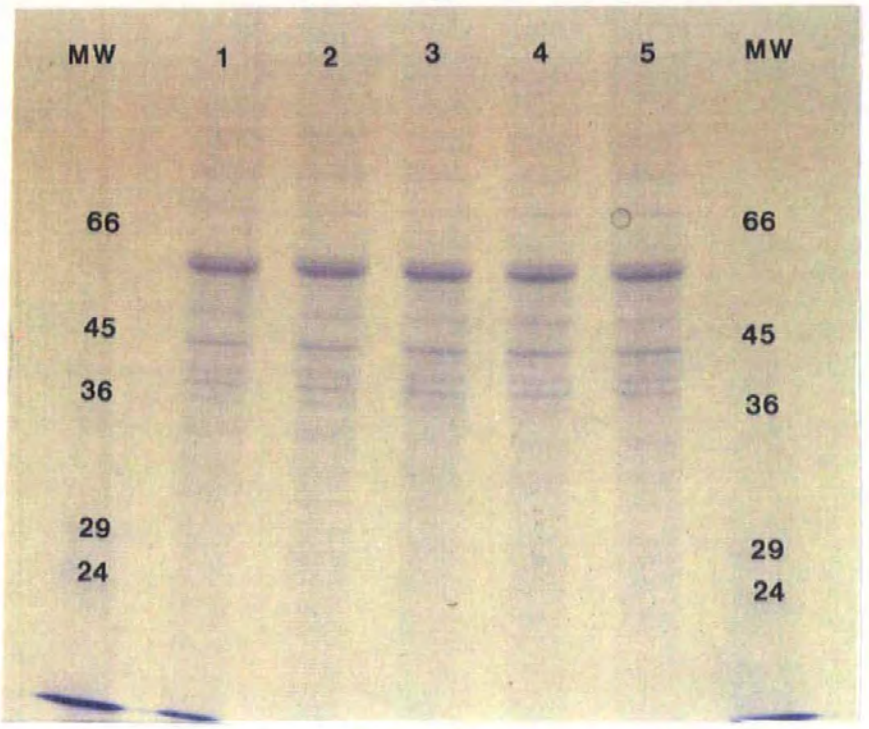
A profile of the cell-associated and extracellular proteins of *R. salmoninarum* was made using sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE). Bacteria from 4 week-old broth cultures (250 ml) of *R. salmoninarum* isolates ATCC33209, DR143, MT444, NCIMB1116, and PPD were harvested by centrifugation at 5,000 x g for 15 min., and washed 3 times with PBSA. The culture supernatants, containing extracellular products (ECP) of the bacteria, were concentrated by precipitation with ammonium sulphate at 90% saturation.

Bacterial cells and concentrated ECP were suspended in 2x SDS-PAGE reducing sample buffer, and subjected to electrophoresis in 10% polyacrylamide gels in conjunction with molecular weight markers. The were visualized by coomassie blue staining.

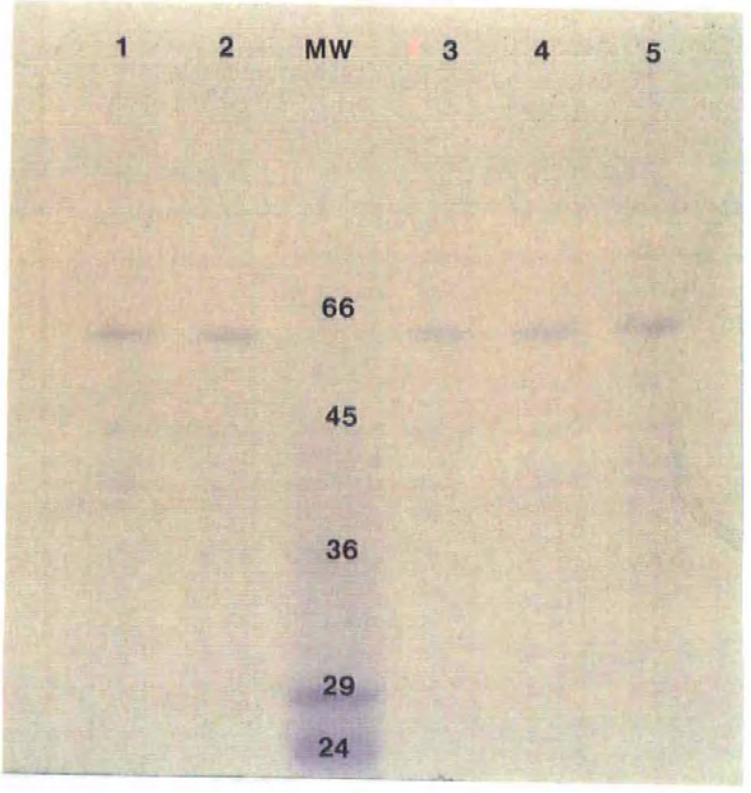
The results of the SDS-PAGE of *R. salmoninarum* proteins can be seen in Fig. 4.1. On examination, the protein profiles of all *R.salmoninarum* isolates analysed, appear to be identical, both in the sonic whole cell preparation and in the ECP fraction. This fact would tend to support the body of experimental work that has lead to the conclusion that *R.salmoninarum* is a genospecies, with all isolates showing uniformity in both biochemical and antigenic properties (Bullock *et al.*, 1975; Austin and Rodgers, 1980; Embley *et al.*, 1983; Bruno and Munro, 1986) Also visible on both gels, is the major surface antigen (MSA, p57 or haemagglutinin) of approximately 57 kilodaltons molecular weight, this protein is by far the most abundant molecule in both preparations. Also visible in the sonic whole cell preparations, are distinct protein bands of approximately 45 kDa and 36 kDa which may correspond to the putative breakdown products of the p57 protein reported by Rockey *et al.* (1991) and Griffiths and Lynch (1991).

Figure 4.1 SDS-PAGE analysis of whole cell and concentrated ECP from five strains of *R.salmoninarum*. Showing the electrophoretic profiles (10% gel) of whole cell preparations (**A**) and concentrated ECP (**B**). Lane numbers in both gels are comparable. Lane 1: *R.salmoninarum* ATCC33209, Lane 2: *R.salmoninarum* DR143, Lane 3: *R.salmoninarum* MT444, Lane 4: *R.salmoninarum* NCIMB 1116, Lane 5: *R.salmoninarum* PPD. **MW**= molecular weight markers (kilodaltons)

A



B



4.7.2 IMMUNOBLOTTING.

The cell-associated and extracellular proteins of *R. salmoninarum* were also analysed by immunoblotting. Proteins were separated by SDS-PAGE and subjected to electrotransfer on to a nitrocellulose membrane. Immunoblotting was performed using either rabbit anti-*R. salmoninarum* ATCC33209 whole cell serum or rabbit anti-*R.salmoninarum* ATCC33209 ECP serum as the primary antibody at a concentration of 1:250, and swine anti-rabbit immunoglobulins peroxidase conjugate (P217, Dako) as the secondary antibody at a concentration of 1:500. Bound antibody was visualized using DAB/$NiCl_2$ colour development.

The results of the immunoblotting of *R. salmoninarum* proteins can be seen in Fig. 4.2. Once again, the profiles of all *R.salmoninarum* isolates analysed appear identical, with the p57 protein being the most abundant antigen in both whole cell and ECP preparations. Putative breakdown products of 45 kDa and 36 kDa are again visible, and in addition there is a band at 20 kDa.

4.8 STUDY OF THE HAEMOLYTIC AND PROTEOLYTIC PROPERTIES OF *R.salmoninarum*.

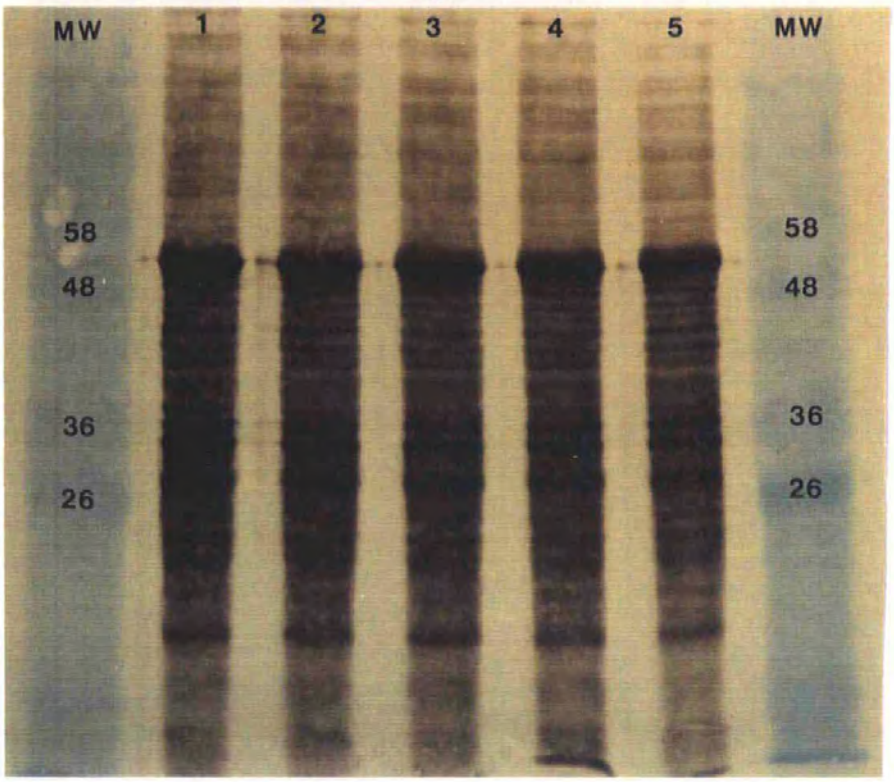
4.8.1 CULTURE OF *R. salmoninarum* ON SUBSTRATE-CONTAINING GROWTH MEDIUM.

The 5 *R. salmoninarum* isolates were inoculated on to MHC agar containing: a)5% washed rainbow trout erythrocytes, b)5% washed horse erythrocytes, or c)1% casein. All plates were incubated 15°C until examination.

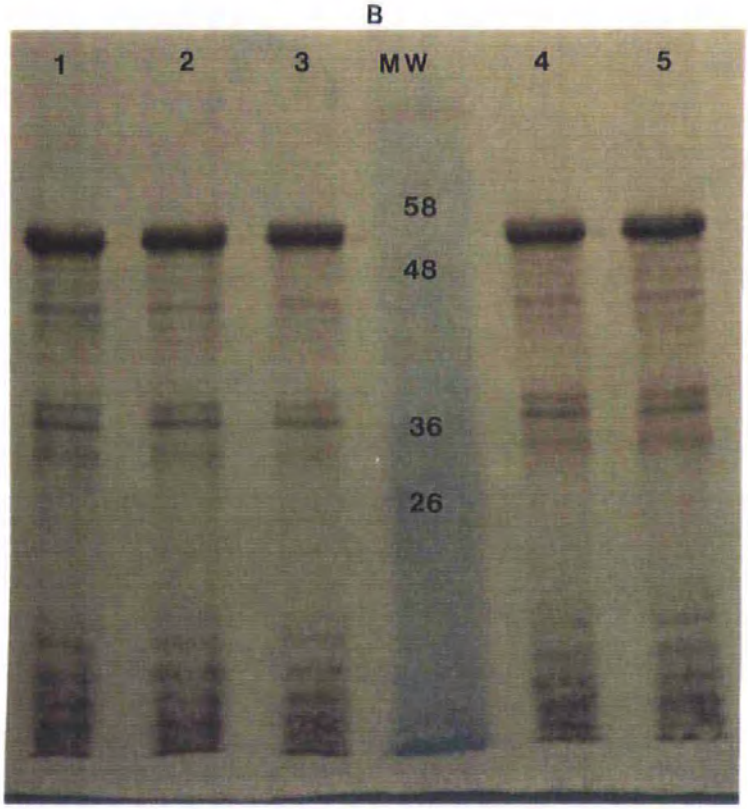
Inoculated plates containing trout erythrocytes, were found to deteriorate rapidly. After 4-5 days, the blood cells were seen to have lysed and the medium adopted a dark brown colour as a result of the lysis. It was therefore impossible to detect any zones of haemolysis attributable to the bacteria. Horse erythrocyte-containing plates withstood incubation up to 4 weeks before deterioration was evident. These plates were examined

Figure 4.2 Immunoblotting analysis of whole cell preparations and concentrated ECP from five strains of *R.salmoninarum*. Showing the protein antigens present in electrophoretically separated (10% gel) whole cell (**A**) and concentrated ECP (**B**) preparations of the bacterium. Proteins transferred on to nitrocellulose were immunostained with rabbit anti-*R.salmoninarum* ATCC33209 whole cell serum (**A**) and rabbit anti-*R.salmoninarum* ATCC33209 ECP serum (**B**) as the primary antibody, and peroxidase-conjugated swine anti-rabbit immunoglobulins (P217, Dako) as the secondary antibody. Antibody binding was detected with DAB/$NiCl_2$ colour development. **MW**=molecular weight markers (kilodaltons)

A



B



at daily intervals, but no zones of haemolysis were detected.

Plates containing the casein substrate were also examined daily for zones of proteolysis, after 6 weeks, these plates were flooded with 5% acetic acid to facilitate the precipitation of casein and to increase the sensitivity of the determination of proteolysis.

4.8.2 OVERLAYS OF BACTERIAL GROWTH WITH AGAROSE/ SUBSTRATE.

The next method adopted for the determination of the haemolytic and proteolytic activities of *R. salmoninarum*, was to overlay established bacterial growth with substrates suspended in agarose.

Low gelling-point agarose ('Seaplaque', FMC products) was dissolved in sterile PBSA at a concentration of 0.75% w/v by heating in a microwave oven. This agarose solution was maintained at 25°C until use.

Substrate/agarose mixtures were prepared immediately prior to use by the addition of washed horse or rainbow trout erythrocytes to give a final concentration of 10% or by the addition of a 10% casein solution to give a final concentration of 2%.

Overlays of 21 day-old MHC plate cultures of the five isolates of *R. salmoninarum* were made by gently pipetting 5 ml of the agarose/substrate mixture on to the surface of the plate and allowing the agarose to set. Overlayed plates were incubated at 15°C and examined daily over a two week period for indications of haemolytic or proteolytic activity. As before, casein-containing plates were flooded with 5% acetic acid prior to final examination.

As found in the previous experiment, the integrity of rainbow trout erythrocytes were found to be limited, deterioration occurring within 4-5 days, horse erythrocytes were found to be unaffected.

Using this method, neither haemolytic or proteolytic activity was detected in any of the *R. salmoninarum* isolates.

4.8.3 GEL DIFFUSION ASSAYS.

Gel diffusion assays of the haemolytic and proteolytic activities of *R. salmoninarum* were also performed, using substrate-containing agarose, prepared as in 4.8.2 (above).

Assay plates were made by pipetting 15 ml of molten agarose containing blood or casein into sterile petri dishes and allowing the agarose to set. Wells were then cut in the gel with a sterile cork-borer of 5 mm diameter.

Assays were performed by the addition of 50μl of sample to the wells in the substrate containing gels followed by incubation in a 'wet box' consisting of a plastic food container lined with moistened paper tissues. Plates were examined daily for 1 week for indications of haemolytic or proteolytic activity. Prior to final examination, casein-containing plates were flooded with 5% acetic acid.

Initial assays consisted of samples of 21 day old broth cultures of the 5 *R. salmoninarum* isolates from which there was no detectable haemolytic or proteolytic activity.

A second set of assays was performed using samples taken at different times during the culture of *R. salmoninarum* in an attempt to detect any haemolytic or proteolytic molecules produced for a limited time period during the culture of the bacterium. Samples were taken from *R. salmoninarum* cultures with visible turbidity at 21, 28, 35, and 42 days and assayed for as described above using uninoculated culture medium as a control. No detectable activity was observed.

The final set of assays was performed using the 42 day old cultures separated by centrifugation into cellular and extracellular fractions. The *R. salmoninarum* cells were washed 3 times and resuspended in 5ml PBSA in which they were subjected to sonic disruption using a Heat Systems sonicator (Heat Systems-Ultrasonics inc. Farmingdale, N.Y. U.S.A.). Sonication was performed in 5 bursts of 1 min. duration, interspersed with 1 min. intervals on ice to facilitate cooling. Sonicated cell preparations were kept on ice until use. Extracellular fractions of *R. salmoninarum* cultures were concentrated by

ammonium sulphate precipitation at 90% saturation and resuspended in 5 ml of PBSA. Aliquots of 4 ml from both the sonicated cell preparation and the concentrated extracellular fraction were removed and the material stored at -20°C. for future use.

Samples of the sonic cell preparation and concentrated extracellular fraction were assayed using the gel diffusion assay, but again there were no indications of either haemolytic or proteolytic activity.

4.8.4 MICROTITRE PLATE ASSAYS

The detection of haemolytic and proteolytic activities of *R. salmoninarum* was also attempted using substrate suspensions placed in the wells of plastic microtitre plates.

Assays for haemolysis were performed by the addition of the test sample to an erythrocyte suspension followed by a period of incubation, the presence of haemolytic activity is indicated by the release of the red coloured cell contents in to the surrounding medium. A negative result is indicated by the settling of the intact erythrocytes on the bottom of the well in the form of a 'button', surrounded with uncoloured medium.

To assay for haemolytic activity of *R. salmoninarum*, washed horse or rainbow trout erythrocytes were suspended at a concentration of 1% in sterile PBSA and 50μl of this suspension dispensed in to each sample well. A control well was also set up using 50μl. of this suspension.

The determination of proteolytic activity was also performed using microtitre plates, and by the exploitation of a 'colour release' method. Chromogenic derivatives of proteins such as albumin, casein and collagen are commercially available. Detection of protease activity with these chromogenic substrates is convenient because it depends on the solubility of low-molecular-weight coloured peptides in a supernatant fluid after the precipitation of large fragments of the azoprotein. For the assay of *R. salmoninarum* proteolytic activity, azocasein was dissolved at a concentration of 2% in PBSA and 50μl of this solution was placed in each sample well. A control well, containing 50μl of azocasein solution was also

prepared.

The first set of microtitre plate haemolytic and proteolytic assays was performed using 50μl samples taken from 21, 28, 35 and 42 day-old broth cultures of *R. salmoninarum*. Samples were mixed thoroughly with the substrates and the plates incubated at 15°C for 1 week. The plates were examined daily for haemolytic activity, and at the end of the incubation period, the azoprotein was precipitated by the addition of 20% trichloroacetic acid (TCA).

Haemolysis was observed in all the wells containing rainbow trout erythrocytes after 3 days, however, haemolysis in the control well indicated that this was the result of erythrocyte deterioration rather than an indication of activity from the samples added. There was no detectable haemolytic activity in the wells containing horse erythrocytes, and no detectable proteolytic activity in the azocasein-containing wells.

The microtitre plate assay for haemolysis and proteolysis was repeated using the concentrated fractions from the 5 *R. salmoninarum* isolates used in the final gel diffusion assay described above. Again, the rainbow trout erythrocytes were found to be labile, but no detectable haemolytic or proteolytic activity could be found in either the sonicated cells or the concentrated extracellular fraction.

4.8.5 ZYMOGRAMS OF ELECTROPHORETICALLY SEPARATED *R.salmoninarum* PROTEINS.

The final method used for the detection of proteolytic and haemolytic activities in *R. salmoninarum*, involved the production of zymograms for bacterial proteins separated by polyacrylamide electrophoresis. This method consists of the overlaying of electrophoresis gels with substrate containing agarose, with the consequent detection of enzymes by virtue of visible substrate breakdown within the agarose overlay (Titball, 1983).

4.8.5.1 SDS-PAGE

Concentrated cell sonicates and extracellular components were prepared from both 21 and 42 day-old cultures of the 5 *R. salmoninarum* isolates as previously described. Each preparation was then subjected to SDS-PAGE in 10% gels. After electrophoresis, the SDS contained within the gel was removed by washing, firstly in 50 ml. of 1% triton X100 for 15 min followed by two 10 min. washes in PBSA. After washing the surface of the gel was gently blotted with a medical wipe and placed at the bottom of a plastic food container lined with moistened paper tissues. Agarose, containing washed horse or rainbow trout erythrocytes, or casein, was prepared as in 4.8.2 above. Overlays were formed by gently pipetting 10 ml. of agarose/substrate on to the surface of the polyacrylamide gel. After the agarose had set, the lid of the food container was replaced and the overlays incubated for 1 wk. at 15°C. The overlayed gels were examined daily for signs of haemolytic or proteolytic activity. As in previous experiments, casein containing gels were flooded with 5% acetic acid prior to final examination. Once again, the rainbow trout erythrocytes were found to deteriorate after 3-4 days, and as with all the previous experiments, there was no detectable lysis of horse erythrocytes or breakdown of casein.

4.8.5.2 NATIVE PAGE

As the denaturing conditions present during SDS-PAGE could conceivably have affected the activity of any haemolytic or proteolytic molecules, the zymograms were repeated using the same *R. salmoninarum* samples subjected to native PAGE. Native PAGE was performed in the same manner as SDS-PAGE but all reagents used for the electrophoresis lacked SDS. In addition, the sample buffer lacked the reducing agent β-mercaptoethanol.

After electrophoresis, the polyacrylamide gels were briefly washed in PBSA and treated in an identical way to the first zymogram experiment. Once again, deterioration of the rainbow trout erythrocytes was observed, but there was no detectable activity against horse erythrocytes or casein.

4.9 DISCUSSION.

It can be clearly seen from the results obtained for the experimental work described in this chapter, that *R.salmoninarum* is a difficult microorganism to study. The problems encountered even during this initial characterization of the bacterium, serve not only to explain the paucity of general bacteriological knowledge concerning *R.salmoninarum* and its constituent macromolecules, but also serve to indicate, a new direction to further analyses of the bacterium as will be attempted in this research project.

Initially, *in vitro* culture of *R.salmoninarum* proved extremely troublesome, cultures were slow growing and invariably suffered from overgrowth with other microorganisms especially fungi. The KDM growth medium was found to be prone to contamination, although *R.salmoninarum* grew reasonably well using this formulation. The number of useful cultures obtained using KDM medium was small, and indeed, four of the initial isolates of *R.salmoninarum* were lost from the collection as a result of gross contamination. Even when the so-called selective SKDM medium was utilized to culture the isolates of the bacterium, contamination occurred on a regular basis.

Consultation with Dr. David Bruno at the SOAFD laboratory in Aberdeen, resulted in the adoption of MHC medium as the formulation for routine *R.salmoninarum* culture. An advantage of this medium are is that it is easily prepared, without the need for expensive serum or antibiotics, and that there are no additions to the medium after sterilization. Such additions provide opportunities for contamination, especially if the medium is being made and dispensed in anything other than a dedicated, sterilizable media preparation area.

A collection of 13 *R.salmoninarum* isolates was initially established, as a source of DNA for gene cloning experiments and other macromolecules for biochemical characterization. Prior to the commencement of experimental work, it was considered that this number of isolates would be both manageable, and, reflect any genetic differences in isolates due to factors such as geographical location, species of salmonid infected and number of

laboratory passages. However, as a result of the difficulties encountered in routine culture of *R.salmoninarum* a smaller, more manageable number of 5 different isolates was chosen.

The five *R.salmoninarum* isolates were subjected to a series of biochemical and immunological characterization experiments, in an attempt to find putative determinants of pathogenicity whose further analysis could be facilitated by gene cloning techniques. Experiments performed on the *R.salmoninarum* isolates were designed to establish the presence of such molecules and investigate detection techniques that could be used in cloning experiments. SDS-PAGE and immunoblotting analysis of both *R.salmoninarum* whole cells and extracellular products (ECP) revealed a large number of potential antigens as candidates for gene cloning studies. Along with the 57 kDa major surface antigen, other uncharacterized proteins were detectable with rabbit antiserum raised against both *R.salmoninarum* whole cells and ECP.

The detection of both proteolytic and haemolytic activities in *R.salmoninarum* proved more difficult, in fact, despite the use of several different assay techniques, no such activities were found. A number of possible reasons exist for the inability to detect these activities, and these fall into two categories; deficiencies in the assay methods used, and, factors influenced by the biochemical and genetic properties of *R.salmoninarum* itself. Deficiencies in the assay methods utilized are hard to visualize, as substrates for both proteolytic and haemolytic activities were selected on the basis of their use in previous biochemical characterization experiments with *R.salmoninarum* (Austin and Austin, 1987) and on methods used to detect similar properties in other bacteria (Cruikshank *et al.*,1975). Aspects of the biochemical and genetic nature of *R.salmoninarum* however, may explain the failure to detect such properties. The molecules conferring proteolytic and haemolytic activity may be labile, and the long incubation periods required for the culture of the bacterium may mean that these molecules are inactive at the time of assay. It has already been shown that the major surface antigen degrades rapidly both *in vivo* (Griffiths

and Lynch, 1991) and *in vitro* (Rockey *et al.*, 1991), perhaps the same process applies to the proteolytic and haemolytic molecules of *R.salmoninarum.* The production of pathogenicity determinants such as haemolytic and proteolytic molecules, is often under strict genetic control (Finlay and Falkow, 1989; Miller *et al.*, 1983) and the expression of the genes coding for such molecules is often regulated by host stimuli. Growth of *R.salmoninarum in vitro* may not stimulate the production of haemolytic and/or proteolytic molecules, or equally their production may be limited to a short period during the growth cycle.

It may also be the case, that present formulations of culture media for *R.salmoninarum* are sub-optimal, e.g. they do not stimulate the production of the full number of macromolecules found *in vivo.*

Answera to all of these questions may be answered by the application of gene cloning techniques to *R.salmoninarum*, and despite the failure to detect proteolytic and haemolytic activities in the five isolates of *R.salmoninarum* tested, these molecules will, along with the large number of protein antigen components, be the target for gene cloning experiments. This work, the main thrust of the present research project, forms the basis of the rest of this thesis.

CHAPTER 5: THE CONSTRUCTION OF *R. salmoninarum* GENOMIC GENE LIBRARIES IN *E. coli* CLONING VECTORS.

5.1 INTRODUCTION.

The process fundamental to this research project, is the transfer of fragments of *R.salmoninarum* genomic DNA into cells of *E. coli K12*, in a manner that allows the genetic sequences to be maintained in the bacterium, facilitating isolation and characterization of genes encoding specific *R.salmoninarum* proteins.

Prior to the introduction of modern gene cloning techniques, many studies were made on the ways in which bacteria can acquire new genetic information. The three classical methods that formed the basis of these studies were, 1.) Transformation, in which bacteria can take up exogenous DNA (Mandel and Higa, 1970; Hanahan, 1983); 2.) Conjugation, in which DNA is transferred directly from one bacterium to another (Williams and Skurray, 1980); and 3.) Bacteriophage-mediated transduction, in which genetic material is transferred from one bacterium to another via a bacteriophage particle (Singer and Berg, 1991).

Whilst characterization of these processes was in progress, many attempts were made at introducing foreign DNA into both prokaryotic and eukaryotic cell types. Little progress was made in this field, due to three basic problems. Firstly, DNA introduced into bacterial cells can be degraded by host-controlled restriction and modification systems (Glass, 1982). Secondly and most important, the exogenous DNA could not replicate in its new host and therefore was not maintained within the host cell population, and thirdly, where detection of DNA uptake was reliant on gene expression, failure could be due to lack of accurate transcription and translation of the nucleotide sequence or failure of any post-translational modification.

The maintainance of foreign DNA sequences within a cell, is dependent on the ability of the molecule to replicate, since in the absence of replication, foreign DNA molecules will

be lost by the dilution brought about by the subsequent multiplication of the host cell population.

In order to be replicated, DNA molecules must contain an origin of replication which is recognised by the host cell, and in bacteria (and viruses) there is typically one such origin per genome. Such DNA molecules posessing an origin of replication are known as replicons. As it is unlikely that exogenous DNA fragments will contain an origin of replication recognised by the host, their survival and maintainence within the host cell is therefore dependent on their combination with a replicon. As the relatively large bacterial genome would be to unwieldy to manipulate in an intact form, and virtually impossible to characterize for use as a replicon in gene transfer experiments, other smaller replicons are utilized in gene cloning technology. The huge amount of microbial genetic studies performed with bacterial species such as *E. coli* facilitated the discovery of more suitable replicons in the form of small plasmids and bacteriophage DNA, which have been widely exploited as vehicles or vectors of foreign DNA in gene cloning (Pouwels *et al.*, 1985). Small plasmids and bacteriophage are the most suitable cloning vectors, as they are replicons in their own right, their replication and maintainence does not require integration into the host genome, and, their DNA can be readily isolated in an intact form. Vectors are also particularly useful for the carriage of foreign DNA in gene cloning experiments as relatively simple methods are available for purifying the vector molecule, complete with its foreign DNA insert, from recombinant host cells. Thus not only does the vector provide the replicon function, but it also permits the easy bulk preparation of the foreign DNA sequence, free from interfering host cell DNA (Singer and Berg, 1991).

Composite DNA molecules consisting of a cloning vector and a foreign DNA insert, are termed recombinant molecules, and hence gene cloning techniques are often referred to as part of the recombinant DNA technology. Gene cloning or molecular cloning, are however the more appropriate terms, as the process results in a line of genetically

identical organisms, all of which contain the recombinant molecule, which can be propagated and grown in bulk, hence amplifying the recombinant DNA and any gene product whose synthesis it directs.

The formation of a recombinant DNA molecule, and hence the evolution of modern gene cloning techniques, required the means for: a) a method for cutting and joining DNA; b) a way of monitoring the cutting and joining processes; and, c) a method for introducing recombinant DNA molecules into the host cell. Historically, these three techniques were all developed in the late 1960s, early 1970s with the discovery of restriction endonucleases and DNA ligase, the development of agarose-gel electrophoresis and the development of a simple and relatively efficient method of transforming *E. coli* bacteria, respectively. As a result of these advances, the first gene cloning experiments were performed by Jackson *et al.* in 1972, and Lobban and Kaiser in 1973.

The work presented here, namely, the application of gene cloning to the study of *R. salmoninarum*, commenced in a manner similar to other such studies with bacteria. The genomic DNA of *R. salmoninarum* was isolated and fragmented in a random manner, the fragments produced were inserted into a cloning vector to form a set of recombinant molecules, the inserts of which, in their totality, represented the whole genome of the bacterium. The recombinant molecules were then introduced in to *E.coli* cells, where it was hoped that genes encoding *R. salmoninarum* proteins would be expressed, thus enabling further analysis of the gene sequences and their products.

After an initial description of the methods employed to isolate genomic DNA from *R. salmoninarum*, this chapter represents the experimental work undertaken to form a set of recombinant molecules containing DNA fragments representing the whole genome of the bacterium. This collection of fragments, contained within a cloning vector, is known as a gene bank or gene library, so called because it is from this collection that the desired clone is withdrawn for further study.

5.2 ISOLATION AND PURIFICATION OF *R. salmoninarum* DNA.

Prior to the construction of *R. salmoninarum* gene libraries, genomic DNA from the bacterium was isolated and purified. As there were no published methods for specific isolation and purification of *R. salmoninarum* DNA, the methods utilized had to be based on techniques adopted for other bacterial species.

5.2.1 LYSIS OF BACTERIAL CELLS.

R. salmoninarum cells were recovered from a 1 litre MHC broth culture by centrifugation at 5,000 x g for 20 min. at 4°C, and washed twice in PBSA to remove any potentially interfering cell debris or culture medium. Lysis was carried out by resuspending the washed cells in 30 ml of TE containing 0.5% SDS and 25 μg/ml protease K. This mixture was then placed at 56°C until lysis had occurred.

5.2.2 PURIFICATION OF *R. salmoninarum* DNA.

Two different isolation and purification methods were performed on the *R. salmoninarum* DNA liberated by bacterial lysis.

5.2.2.1 MARMUR PROCEEDURE.

The first isolation and purification method applied to *R. salmoninarum* DNA was a modification of the proceedure of Marmur (1961), the full details of which are given in the materials and methods section of this thesis (see 3.5.1.1, above). Deproteinization was performed by treatment with sodium perchlorate followed by extraction with organic solvents (chloroform/isoamyl alcohol). The nucleic acids were then precipitated with ethanol and recovered by spooling on to a glass rod. The recovered nucleic acids were redissolved in saline-citrate and further purified by repeated extraction with organic solvents and ethanol precipitation as above. The final precipitation and recovery was made with isopropanol and the DNA finally dissolved in a 1 ml volume of TE buffer.

5.2.2.2 ULTRACENTRIFUGATION METHOD.

An alternative method of DNA isolation from *R. salmoninarum* lysates consisted of equilibrium ultracentrifugation in caesium chloride/ethidium bromide gradients (CsCl/EtBr).

Bacterial lysates were subject to centrifugation at 15,000 x g for 30 min. at 20°C. To the supernatant fraction was added a 0.1 x volume of 3M sodium acetate and a 0.6 x volume of isopropanol, this mixture was left for 15 min. at room temperature, after which the precipitated DNA was recovered by centrifugation at 13,000 x g for 10 min. at 20 °C. The resultant DNA-containing pellet was drained, dried and dissolved in a suitable volume of TE buffer prior to purification by ultracentrifugation. DNA was purified by CsCl/EtBr ultracentrifugation in either a Beckman L-7 ultracentrifuge or a Beckman L-100 micro-ultracentrifuge as described in 3.5.4.1 above.

Although the Marmur proceedure was found to be quicker than isolation and purification by ultracentrifugation, the latter was found to be more convenient and to provide a higher yield of purified DNA. Therefore it was decided that CsCl/EtBr gradient ultracentrifugation would be the routine method for the isolation and purification of *R. salmoninarum* DNA during this research project.

5.3 FRAGMENTATION OF *R.salmoninarum* GENOMIC DNA.

After the isolation and purification of *R. salmoninarum* genomic DNA, the next step in constructing a gene library of the bacterium is the production of a set of appropriately sized random fragments of this DNA for insertion into the chosen cloning vector. One method of random DNA fragmentation that may be used, would be mechanical shearing, where the DNA is physically broken using a method such as ultrasonication. However, although this method produces truly random DNA fragments, the process is uncontrollable with respect to the nature of the ends of the newly-produced fragments, which may be incompatible to those on the cloning vector to which they will be subsequently joined.

A more commonly used proceedure for the random fragmentation of DNA for gene library formation, involves the use of restriction endonucleases (restriction enzymes). A number of fragmentation strategies utilizing restriction enzymes exist, using a single enzyme or a combination of enzymes to produce DNA fragments suited for cloning experiments. The two-enzyme method of Maniatis *et al.* (1978), involves the digestion of DNA with two unrelated restriction enzymes such as *Hae III* and *Alu I.* These enzymes have tetranucleotide recognition sequences, which therefore occur frequently in the target genomic DNA, and in a reaction taken to completion (a limit digest) will produce a large number of relatively small fragments. However, by using the enzymes in a partial DNA digest, the result is a random set of larger fragments. A disadvantage of this method, is that the DNA fragments produced posess flush or 'blunt' ends, which may require modification by small 'linker' molecules to produce compatible ends for ligation to an appropriate cloning vector. A slightly less random set of DNA fragments can be produced using a convenient simplification of this method, involving a single restriction enzyme which cuts frequently such as *Sau 3A* or *Mbo I.* However, although the fragments produced by this method, are of a slightly less random nature than those produced using the two-enzyme method, it has the great advantage that the fragments produced by *Sau 3A* have cohesive 'sticky' ends compatible with vector DNA cleaved with the restriction enzyme *Bam HI.* This enables DNA fragments produced by *Sau 3A* digestion to be inserted into a wide number of different cloning vectors, without the need to modify the ends of the fragments.

Genomic DNA fragments for gene library construction may also be produced using single restriction enzymes posessing usually hexanucleotide recognition sequences. But although a series of limit digests of the target DNA with enzymes such as *Pst I, Bam HI,* or *Eco RI* may produce suitably sized fragments with ends compatible for ligation to the vector of choice, the set of fragments will not be random. The possibility also exists that a gene

or other sequence of interest may contain a recognition site for one of these enzymes, and consequently will become extremely difficult if not impossible to isolate from any gene library constructed using that enzyme.

5.3.1 PARTIAL DIGESTION OF DNA WITH *Sau 3A*.

As mentioned above, the partial digestion of genomic DNA with the restriction enzyme *Sau 3A*, results in the production of a random set of DNA fragments. The size of the fragments produced by enzyme digestion can be regulated by varying the reaction conditions in a set of 'pilot' reactions, determining the size of the reaction products by agarose gel electrophoresis, and the use of the appropriate reaction conditions in a scaled up enzyme digestion.

Approximately 1μg amounts of *R.salmoninarum* genomic DNA, were subject to *Sau 3A* digestion at 37°C with varying amounts of enzyme produced by a doubling dilution series. After 20-30 min., the reactions were terminated via the inactivation of the enzyme by placing the reaction tubes at 65°C for 10 min. The reaction products were then subjected to agarose gel electrophoresis (0.5% gel) in conjunction with DNA molecular size markers (Fig 5.1). The enzyme concentration producing DNA fragments of an appropriate size for cloning was determined, and this concentration utilized in a scaled up partial digest.

5.4 CONSTRUCTION OF *R.salmoninarum* GENE LIBRARIES IN BACTERIOPHAGE LAMBDA VECTORS.

Bacteriophage λ, is a much studied virus of *E. coli*. As the result of many *in vitro* and *in vivo* manipulations of the λ genome, there are now available a large number of λ-based cloning vectors, which provide the easy and efficient means for the routine construction of gene libraries from genomic DNA. The λ genome is a linear duplex DNA molecule of approximately 49 kbp. At each end of the genome are short, single stranded cohesive termini, complememtary in sequence (*cos* site), by which the the DNA joins together to adopt a circular structure once injected into the host cell. The central region of the

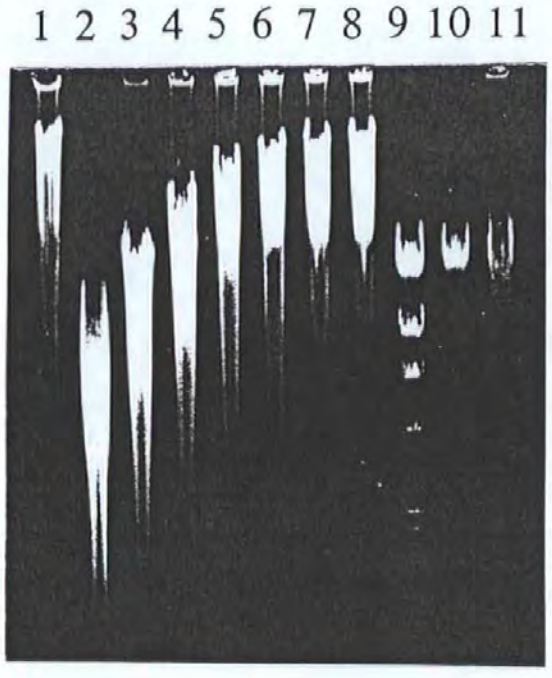


Figure 5.1. Fragmentation of *R. salmoninarum* genomic DNA by controlled *Sau 3A* partial digestion. Agarose gel electrophoresis on a 0.5% gel. Lane 1: Uncut *R.salmoninarum* DNA. Lanes 2-8: Partial digests of *R.salmoninarum* DNA with doubling dilutions of *Sau 3A* (Lane 2= 1 unit, Lane 8= 0.0156 units. Lane 9: Lambda/*Hind III* molecular size markers. Lane 10: Wild-type lambda DNA (48.6 kbp.)

molecule (the 'stuffer' fragment), nearly 40% of the λ genome, is inessential for vegetative propagation of the phage, and it is in this region, that foreign DNA can be accomodated. Vectors based on phage λ used for cloning experiments can accept between 0 to 23 kbp of foreign DNA.

The derivatives of wild-type λ produced for cloning purposes, either have a single target site at which foreign DNA can be inserted (insertional vectors), or have a pair of sites defining a portion of the stuffer fragment that can be removed and replaced with foreign DNA (replacement vectors). The capacity of λ vectors for foreign DNA is determined by the efficient packaging of the genome into the mature phage particle, only genomes in the 35-52 kbp range are packaged efficiently and hence will be propagated by the infectious life cycle of the phage. Wild type λ can therefore accomodate only about 5% more than its normal complement of DNA, hence, vector derivatives of the phage have deletions to increase the space available for foreign DNA. This size requirement for the efficient λ packaging provides a powerful positive selection method for recombinant molecules made in replacement type vectors. For, if the central 'stuffer' fragment is removed from the genome and discarded, then the deleted vector genome will only be packaged and give rise to infective virus if a new, foreign fragment of DNA is inserted.

Phage DNA molecules, recombinant or otherwise, can be introduced into the host bacterial cell as naked DNA in much the same manner as transformation with plasmids. This process, known as transfection, is not the method of choice for cloning experiments as the efficiency of the process ($< 10^4$ recombinant clones per μg of lambda DNA) is often poor. Higher efficiencies of recombinant λ introduction, are achieved by the process known as *in vitro* packaging, where the recombinant phage genomes are packaged in to phage coats, which are then able to infect the bacterial cell in the normal manner. The packaging procedure is more efficient when phage genomes are joined end to end via their *cos* termini to form concatenated DNA molecules, it is therefore important to ensure that

when recombinant phage genomes are constructed, that the conditions of the DNA ligation reactions chosen are favourable, to enable this concatenation to occur.

Two different λ-derived cloning vectors were used to construct *R.salmoninarum* genomic gene libraries for this research project; the large capacity relacement vector λL47.1 and the insertional expression vector λgt11. Diagrams showing the strategies employed to construct gene libraries in these vectors can be seen in Fig. 5.2.

5.4.1 CONSTRUCTION OF A *R.salmoninarum* GENE LIBRARY IN THE BACTERIOPHAGE VECTOR λL47.1 .

5.4.1.1 λL47.1 .

A diagramatic representation of the bacteriophage cloning vector λL47.1 is shown in figure 5.3). The vector is a replacement type λ derivative (Loenen and Brammar, 1980), whose development came as the result of the many research endeavours of the late 1970's that attempted to construct improved λ-based cloning vehicles. Phage λL47.1 fulfills two of the aims of these researchers, as it has a large capacity for inserted foreign DNA and the positive selection of recombinant phage can be achieved without the need to physically remove or destroy the central portion of the λ genome in order to accommodate the foreign DNA insert. The vector contains double cleavage sites for the restriction enzymes *Eco RI, Hind III* and *Bam HI,* and a capacity for inserted DNA ranging between 4.7 to 24 kbp.

to foreign DNA in the presence of the stuffer fragment, is based on the fact that wild-type λ does not grow on *E. coli* strains lysogenic for the bacteriophage P2. This phenotype is known as Spi$^+$ (sensitive to P2 interference) is due to the activity of the *red* and *gam* genes on the phage genome. Mutant λ, with defects in both genes are spi$^-$ and can undergo a lytic life cycle in *E. coli* P2 lysogens. The products of *red* and *gam* are responsible for phage replication and propagation, but in their absence their role can be fulfilled by the

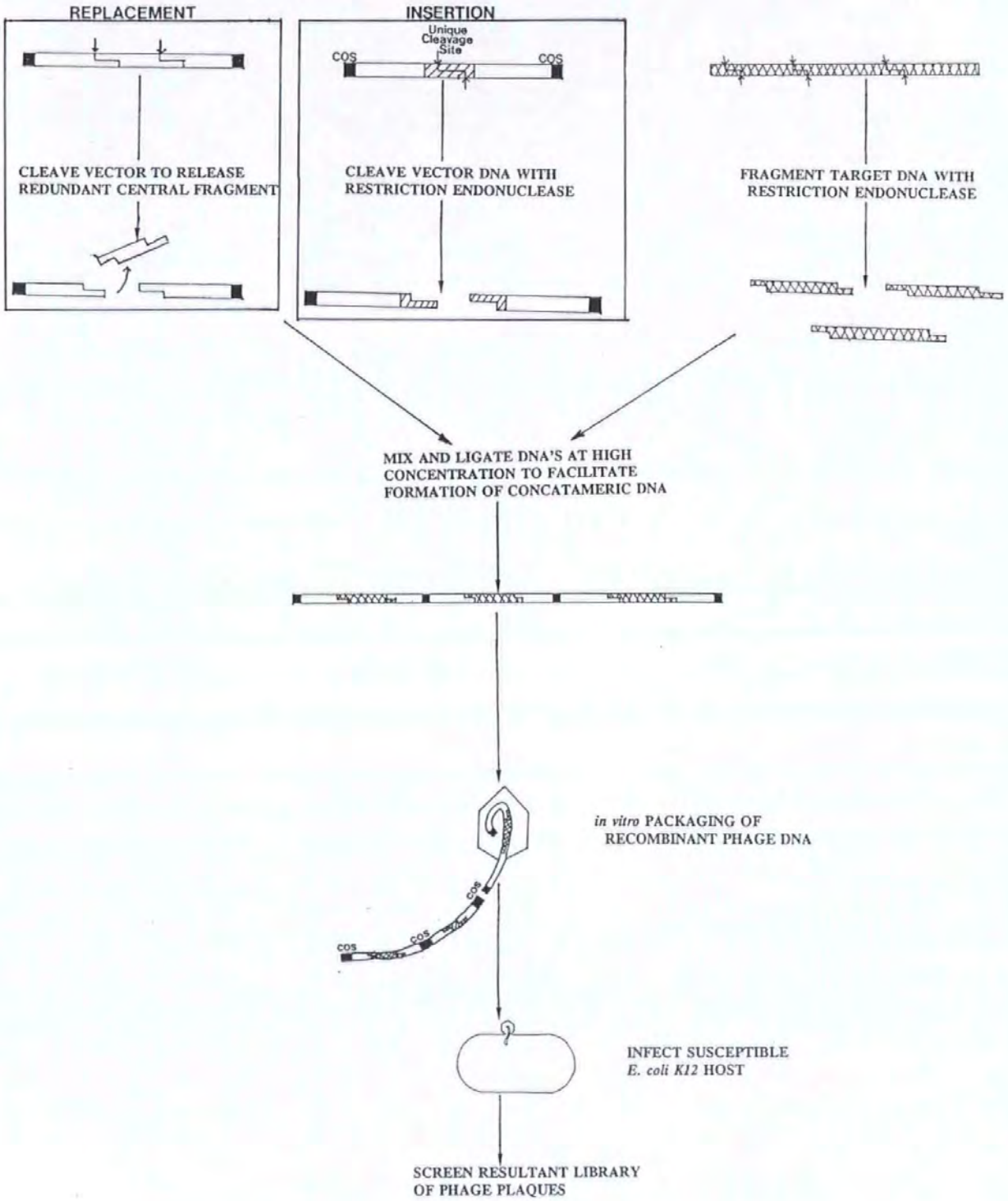
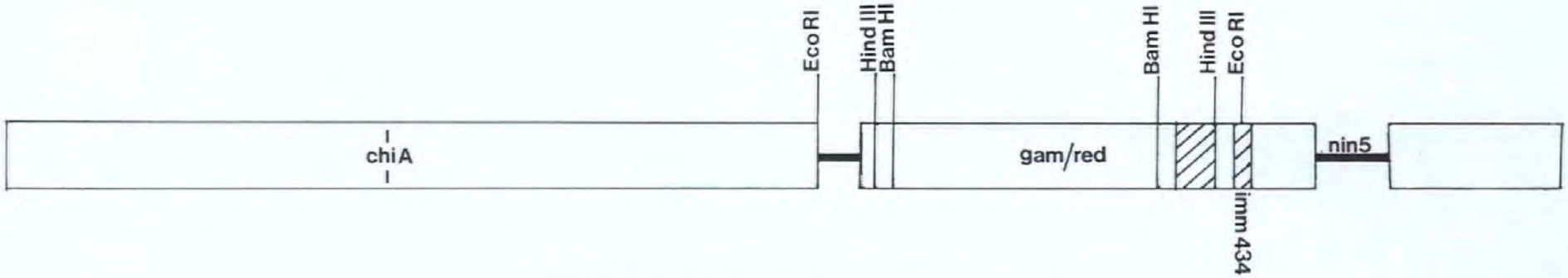


Figure 5.2 Strategy for the construction of gene libraries in replacement-type and insertion-type λ-based cloning vectors.



λL47.1 (Loenen and Brammar, 1980)
40.6 kbp.

Figure 5.3. Bacteriophage cloning vector L47.1. Restriction endonuclease cleavage sites for replacement cloning of foreign DNA are indicated as well as other important regions of the genome. Spaces spanned by thickened lines indicate deleted regions, whilst shaded areas indicate DNA from a non-λ source.

host-specific recombination enzyme RecA, as long as the DNA molecules contain sequences known as *chi* sequences (crossover hotspot instigator). In λL47.1, the *red* and *gam* genes are contained on the central stuffer fragment, whilst the *chi* sequences have been introduced on the left arm of the genome. Vector and recombinant λL47.1, characterized by the presence or absence of the *red* and *gam* genes respectively, can be distinguished by the infection of a Rec A^+ strain of *E. coli* K12, lysogenic for P2, where recombinant phage undergo a lytic life cycle and produce plaques on lawns of host bacteria, whilst unaltered vector phage do not.

5.4.1.2 CONSTRUCTION OF A λ47.1/*R.salmoninarum/Sau3 A* GENE LIBRARY.

Approximately 5μg of *R.salmoninarum* ATCC 33029 genomic DNA, was fragmented by *Sau 3A* digestion (see 5.2.1, above) under reaction conditions favouring the production of DNA fragments with an average size of about 15 kbp.

Phage λL47.1 DNA, at a concentration of 2μg was cleaved by digestion with the enzyme *Bam HI* at 37°C for 5 h. Cleavage of the vector was confirmed by agarose gel electrophoresis in conjunction with molecular size markers, ensuring the presence of the left arm of the vector (24 kbp), the right arm (10.5 kbp) and the central 'stuffer' fragment (7 kbp).

Approximately 1μg of cleaved vector DNA was mixed with 3μg of *R.salmoninarum* DNA fragments and ligated using T4 DNA ligase at 15°C for 18h. Ligation was confirmed by the presence of higher molecular weight DNA observed after agarose gel electrophoresis in a 0.5% gel. Ligated DNA was packaged using a 'Gigapack' *in vitro* packaging system (Stratagene, La Jolla, CA., U.S.A.) according to the manufacturers instructions.

A serial ten-fold dilution series of the packaged bacteriophage particles was made in SM buffer and these dilutions plated out on the *E.coli* K12 P2 lysogen Q359 as described in 3.3.1. (above).

After incubation, recombinant phage were recovered by removing the soft agar overlay

containing the phage plaques and placing it into a microcentrifuge tube. The overlay was then centrifuged at 6,500 x g for 10 min. after which, the supernatant fluid containing bacteriophage particles constituting the *R.salmoninarum* / λL47.1 amplified gene library, was collected, treated with a drop of chloroform to kill any remaining *E. coli* K12, titrated and stored at 4°C.

5.4.2 CONSTRUCTION OF A *R. salmoninarum* GENE LIBRARY IN THE BACTERIOPHAGE EXPRESSION VECTOR λgt11.

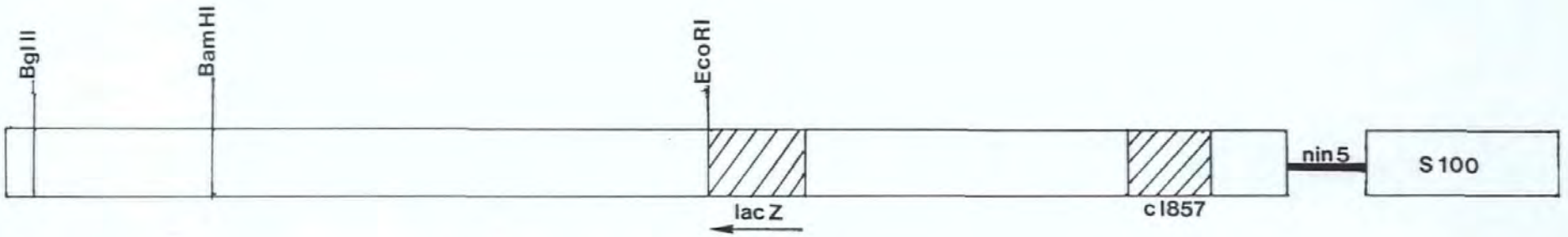
5.4.2.1 λgt11.

The structure of the λgt11 genome is shown in fig 5.4. It is a λ-derived insertional vector, containing a unique *Eco RI* site within the *lac Z* gene for the insertion of up to 7.2 kbp. of foreign DNA. The phage vector produces a temperature-sensitive repressor (*cI*857) which is inactivated at 42°C. and carries an amber mutation (S100) that renders it lysis-defective in hosts lacking the amber repressor *sup F* (Huynh *et al.*, 1985).

Because the site of insertion for foreign DNA in λgt11 is within the structural gene for β-galactosidase, foreign DNA sequences in this vector have the potential to be expressed as fusion proteins with β-galactosidase. In a number of cases, fusion of a foreign protein to a stable *E. coli* protein enhances the stability of the foreign protein.

The *lac Z* gene in λgt11 is derived from the *lac* operon of *E. coli*, and posesses a strong promoter, couple this with the high copy number attained during λgt11 lytic infection and the potential exists for the production of a significant amount of fused foreign protein. Foreign proteins may also be expressed in λgt11 without being fused to β-galactosidase. This may be due to expression utilizing the *lac Z* promoter (*lac* dependent) or as a result of transcription and translation of a DNA sequence utilizing promoter regions contained within the DNA insert (*lac* independent).

An extremely valuable feature of gene libraries constructed in λgt11 (and one of the reasons why this vector was chosen for cloning experiments with *R. salmoninarum* DNA),



λgt 11 (Huynh *et al.*, 1985)
43.7 kbp.

Figure 5.4 Bacteriophage cloning vector λgt 11.
Restriction enzyme cleavage sites are shown, including the unique *Eco RI* site for the insertion of foreign DNA into the *lacZ* gene for the expression of foreign DNA as fusion proteins. Also shown is the *cI857* temperature-sensitive repressor region, and the S100 amber mutation.The space spanned by a thickened line indicates a deleted region, whilst shaded areas indicate non-λ DNA.

is that antibodies can be used as probes to detect and isolate recombinant clones producing proteins of interest. The vector was also chosen because of the particular attention paid during its design to the problems associated with the production of foreign proteins in *E. coli*. The first problem is to achieve an adequate level of expression of the foreign DNA sequence. Having a foreign DNA sequence fused to β-galactosidase gene sequences, enhances the possibility that the foreign DNA will be expressed efficiently in the *E. coli* host. The second problem is to be able to control the production of proteins encoded by the foreign DNA sequence. This is important when the foreign protein is toxic to the host cell and might kill it before sufficient amounts of antigen are produced. This problem has been minimised by using host cells producing large amounts of *lac* operon repressor (the product of the *lacI* gene) to prevent *lacZ*-directed expression of the fusion protein during the initial hours of plaque formation. When the number of cells surrounding the plaque is sufficiently large, *lacZ*-directed expression is induced by inactivating the repressor with the gratuitous inducer molecule isopropyl β-D-thiogalactopyranoside (IPTG). In this way, detectable amounts of the foreign antigen can be produced, even if the antigen is toxic to the cells. The third problem associated with the production of foreign proteins in *E. coli* is their stability. The *lon* protease is one of several proteases responsible for the generally low stability of foreign or otherwise abnormal proteins in *E. coli*. These proteases frequently make it difficult to accumulate detectable amounts of antigen in wild-type cells. For this reason, a *lon*$^-$ strain is used as a host so as to increase the stability of foreign proteins for the screening proceedure.

5.4.2.2 CONSTRUCTION OF A λgt11/*R.salmoninarum/Eco RI* GENE LIBRARY.

Approximately 5μg of *R.salmoninarum* ATCC 33209 genomic DNA was cleaved with the restriction enzyme *Eco RI* for 18 h. at 37°C. utilizing the manufacturers supplied reaction buffer.

Eco RI-cleaved λgt11 was obtained in the form of alkaline phosphatase phosphorylated

vector arms (Stratagene, La Jolla, CA., USA).

A 1μg quantity of vector arms was mixed with 3μg of *Eco RI*-cleaved *R.salmoninarum* DNA and ligated using T4 DNA ligase. The ligation reaction was performed at 15°C for 18 h. in a small volume of reaction mixture to encourage the formation of concatenated λ recombinants. The ligation reaction was confirmed by agarose gel electrophoresis, and the ligated DNA packaged using the 'Gigapack' *in vitro* packaging kit. A tenfold dilution series of the packaged bacteriophage particles was made in SM buffer and samples were plated on *E. coli* K12 strain Y1088.

After incubation, recombinant phage, constituting a *R.salmoninarum* / λgt11 gene library were recovered from the soft agar overlay by centrifugation (see 5.3.2), treated with a drop of chloroform to kill remaining *E. coli* cells, titrated and stored at 4°C.

5.5 CONSTRUCTION OF *R.salmoninarum* GENE LIBRARIES IN PLASMID VECTORS.

Plasmids, are extrachromosomal genetic elements which can replicate independently within a host cell. They are normally dispensable to the cell, but their presence may be advantageous, e.g. they may convey resistance to antibiotics upon a bacterium (Helinski, 1979).

The plasmids used as cloning vectors are usually double-stranded, closed circular DNA molecules ranging between 2 to 8 kbp., with a usable capacity for inserted foreign DNA of up to about 10 kbp. The majority of the plasmid vectors constructed, have been for use in *E. coli* (Pouwels *et al.*, 1985).

An ideal plasmid for cloning purposes would have the following 3 properties: a) a low molecular weight; b) the ability to confer readily selectable phenotypic traits on host cells; and, c) single cleavage sites for a large number of restriction enzymes, preferably within genes with a readily scorable phenotype.

There are several advantages of a low molecular weight. First, the plasmid is much easier

to handle, i.e. it is more resistant to damage by shearing, and is readily isolated from host cells. Second, low molecular weight plasmids are usually present as multiple copies, not only facilitating their isolation but this also leads to gene dosage effects for all cloned genes. Finally, with a low molecular weight, there is less chance that the vector will have multiple restriction enzyme cleavage sites.

After a piece of foreign DNA is inserted into a plasmid vector, the resulting recombinant molecules can be transformed into a suitable bacterial host. Since transformation efficiency is generally low, it is essential that the recombinants have some readily selectable phenotype, usually resulting from a gene carried on the vector, e.g. antibiotic resistance, but it could equally be produced by a gene cloned on the inserted DNA. Plasmids with biological markers for antibiotic resistance and genes for the production of readily detectable enzymes such as β-galactosidase, are even more versatile for cloning purposes.

Possessing only one site of cleavage for a given restriction enzyme, allows the vector to be cut only once to accept foreign DNA. The advantage gained by cloning in a vector where the insertion of foreign DNA inactivates a gene whose phenotype is readily scorable, is that it is possible to distinguish recombinants from non-recombinants, whose vector molecules have merely re-annealed. Vector re-ligation can be a common occurrence, even if the target DNA is present in proportionally large amounts. However the treatment of cleaved vector DNA with alkaline phosphatase can prevent overt recirculation events during ligation reactions.

Of course, readily detectable insertional inactivation is not essential if the foreign DNA insert confers a new phenotype on the host cell, as this new phenotype can be used in the selection procedure.

There is an inherent ability to 'custom build' plasmid vectors and still maintain a low molecular weight, this stems from the fact that there is a relatively small sequence of DNA required for plasmid replication in those naturally occurring *E. coli* plasmids Col

E1 and pMB1, on which the majority of plasmid cloning vectors have been based. There is a huge variety of plasmid vectors avilable for cloning experiments in *E. coli* K12, and more are constructed each year. Two different plasmid vectors were chosen for the construction of *R.salmoninarum* gene libraries, these were the versatile high copy-number expression plasmid pUC18, and an equally versatile low copy-number plasmid pGD103. A general strategy for gene library construction in plasmid vectors is shown in Fig 5.5.

5.5.1 PLASMID VECTOR pUC18.

The structure of the plasmid cloning vector pUC18 is shown in fig 5.6. pUC18 is a widely used member of the pUC family of general purpose plasmid vectors for *E. coli* utilizing the replicon of pMB1. At a size of 2.7 kbp, the plasmid carries the selectable phenotype of resistance to the antibiotic ampicillin, and the *lac Zα* region of *E. coli* containing unique cleavage sites for a number of restriction enzymes. Insertion of foreign DNA into the *lac Zα* region, renders recombinants *lac*$^-$, enabling their selection via *lac* gene complementation (blue-white screening) in *E. coli K12* hosts such as JM109 or XL1-blue which are deleted for *Lac Zα*. The large number of unique cloning sites, and the small size of the pUC18 molecule, make it an ideal cloning vector. In addition, the strong induceable *lac* promoter and the potential high copy number of up to 700 copies of the plasmid per host cell, facilitates increased production of foreign proteins.

5.5.1.1 CONSTRUCTION OF A pUC18/*R.salmoninarum/Sau 3A* GENE LIBRARY.

Approximately 5μg of *R.salmoninarum* MT444 genomic DNA was partially cleaved with *Sau 3A*, under conditions favourable to the production of DNA fragments of a size of 10 kbp and under. At the same time, 2μg of pUC18 DNA was cleaved with the restriction enzyme *Bam HI* at 37°C for 5 h. in the manufacturers supplied buffer system. Vector cleavage was confirmed by agarose gel electrophoresis in a 0.5% gel. To suppress vector/vector ligation and recircularization events, the cleaved plasmid DNA was then treated with alkaline phosphatase as described in 3.6.2 (above).

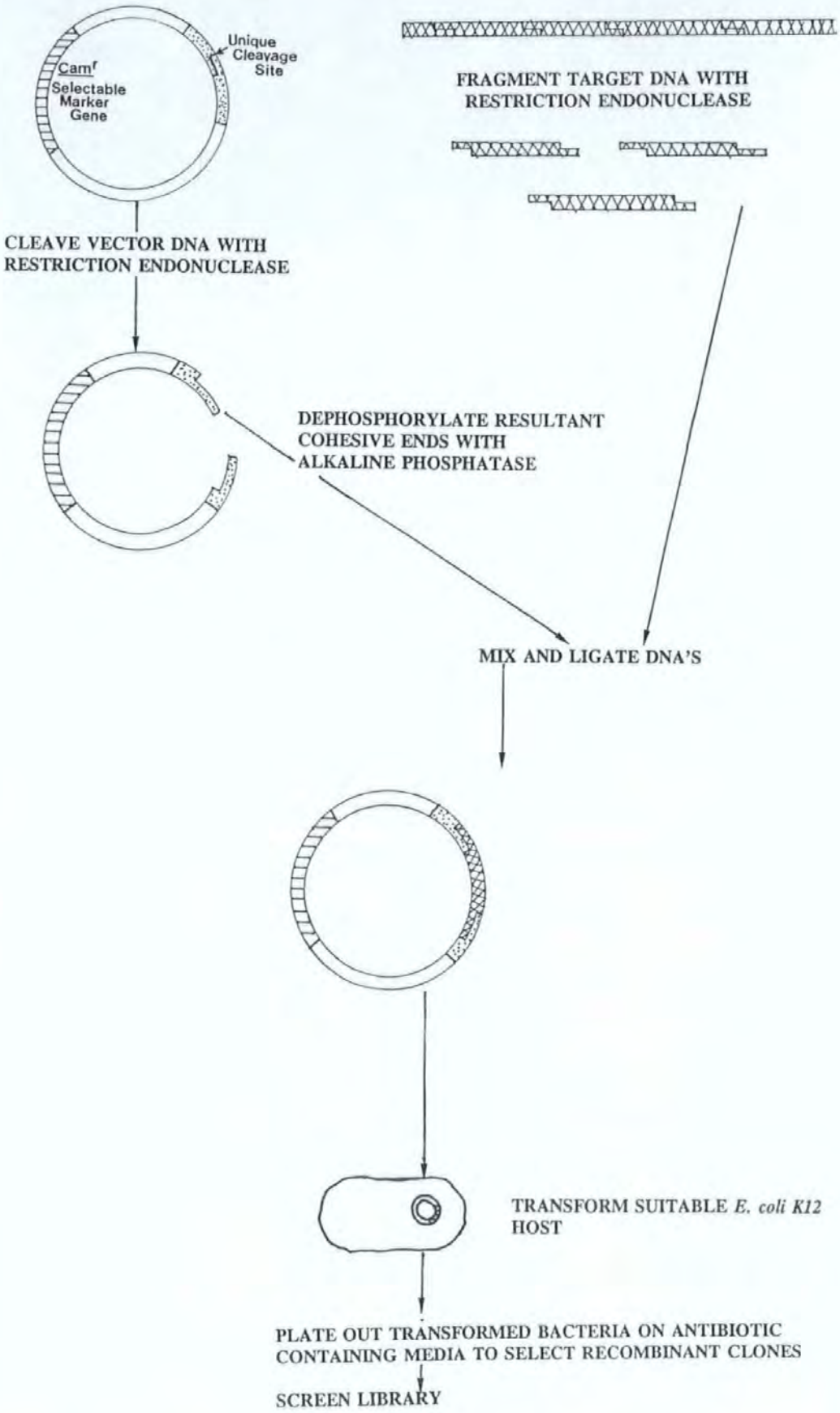


Figure 5.5. Strategy for the construction of gene libraries in plasmid cloning vectors. The plasmid shown possesses a selectable marker gene coding for resistance to the antibiotic chloramphenicol (*cam*^{r}) and a unique restriction endonuclease cleavage site for the insertion of foreign DNA.

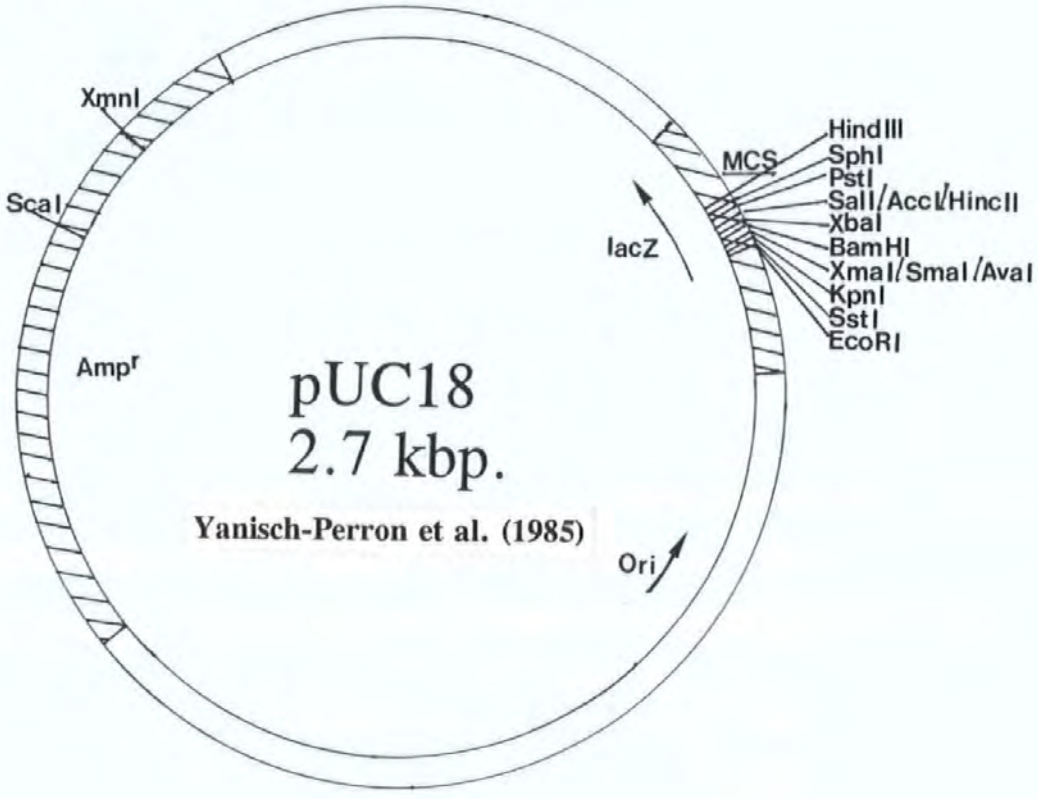


Figure 5.6. Plasmid cloning vector pUC18.
Unique restriction enzyme cleavage sites for the cloning of foreign DNA are shown including the multiple cloning site (MCS). The origin of replication (Ori) is shown, along with the selectable markers, the genes for resistance to the antibiotic (Amp^r) and for the enzyme β-galactosidase (*lacZ*).

R.salmoninarum DNA fragments were ligated to cleaved pUC18 at 15°C for 18h at a target:vector DNA ratio of 2:1, and the ligation confirmed by agarose gel electrophoresis. The ligated DNA mixture, which would later be used to form a gene library was stored at 4°C.

5.5.2 PLASMID VECTOR pGD103.

The plasmid cloning vector pGD103 (Fig. 5.7), is derived from the low copy number plasmid pLG339, which permints the propagation of cloned DNA segments at low gene dosage levels. This vector may therefore be used to clone genes encoding products that that are toxic to the host when cloned in a vector with a high copy number. The pGD103 selectable phenotype is resistance to the antibiotic kanamycin, whilst the major structural difference between pGD103 and its parent pLG339, is the inclusion of the *lac Zα* region from pUC8. As with pUC18, this increases the usefulness of the plasmid as a cloning vector, both by increasing the number of unique cloning sites available, and, the selection of recombinants by virtue of their *lac*$^-$ phenotype in *E. coli K12* host strains such as JM109 and XL1-blue.

5.5.2.1 CONSTRUCTION OF A pGD103/*R.salmoninarum*/*Sau 3A* GENE LIBRARY.

Approximately 5μg of *R.salmoninarum* NCIMB1116 DNA was was partially cleaved with *Sau 3A*, under conditions favourable to the production of DNA fragments of a size of 10 kbp and under. At the same time, 2μg of pGD103 DNA was cleaved with the restriction enzyme *Bam HI* at 37°C for 5 h. in the manufacturers supplied buffer system.

Vector DNA molecules were dephosphorylated with alkaline phosphatase, before the cleaved DNAs were ligated using T4 DNA ligase at 15°C for 18 h. and the successful ligation confirmed by agarose gel electrophoresis. The ligated DNA mixture, which would later be used to form a gene library, was stored at 4°C.

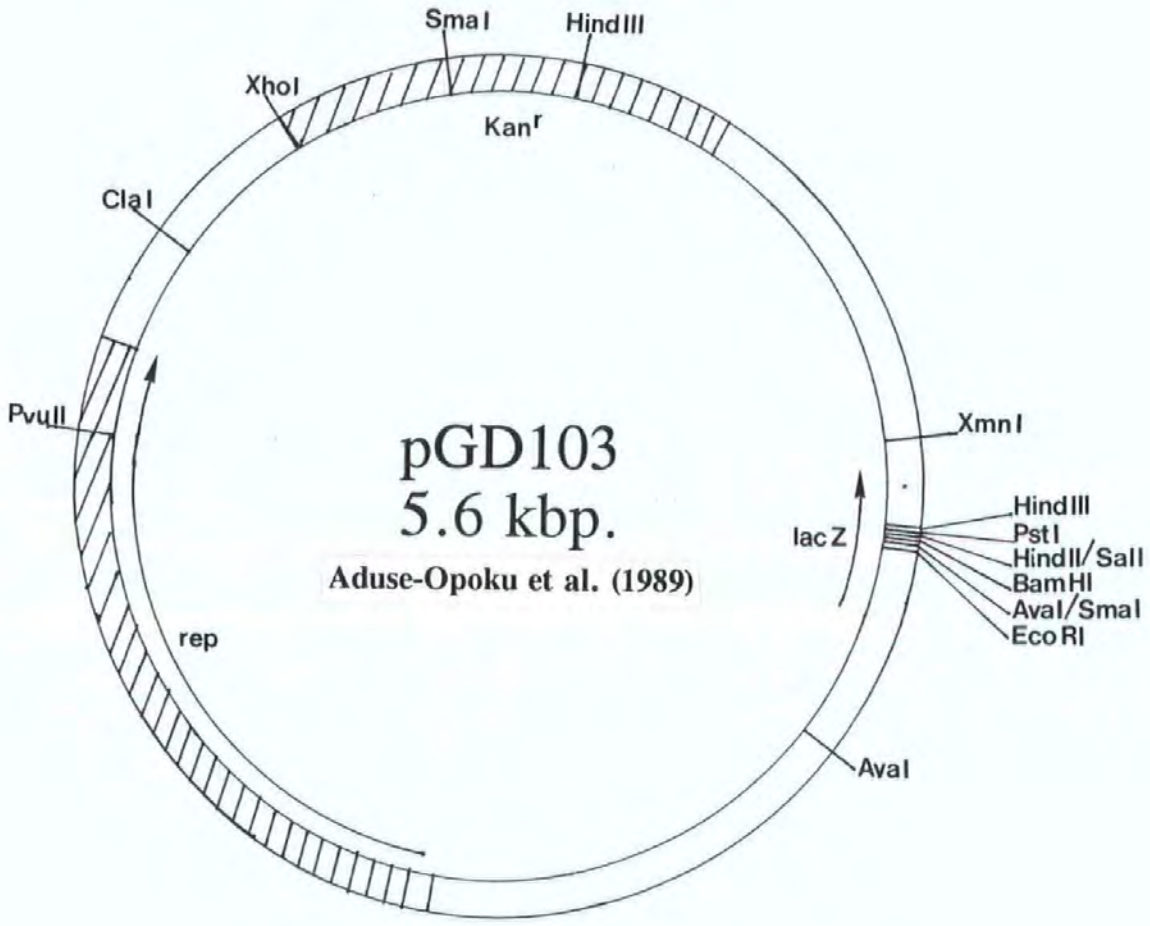


Figure 5.7. Plasmid cloning vector pGD103.
As well as restriction enzyme cleavage sites, genes for selectable markers kanamycin resistance (*kan*^{r}) and β-galactosidase (*lacZ*) are shown. Also shown is the region containing the origin of replication from the plasmid pLG339 (rep).

5.6 CONSTRUCTION OF A *R.salmoninarum* GENE LIBRARY IN THE COSMID VECTOR pHC79.

5.5.1 COSMID VECTORS.

Cosmid cloning vectors were first developed by Collins and Hohn (1980) to overcome the technical difficulties of introducing large pieces of DNA into *E. coli* by the processes of transformation or transfection. Most protocols for the preparation of competent *E. coli* cells, allow efficient transfer of DNA molecules up to 10-15 kbp, but attempts to use molecules larger than this, unless incorporated in to a bacteriophage λ vector, results in the dramatic reduction of transformation efficiency. Collins and Hohn introduced the bacteriophage λ *cos* sequence into a conventional 4-5 kbp plasmid cloning vector. This allowed the use of the λ *in vitro* packaging protocols, completely overcoming the size effects seen in transfection and transformation. The *cos* sequence, and about 200 bp of DNA either side, is required for packaging DNA into mature λ phage particles. There are no other specific λ DNA segments that are necessary for this, and for successful packaging of DNA molecules, there must be 2 compatable *cos* sequences contained on the same molecule, separated by 35-52 kbp, which are the minimum and maximum, respectively, packaging limits of λ phage. Because of the small amount of plasmid DNA present in a cosmid vector, the capacity for foreign DNA is large (30 to 45 kbp). Such a large capacity vector is invaluable for genomic library formation, as theoretically the number of individual recombinants that need to be screened to acheive a high probability of isolating the gene of interest, is smaller compared to gene libraries constructed with other types of cloning vectors, which can only accomodate smaller DNA inserts. The individual steps of cosmid cloning are as follows. Cosmid vector DNA is first linearized by digestion with a suitable restriction enzyme. The foreign DNA to be inserted is partially cleaved to form fragments of an appropriate size containing compatible protruding ends. Ligation of the mixed DNA with DNA ligase yields a wide range of

recombinant molecules, and the use of high DNA concentrations favours the formation of concatermers.The ligated DNA is packaged into mature λ particles by employing an *in vitro* packaging protocol and the subsequent phage suspension is used to infect a suitable λ-sensitive *E. coli* strain. The cosmid, which does not carry any other λ functions, is circularized *in vivo,* and replicates without producing plaques. Under these conditions, only plasmid-specific functions, such as the origin of DNA replication, and the selective biological markers are active. These markers, normally antibiotic resistance markers, can then be used for the selection of recombinant cosmids. A general strategy for gene library construction in cosmid vectors is shown in Fig. 5.8.

5.6.2 COSMID pHC79.

A diagramatic representation of the cosmid vector pHC79 is shown in Fig 5.9. This vector was constructed by replacing a 207 bp. *Sau 3A* fragment of the plasmid pBR322 with a 1.78 kbp. *Bgl II* fragment from the genome of λ charon 4A which contains the *cos* region. Other plasmid sequences, such as the genes for resistance to ampicillin and tetracycline, have not been altered, and are available as selective biological markers for recombinant screening. This cosmid is regarded as one of the most versatile cosmid vectors, as it contains several useful restriction enzyme cleavage sites for the insertion of foreign DNA. Cosmid pHC79, maintains a low copy number whist replicating in its *E. coli* host (1-5 copies). It was because of this fact, along with the smaller size of the resultant gene library, that pHC79 was chosen for gene library construction, for if the inserted DNA expresses a protein, that is toxic to the *E. coli* host cell, the increased gene dosage effect from high copy number vectors such as pUC18 may result in the rapid death of the *E. coli* clone.

5.6.3 CONSTRUCTION OF A pHC79/*R. salmoninarum/Sau 3A* GENE LIBRARY.

A 5μg quantity of *R. salmoninarum* PPD genomic DNA was partially digested using the restriction enzyme *Sau 3A* (see 5.2.1), under the appropriate conditions for the formation

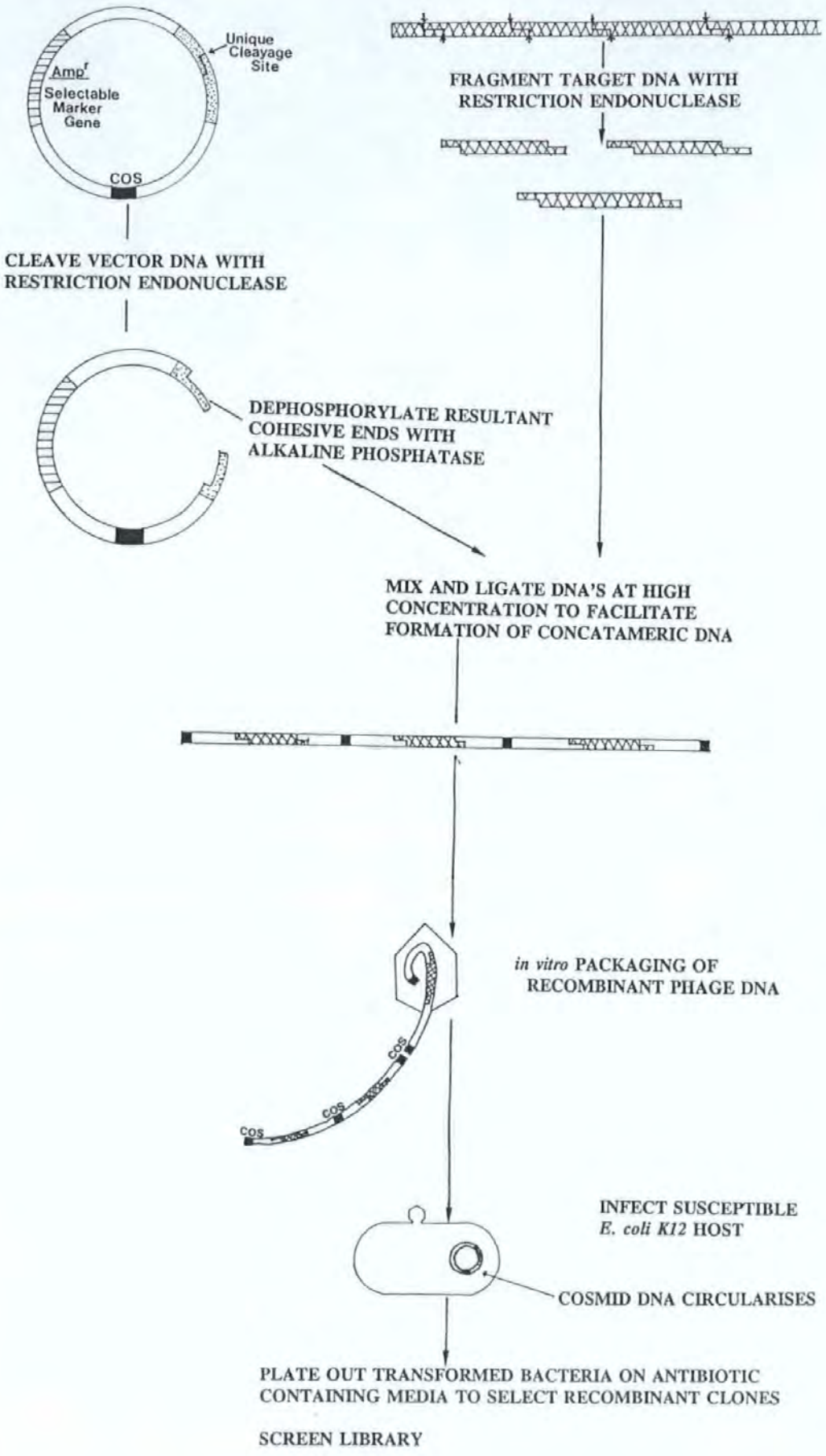


Figure 5.8. Strategy for the construction of gene libraries in cosmid cloning vectors. The cosmid shown has a selectable marker gene for resistance to the antibiotic ampicillin (*Amp*^{r}) and a unique restriction endonuclease cleavage site for the insertion of foreign DNA. Also shown are the cohesive termini (cos region) originating from the λ genome.

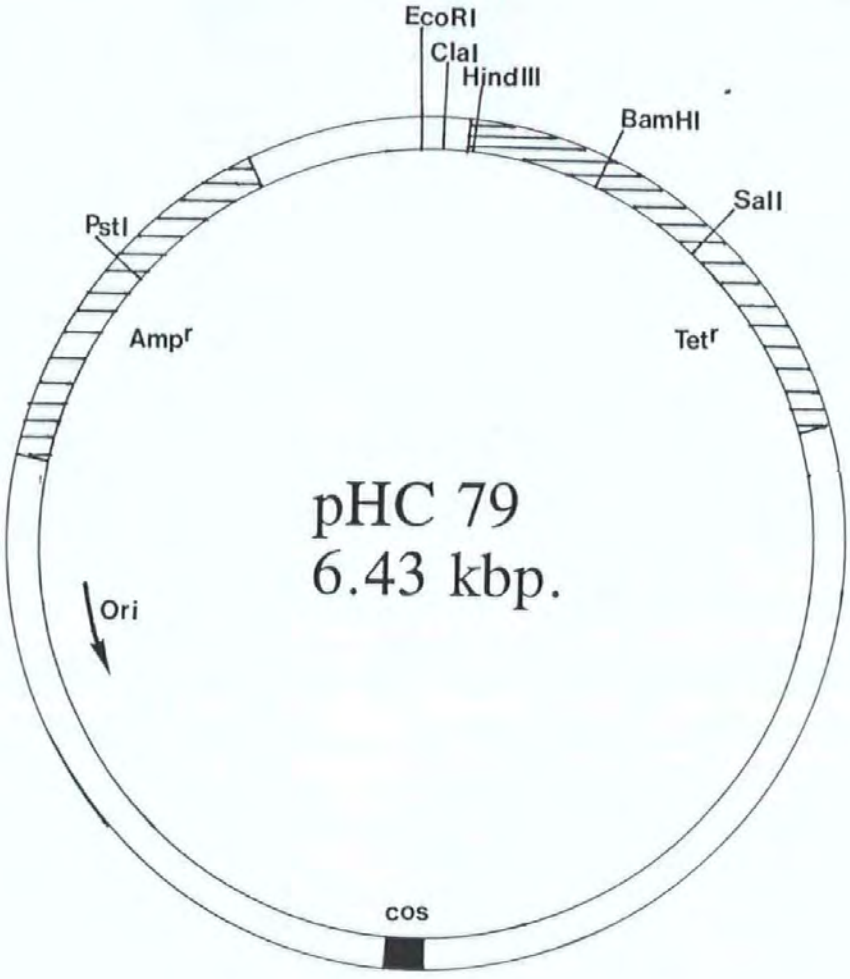


Figure 5.9. Cosmid cloning vector pHC79.
Unique restriction enzyme cleavage sites are shown along with genes encoding the selectable markers of resistance to the antibiotics ampicillin (Amp^r) and tetracycline (Tet^r). The cos region from bacteriophage λ is also shown (cos) along with the origin of replication (Ori).

of DNA fragments in the size range 30 to 45 kbp.

Approximately 3μg of pHC79 DNA was cleaved using the restriction enzyme *Bam HI* at 37°C. for 5 h. followed by treatment with alkaline phosphatase as described in 3.6.2 (above).

R. salmoninarum DNA fragments (3μg) were mixed with approximately 1μg of cleaved pHC79 DNA and ligated for 18 h. at 15°C using T4 DNA ligase and in a relatively small buffer volume to encourage the formation of concatemers.

The ligated recombinant DNA was then packaged into mature phage particles by *in vitro* packaging. This collection of recombinant DNA molecules was stored at 4°C. over chloroform for future use.

5.6 DISCUSSION

The construction of gene libraries from *R. salmoninarum* is the starting point of this research project. Although the methods employed are today well established, gene library construction can be seen as a crucial phase of any gene cloning based study of *R. salmoninarum* in that the success of such a study i.e. the isolation and characterization of macromolecules important in BKD infection could well be influenced by the judicious choice of cloning vector.

Twenty years on from the first cloning experiments, there is now a bewildering range of cloning vectors based on bacteriophage, plasmids and combinations thereof for use in the construction of gene libraries. Although the choice of an appropriate vector may appear a difficult task, in reality, such a choice is both driven and guided by a small number of factors.

Firstly, it is desirable that the vector chosen allows the construction of a conveniently sized gene library, one where only a relatively small number of individual recombinant bacterial or viral clones have to be screened to be sure of a high probability of obtaining the gene sequence of interest. As each type of cloning vector has a certain capacity for

foreign DNA inserts, and the genome of interest is of a finite size, it is possible to calculate, prior to any experimental work, the number of individual recombinants that would have to be screened to ensure the high probability of obtaining a given DNA sequence. This calculation, that relates the probability of including any given DNA sequence in a random library of independent recombinants, is made using the formula of Clarke and Carbon (1976):

$$N = \frac{\log(1-P)}{\log(1-x/y)}$$

Where, P= the desired probability, x=size of foreign DNA insert (base pairs), y=the size of the genome (haploid) under study (base pairs) and N= the number of individual recombinants.

Therefore it can be seen, that the size of a gene library is influenced by both the capacity of the cloning vector chosen and the size of the genome under study, and indeed, it has been this interrelationship that has driven the development of a number of cloning vectors. In the present work, the target genome is bacterial in origin and therefore considerably smaller than those found in eukaryotes, therefore, even gene libraries constructed with small capacity vectors such as plasmids will be of a manageable size.

Another major consideration to be made when undertaking genomic library construction is whether, as is the case in this research project, gene expression is required. In the proposed molecular study of *R. salmoninarum* the target gene sequences are unknown and the encoded proteins totally or virtually uncharacterized, so ultimately, the success of the present work depends on the stable expression of cloned gene sequences in the *E. coli* host. Again, it is fortunate that the DNA sequences used in this project are bacterial in origin and are more suited to expression in *E. coli*. In anticipation of any incompatibility between the machinery of gene expression in *R. salmoninarum* and *E. coli*, gene libraries of *R. salmoninarum* have been made in cloning vectors possessing strong

promoter regions, which if placed in frame with *R. salmoninarum* gene sequences could drive the expression of *R. salmoninarum* macromolecules.

Another consideration in the choice of cloning vector for the construction of *R. salmoninarum* gene libraries is the possibility that genes for the target molecules, i.e. cell-associated antigens, proteolytic enzymes and pathogenic toxins, if expressed, may kill the *E. coli* host or severely impair its metabolism, hence preventing the cloning process. To this end, cloning vectors with low copy numbers, such as pGD103 and pHC79 were employed in gene library construction. It was hoped that even if toxic molecules were produced in the *E. coli* host, that a gene dosage effect would be prevented.

Familiarity with certain cloning vectors, often influences their choice for gene library construction. Although there are a plethora of various cloning vectors available commercially to the research worker, it is often the case that the vector(s) employed are those in regular use in his/her laboratory and have been used with previous success by his/her co-workers. In this way, resources may be conserved and any practical problems anticipated and overcome.

The construction of gene libraries from *R. salmoninarum* DNA was found to be relatively straightforward. The use of well established techniques enabled the formation of a number of libraries from which gene sequences encoding *R. salmoninarum* macromolecules could be recovered. The experimental work described above, however, is not a true chronological record of gene library construction. For the sake of clarity, the construction of gene libraries in each type of vector is described. In practice, a library was first constructed and then screened for recombinant phage or bacteria expressing *R. salmoninarum* antigens, or possessing a haemolytic or proteolytic phenotype. If the screening process was unsuccessful, a new library was made in a different vector and this library screened as before. The means by which each library was screened, the chronological order of library construction and screening, and the outcome of each

screening proceedure, is the subject of the next chapter.

CHAPTER 6. SCREENING OF *R.salmoninarum* GENE LIBRARIES CONSTRUCTED IN *E. coli K12* HOST/VECTOR SYSTEMS.

6.1 INTRODUCTION.

Once a gene library has been constructed and is represented by a collection of bacterial or viral clones, the next task in a gene-cloning study, is to find the desired clone or clones contained within this population. For the purposes of this research project, the desired clones will be those containing and expressing genes coding for *R.salmoninarum* proteins.

At first sight, the task of finding the individual desired clone among many thousand bacterial colonies or bacteriophage plaques may appear to be a formidable one, however, by using unique properties of the gene sequence of interest, an individual recombinant may be distinguishable. Two detectable and unique properties of the clone of interest can be used in its identification, and on which the successful search (screening) of a particular gene library depend; one property being the structure of the target DNA insert i.e. its nucleotide sequence, and the other its function i.e. the product of expression of a cloned gene (Old and Primrose, 1989; Sambrook *et al.*, 1989).

Complementary single-stranded polynucleotides, whether DNA or RNA, can anneal together (hybridize) with high specificity regardless of the presence of a great excess of unrelated sequences, and this fact can be exploited in a number of ways to find specific sequences within a large number of recombinant DNA molecules (Kaiser and Murray, 1985). The simplest way of screening gene libraries for complementary nucleic acid sequences, whether they are contained within a population of plasmid containing bacterial colonies or bacteriophage plaques, is to detect the recombinant DNA once it has been transferred to an immobilizing matrix support such as a nitrocellulose or nylon membrane. The colonies or plaque products are transferred to the membrane directly from an agar plate, in the case of bacterial colonies these are lysed prior to tranfer. The DNA is denatured, and the matrix incubated in a solution containing a labelled nucleotide probe

under conditions that permit annealing of complementary strands. In this manner, many thousands of recombinant clones can be screened rapidly and efficiently for the presence of a desired DNA sequence, which is identified by the detection of the labelled annealed probe.

Gene libraries may also be screened in this manner, without prior knowledge of the nucleotide sequence of interest, if the amino acid sequence of the polypeptide product of the desired gene is known. By using the genetic code, it is possible to deduce a corresponding nucleotide sequence and to synthesize it chemically, and use this product as a probe for screening gene libraries.

However, it is often the case, and this applies to the present study of *R.salmoninarum*, that there is simply no available probe for direct screening of recombinant clones. In such a case, indirect methods of screening may be utilized. These indirect methods form the basis of the screening procedures adopted for *R.salmoninarum* genomic gene libraries in *E. coli K12* host/vector systems and are based on selection of a given phenotype.

The expression of a gene contained within a recombinant DNA insert, will under some circumstances impart to a host cell a distinct and ultimately detectable phenotype. Screening techniques based on phenotype selection enable the identification and isolation of a specific clone, just as the standard selectable markers included in many cloning vectors permit the detection of transformed cells. In practice, there are two means of screening gene libraries via phenotypic selection; a) biochemical means, where the target gene product is an enzyme and may be detected using the appropriate substrate either natural or synthetic, and b) Immunochemical means, where a specific antibody has been raised against the target molecule. Both methods can be designed to screen large numbers of colonies or plaque products in a short period of time, and can be very selective when used with appropriate host/vector controls are utilized.

This chapter describes chronologically, the screening of *R. salmoninarum* gene libraries

constructed in bacteriophage, plasmid and cosmid cloning vectors. The gene libraries were screened using both biochemical and immunochemical phenotypic selection methods either for the expression of haemolytic and proteolytic activity or the expression of *R. salmoninarum* antigens recognised by rabbit antiserum raised against both whole cells and the extracellular products (ECP) of the bacterium.

6.2 IMMUNOLOGICAL SCREENING OF A λgt11/ *R.salmoninarum /EcoRI* GENE LIBRARY.

Gene libraries constructed using the insertion-type, lambda-derived, expression vector λgtll, whether utilizing genomic DNA or cDNA, are screened immunologically as recombinant phage plaques on a lawn of *E. coli K12* host bacteria. Proteins released by the lysis of cells within the plaques are immobilized on a nitrocellulose membrane placed over the lawn. Protein bound to the nitrocellulose membrane is probed with antibodies raised against the antigen(s) of interest, and antibody binding is revealed in a second step by probing the membrane with a second, labelled antibody.

The *E. coli K12* strain used for the screening of λgt11 gene libraries is Y1090. The features of this strain that make it suitable for this purpose are:

i) The *lac* repressor, which prevents *lacZ*-directed gene expression until it is derepressed by the addition of the gratuitous inducer, IPTG;

ii) A deficiency in the *lon* protease, which increases the stability of recombinant proteins expressed in the cell;

iii) The amber suppressor *supF* gene, to suppress the phage mutation causing defective lysis (*S*100).

The number of individual phage plaques that needed to be screened to constitute a λgt11/ *R.salmoninarum/EcoRI* gene library was estimated by using a probability value of 0.99 (99% probable), taking 4 kbp as the average insert size, and the size of the *R.salmoninarum* genome as 3×10^6 bp. Using these in the formula described in 5.6

(above), the value for N, or the number of phage plaques was calculated at 3452. As the values used in the calculation were only estimates, and the value for N only refers to foreign DNA sequences stably maintained within their respective vectors and host cells, the number of recombinant plaques chosen to be screened at each library formation was much larger than the calculated figure for N, and was in the order of 10,000.

Recombinant phage suspension was plated out on to an overnight culture of *E. coli K12* Y1090 in proportions favouring the formation of approximately 1,000 plaques per petri dish. Phage and host bacteria were mixed in 3 ml molten soft agar containing 40μl of a 24mg/ml solution of IPTG.

Once the agar overlay had set, the plates were incubated for 2 hours at 45°C to inactivate the *cI857* temperature-sensitive repressor and prevent the adoption of a lysogenic life cycle by the bacteriophage. After 1 hour at this temperature, the plates were transferred to 37°C for 18 hours, to facilitate plaque formation. During the incubation periods, plates were placed in a 'wet box' environment to prevent drying of the agar overlay, which could result in the unwanted adhesion of the overlay to the nitrocellulose membrane during the subsequent screening proceedure.

After incubation, plates were overlayed with nitrocellulose membranes as follows. Gridded, 82 mm nitrocellulose filters (BA 85/23 Schleicher and Schuell, Dassel, Germany) were carefully numbered and marked at the outer edges with ball-point pen ink. Using surgical gloves and blunt plastic forceps to prevent contamination of the membrane, the filters were placed, gridded side downward, on to the plaque-containing agar plates. To follow the orientation and hence aid the recovery of any immunopositive plaques, each plate was numbered and marked on the underside in a position corresponding to the marks on its respective filter.

Plates overlayed with filters were returned to the incubator for 1 hour at 37°C, after which time the nitrocellulose membranes were carefully removed using forceps and placed

in a 50 ml volume of PBSA. The filters were washed for five minutes in PBSA with gentle agitation. The PBSA was poured off and replaced with 50 ml of PBSA containing 2% w/v skimmed milk powder as a membrane blocking reagent (PBSM). Blocking was carried out with gentle agitation for 1 hour, at which time the blocking reagent was replaced with a further 50 ml of PBSM containing the primary antibody (1:200).

Filters were exposed to the primary antibody for 1 hour at room temperature, and washed for 3 x 5 min. in 50 ml volumes of PBSA. After washing, the PBSA was removed and replaced with 50ml of PBSM containing peroxidase-conjugated swine anti-rabbit immunoglobulins (P217, Dako Ltd., High Wycombe, U.K.) as the secondary antibody (1:500). Filters were exposed to secondary antibody for 1 hour followed by 3 x 10 min. washes with 50 ml volumes of PBSA. After washing, antibody binding was detected by immersing the filters in 50 ml of developing solution consisting of 50 ml of 0.06% (w/v) 4-chloro-1-napthol and 0.01% (v/v) hydrogen peroxide.

On eight occasions the λgt11/ *R.salmoninarum/EcoRI* library was screened in this manner, four utilizing rabbit antiserum raised against *R.salmoninarum* ATCC33209 whole cells and four utilizing rabbit antiserum raised against *R.salmoninarum* ATCC33209 ECPs. Both positive and negative controls for each screening were provided by using nitrocellulose filter lifts of λgt11 vector and the respective *R.salmoninarum* antigens spotted on to small squares of nitrocellulose.

Of eighty filters probed during the screening of λgt11/ *R.salmoninarum/EcoRI* gene libraries, twelve were found to contain immunopositive plaque lifts, an example of which can be seen in Fig. 6.1. From these twelve filters a total of 23 immunopositive plaques,designated lifts were detected, designated λRSGT 1-23, all of which were the result of immunoscreening with anti-whole cell antibody.

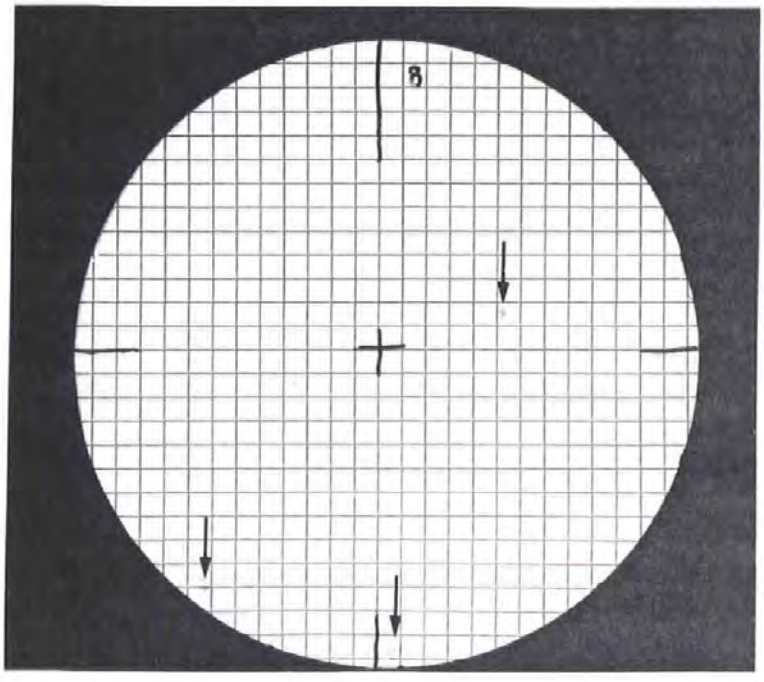


Figure 6.1. Nitrocellulose filter containing immunopositive plaque-lifts from a λgt11/ *R.salmoninarum /EcoRI* gene library. Immunopositive plaque lifts are arrowed. The filter shown was probed with rabbit anti-*R.salmoninarum* ATCC33209 whole cell serum as the primary antibody using the method described in 6.2 (above).

6.2.1 PURIFICATION OF IMMUNOPOSITIVE RECOMBINANT λgt11 BACTERIOPHAGE.

The immunopositive recombinant λgt11 phage plaques designated λRSGT 1-23 were located on their respective agar overlays and recovered by stabbing with a sterile Pasteur pipette. The plaque-containing agar plug was placed into 500 μl of SM buffer in a microcentrifuge tube, agitated by vortexing and left for 1 hour to allow maximal elution of phage from the agar plug. A wire loopful of each phage suspension was then streaked on to LB agar plates overlayed with soft agar containing Y1090 host cells and IPTG, and incubated as in 6.2 (above). After incubation, segments of the agar plate with well separated phage plaques were overlayed with pieces of nitrocellulose, which were subsequently probed with antiserum as before. Four of the recombinant phage, λRSGT 4, 12, 14 and 19 remained immunopositive and these were retained for further analysis.

6.2.2 DETECTION OF IMMUNOREACTIVE ANTIGENS IN THE RECOMBINANT λgt11 CLONES λRSGT 4, λRSGT 12, λRSGT 14 AND λRSGT 19.

Concentrated phage lysates of *E. coli K12* Y1090 infected with the immunopositive λgt11 clones λRSGT 4, 12, 14 and 19 were prepared in the following manner. Each suspension of recombinant phage was diluted and plated so as to give near-confluent lysis on a lawn of Y1090 bacteria. After incubation for 18 hours at 37°C, the overlay containing the phage was carefully removed with a glass spreader, placed in 1.5 ml microcentrifuge tubes and subjected to centrifugation at 13,000 x g for 10 min.. The resulting supernatants were removed and placed in a clean, sterile microcentrifuge tube and stored at 4°C until use. In addition to the recombinant phage lysates, a control phage lysate of Y1090 infected with λgt11 was also prepared.

A 50 μl aliquot of each phage suspension was added to an equal volume of SDS-PAGE sample buffer and subjected to SDS-PAGE along with molecular weight markers in a 10% polyacrylamide gel under denaturing conditions. The electrophoresed gel was then stained

in coomassie blue to visualise the separated proteins. On examination, there was found to be no visible difference between the protein profiles of the recombinant phage lysates and the λgt11 vector lysate. A second set of 50 μl phage lysates were subjected to SDS-PAGE as before, but on this occasion, the resultant separated proteins were subjected to western blotting, transferring them to a nitrocellulose membrane and probing them with rabbit anti-*R.salmoninarum* ATCC33209 whole cell serum as the primary antibody.

On examination of the colour-developed membrane, a strongly immunopositive protein band of a size in the region of 60,000 daltons was observed, in not only the recombinant phage lysates but also the control vector lysate.

As there was a possibility that the immunoreactive band observed was the result of the contamination of the anti-*R.salmoninarum* serum with circulating antibodies produced in the rabbit against antigens of commensal *E. coli*, the immunoblotting experiment was repeated using a primary antibody solution preabsorbed with the control λgt11/ Y1090 lysate. Preabsorbtion was carried out by adding 500 μl of rabbit anti-*R.salmoninarum* ATCC33209 whole cell serum to an equal volume of λgt11/ Y1090 phage lysate and incubating at 37°C for 1 hour. The serum/ lysate mixture was subjected to centrifugation at 10,000 x g for 15 min, and the supernatant used in the immunoblotting proceedure. Once again however, the major immunoreactive band was observed in all electrophoresed lysates.

Because there was no effective and convenient means of discriminating between control and recombinant phage lysates, studies of the recombinant λgt11 phage clones RSGT 4, 12, 14 and 19 were not pursued.

6.3. SCREENING OF A λL47.1/*R.salmoninarum/Sau3A* GENE LIBRARY.

Gene libraries constructed in the replacement-type, lambda-derived cloning vector λL47.1 may be screened in a number of ways. In addition to the immunoscreening proceedure, λL47.1 libraries may also be screened by phenotypic selection methods or by

hybridization with specific nucleic acid probes. Once again, as with λgt11, libraries of recombinant phage are screened in the form of phage plaques on a lawn of host *E. coli K12* bacteria, of which the commonest strain used for this purpose is *E. coli K12* C600. The number of individual phage plaques that needed to be screened to constitute a λL47.1/ *R.salmoninarum* gene library was estimated as in section 6.2 (above) using a 15 kbp average insert size and calculated to be 919 plaques. As before, a higher number of plaques, approx. 15,000, was chosen for each library constructed.

For screening purposes, recombinant phage suspension was plated out on to an overnight culture of *E. coli K12* C600 in proportions favouring the formation of approximately 1,000 plaques per 90 mm plate. Both phage and host bacteria were mixed in molten soft agar at 45°C and poured on to the surface of an LB agar plate, and incubated for 18 h. at 37°C In conjunction with each recombinant phage library, two negative control plates were set up, these consisting of *E. coli K12* C600 infected with the λL47.1 vector.

6.3.1 SCREENING OF A λL47.1/*R.salmoninarum/Sau3A* GENE LIBRARY FOR HAEMOLYTIC ACTIVITY.

The method chosen for the detection of haemolytic activity in the recombinant λL47.1 phage library, was essentially a modification of Kehoe *et al.*,(1983). These authors used an erythrocyte/ agarose overlay to detect genes coding for the α-haemolysin from *Staphylococcus aureus* cloned in recombinant λL47.1.

The λL47.1/*R. salmoninarum/Sau3A* gene library used in this research project was screened using both trout and horse erythrocytes (10%) separately suspended in PBSA/agarose overlays. This was done as follows: Approximately 5 ml volumes of the erythrocyte/agarose mixture (45°C) was carefully poured on to plaque-containing agar plates and allowed to set. These overlayed plates were incubated at a specific temperature for 24 h. Three recombinant phage libraries and their associated negative control plates were overlayed with trout erythrocytes and incubated at 10, 20 and 37°C. Three libraries

and controls were overlayed with horse erythrocytes and incubated at the same temperatures. After incubation, all plates were examined for zones of haemolytic activity. No zones of haemolytic activity were observed on any of the overlayed plates.

6.3.2. SCREENING OF λL47.1/*R.salmoninarum/Sau3A* GENE LIBRARIES FOR PROTEOLYTIC ACTIVITY.

In addition to screening for haemolytic activity, recombinant λL47.1 phage plaques were also screened for the presence of proteolytic activity. Approximately 15,000 recombinant phage plaques were constructed as screened, along with vector phage controls. This time, each plaque-containing agar plate was overlayed with molten agarose/PBSA containing a protease substrate, allowed to set. and incubated for a further 24 h. prior to examination.

6.3.2.1. NATURAL PROTEASE SUBSTRATES.

The natural protease substrates chosen for use in the screening of recombinant clones were casein and gelatin, incorporated into the PBSA/ agarose overlay solution at a concentration of 1% (w/v). As before, plates were overlayed with 5 ml of substrate containing overlay and allowed to set, prior to incubation. On six occasions, the phage library was screened with natural protease substrates, three with casein and three with gelatin. The overlayed plaque-containing plates were incubated at either 10, 20, or 37°C., after which those containing casein as substrate were flooded with 5% (v/v) acetic acid solution, whilst the gelatin-containing plates were flooded with 5% (w/v) tannic acid solution. These acid solutions were utilized to precipitate the protein substrate for the sensitive detection of any zones of proteolysis. No proteolytic activity was detected on any of the overlayed plates.

6.3.2.2. SYNTHETIC PROTEASE SUBSTRATES.

The recombinant λL47.1 phage library was also screened with two synthetic protease substrates. The substrates chosen were chromogenic azocollagen (azocoll) and fluorogenic 4-methylumbelliferyl p-guanidinobenzoate (MUGB).

The library and control plates were overlayed with PBSA/ agarose containing either 0.5% (w/v) azocoll or 20 mM MUGB folllowed by 24 h. incubation. On three occasions the library and control plates overlayed with azocoll and MUGB, these were then incubated at either 10, 20 or 37°C. Azocoll plates were examined for the release of red/purple chromogen in areas of proteolysis whilst MUGB plates were examined under U.V. light for blue-white fluorescence. No proteolytic activity was observed on plates containing either substrate.

6.3.3. IMMUNOSCREENING OF λL47.1/ *R. salmoninarum/Sau 3A* GENE LIBRARIES.

The recombinant λL47.1 phage library and associated vector phage controls was also screened for the presence of *R. salmoninarum* antigens, in a similar manner to the immunoscreening of the λgt11/ *R. salmoninarum/EcoRI* gene library (see 6.2, above). A collection of approximately 15,000 recombinant phage plaques and 2 x 1,000 vector phage plaque controls were set up by infecting *E. coli K12* strain C600 in soft agar overlays on LB agar plates and 18 h. incubation at 37°C. After incubation, 82 mm diameter, gridded nitrocellulose filters were marked, numbered and carefully placed on to the plaque-containing agar plates. After 1 h. incubation at 37°C., the filters were processed as in 6.2 (above) using either rabbit anti-*R. salmoninarum* ATCC33209 whole cell serum or rabbit anti-*R. salmoninarum* ATCC33209 ECP serum as the primary antibody, and peroxidase conjugated swine anti-rabbit immunoglobulins (P217, Dako) as the secondary antibody.

A total of 4 libraries of recombinant λL47.1 phage were screened in this way, none of which were found to contain immunopositive phage plaques.

At this point, the screening of λL47.1/ *R. salmoninarum* gene libraries was curtailed.

6.4. SCREENING OF *R.salmoninarum* GENE LIBRARIES CONSTRUCTED IN PLASMID VECTORS.

Plasmid gene libraries, in the form of a collection of recombinant bacterial clones on agar

plates, can be screened in much the same manner as bacteriophage libraries. The desired DNA sequence may be detected by hybridization with specific nucleic acid probes or the desired gene product detected by phenotypic selection methods such as immunoscreening or the presence of a given biochemical property.

6.4.1 SCREENING OF A pUC18/*R.salmoninarum/Sau3A* GENE LIBRARY.

The number of individual plasmid-bearing bacterial clones that need to be screened so as to constitute a pUC18/*R.salmoninarum/Sau3A* gene library was calculated as in 6.2 (above). The average DNA insert size was taken to be 5 kbp, giving N a value of 2761 recombinant clones. As before, a much larger figure of approximately 10,000 were screened. Prior to the screening for cloned *R.salmoninarum* components, the library was subjected to blue/white screening to ascertain the percentage of non recombinant plasmids. Out of a total of 182 bacterial colonies obtained by transformation, only seven exhibited the blue *lac*$^+$ phenotype, indicating that the plasmid DNA mixture used for transformation contained approximately 96% recombinant DNA molecules. The plasmid mixture was therefore seen to be suitable for library construction.

The recombinant pUC18 library was constructed in the following manner. Approximately 0.25 μg of ligated plasmid mixture (5.5.1.1, above) was used to transform bacteria from an overnight culture of *E. coli K12* strain JM109, using the standard transformation proceedure (3.6.4.3, above). Aliquots of this transformation mixture were spread-plated on to LB agar containing 50 μg/ml ampicillin for selection of pUC18-containing bacterial clones. The plates were incubated at a specified temperature for a specified time period until bacterial colonies of approximately 1-2 mm had grown. In addition to the recombinant pUC18/ *R.salmoninarum* libraries, negative control plates consisting of *E. coli K12* JM109 transformed with pUC18 vector plasmid were also set up.

6.4.1.1 SCREENING OF pUC18/ *R.salmoninarum/Sau3A* GENE LIBRARIES FOR HAEMOLYTIC ACTIVITY.

Plates of recombinant pUC18-containing bacterial colonies, and their associated vector-containing controls, were screened for haemolytic activity using the erythrocyte/ agarose overlay method described in 6.3.1 (above), using 5 ml of overlay per plate of preformed colonies. Overlayed plates were incubated at a specified temperature for a specified time period.

At first, four screenings of the recombinant pUC18 library and controls were made, two with trout erythrocytes and two with horse erythrocytes. Plates were incubated at either 20 or 37°C for 24 h. prior to examination for the presence of zones of haemolysis around any of the bacterial colonies. No haemolytic activity was detected on any of the overlayed plates. Because of the possibility that the initial incubation temperature (37°C) of the transformed plasmid-bearing clones may be too high for stable production of any haemolytic agent from *R.salmoninarum*, a second set of libraries and controls were constructed using an initial incubation temperature of 20°C. These plates were subjected to 72 h. incubation periods to allow the formation of the appropriate size colonies for screening. Four libraries and sets of controls were treated in this manner, and examined at the end of the incubation period for the presence of haemolytic acivity. Once again, no haemolytic activity was detected on any of the overlayed plates.

6.4.1.2 SCREENING OF pUC18/ *R.salmoninarum/Sau3A* GENE LIBRARIES FOR PROTEOLYTIC ACTIVITY.

The recombinant pUC18 library was also screened for proteolytic activity. The method used was exactly the same as described in 6.3.2 (above) using the natural protease substrates casein and gelatin, and the synthetic substrates azocoll and MUGB.

Recombinant bacterial colonies and controls were overlayed with 5 ml of substrate/ agarose mixture and in cubated at a specified temperature for a specified time period.

After incubation, the overlayed plates were examined for signs of proteolytic activity.

As with the screening for haemolytic acivity (see above), transformed bacteria were incubated both at 20 and 37°C prior to overlaying with substrate/ agarose, and at 20 and 37°C after overlaying. No signs of proteolytic activity were detected on any overlayed plate.

6.4.1.3 IMMUNOSCREENING OF pUC18/ *R.salmoninarum/Sau3A* GENE LIBRARIES.

Recombinant pUC18 libraries were also screened immunologically for the presence of *R.salmoninarum* antigens. The method utilized for immunoscreening was essentially that used for the screening of gene libraries constructed in bacteriophage vectors, with the recombinant bacterial proteins being bound to gridded nitrocellulose filters prior to an immunodetection procedure using rabbit antisera raised against *R.salmoninarum* antigens.

Prior to the overlaying of the colonies on agar plates, the bacteria were lysed *in situ* so as to release the cell contents. This was acheived by exposing the bacterial colonies to chloroform vapour for 5 min.

Gridded nitrocellulose filters, marked and numbered for future reference, were carefully placed on top of the colonies and the plates left for 1 h. at room temperature. After which, filters were carefully removed and processed as described in 6.2 (above). During the blocking step in PBSM, visible adhering bacterial debris was removed by irrigating the individual filter discs repeatedly by means of a sterile Pasteur pippette. The filters were probed using either rabbit anti-*R. salmoninarum* ATCC33209 whole cell serum or rabbit anti-*R. salmoninarum* ATCC33209 ECP serum as the primary antiserum, and peroxidase conjugated swine anti-rabbit immunoglobulins (P217, Dako) as the secondary antibody.

The recombinant pUC18 library was screened four times in this way, none were found to contained immunopositive colony lifts. At this point, the screening of pUC18/ *R.*

salmoninarum gene libraries was discontinued.

6.4.2 SCREENING OF A pGD103 /*R.salmoninarum/Sau3A* GENE LIBRARY.

The number of individual plasmid-bearing bacterial clones that need to be screened so as to constitute a pGD103/*R.salmoninarum* gene library was calculated as in 6.2 (above). The average DNA insert size was taken to be 5 kbp, giving N a value of 2761 recombinant clones. As before, the larger figure of approximately 10,000 was decided upon for screening purposes.

Prior to any screening of the recombinant pGD103 library, the ligated plasmid mixture was transformed into the *lac⁻ E. coli K12* strain so as to ascertain the proportion of non recombinant plasmids by blue/white screening. Of 212 clones, 13 were found to exhibit a *lac*$^+$ phenotype, indicating that the transformation mixture contained approximately 94% recombinant plasmids.

A recombinant pGD103 library was constructed as in 6.4 (above), again using 0.25 μg of ligation mixture to transform the *E.coli K12* recipient. For the purposes of recombinant pGD103 library screening protocols, the strain chosen was *E. coli K12* DH1. Aliquots of the transformation mixture were spread-plated on to LB agar containing 25 μg/ml kanamycin for selection of pGD103-containing bacterial clones. The plates were incubated at a specified temperature for a specified time period until bacterial colonies of approximately 1-2 mm had grown. In addition to the recombinant pGD103/ *R.salmoninarum* libraries, negative control plates consisting of *E. coli K12* DH1 transformed with pGD103 vector plasmid were also set up.

6.4.2.1 SCREENING OF A pGD103/ *R.salmoninarum/Sau3A* GENE LIBRARY FOR HAEMOLYTIC ACTIVITY.

Plates of recombinant pGD103-containing bacterial colonies, and their associated vector-containing controls, were screened for haemolytic activity using the erythrocyte/ agarose overlay method described in 6.3.1 (above), using 5 ml of overlay per plate of preformed

colonies. Overlayed plates were incubated at a specified temperature for a specified time period.

At first, the recombinant pGD103 library and controls were screened four times, twice with trout erythrocytes and twice with horse erythrocytes. The overlayed plates were then incubated at either 20 or 37°C for 24 h. prior to examination for the presence of zones of haemolysis around any of the bacterial colonies. No haemolytic activity was detected on any of the overlayed plates. A second set of libraries and controls were constructed using an initial incubation temperature of 20°C These plates were subjected to 72 h. incubation periods to allow colony formation. The library and control plates was screened a further four times in this manner, and examined at the end of the incubation period for the presence of haemolytic acivity. Once again, no haemolytic activity was detected on any of the overlayed plates.

6.4.2.2 SCREENING OF A pGD103/ *R.salmoninarum/Sau3A* GENE LIBRARY FOR PROTEOLYTIC ACTIVITY.

The recombinant pGD103 library was also screened for proteolytic activity. The method used was exactly the same as described in 6.4.1.2 (above) using the same protease substrates, casein, gelatin, azocoll and MUGB.

Libraries and controls were overlayed with 5 ml of substrate/ agarose mixture and in cubated at a specified temperature for a specified time period. After incubation, the overlayed plates were examined for signs of proteolytic activity.

Transformed bacteria were incubated both at 20 and 37°C prior to overlaying with substrate/ agarose, and at 20 and 37°C after overlaying. No signs of proteolytic activity were detected.

6.4.2.3 IMMUNOSCREENING OF A pGD103/ *R.salmoninarum/Sau3A* GENE LIBRARY.

The recombinant pGD103 library was also screened immunologically for the presence of

R.salmoninarum antigens. The method utilized for immunoscreening was identical to the method adopted in 6.1.1.3 (above) using either rabbit anti-*R. salmoninarum* ATCC33209 whole cell serum or rabbit anti-*R. salmoninarum* ATCC33209 ECP serum as the primary antibody, and peroxidase conjugated swine anti-rabbit immunoglobulins (P217, Dako) as the secondary antibody.

The recombinant pGD103 library and control plates were screened four times in this way, none were found to contain immunopositive colony lifts.

At this point, the screening of the pGD103/ *R. salmoninarum/Sau3A* gene libraries was halted.

6.5 SCREENING OF A *R. salmoninarum* GENE LIBRARY CONSTRUCTED IN THE COSMID VECTOR pHC79.

Gene libraries constructed in cosmid vectors are screened as a collection of bacterial colonies containing circularised cosmid DNA molecules, and for all intents and purposes are treated in the same manner as plasmid gene libraries. The large capacity for foreign DNA inserts however, dramatically reduces the theoretical number of individual recombinant cosmid-containing clones (N) that need to be screened for a high probability of detecting the inserted DNA sequence of interest. The value of N calculated for a pHC79/*R. salmoninarum/Sau3A* gene library was calculated as in 6.2 (above). The average DNA insert size was taken to be 37.5 kbp., giving N a value of 366 recombinants. As before, a much larger figure of approximately 4,000 were in fact screened.

The recombinant pHC79/ *R.salmoninarum/Sau3A* library was constructed as follows. Bacterial cells from an overnight culture of *E. coli K12* DH1 were infected with 5 μl of packaged phage suspension (5.6.3, above). Aliquots of the infected bacterial suspension were then spread-plated on to LB agar containing 50 μg/ ml ampicillin for the selection of pHC79-containing bacterial clones. The inoculated plates were incubated for 37°C for

24 hrs, or until colonies of 1-2 mm diameter had grown. In addition to the recombinant pHC79 library, a set of negative control plates consisting of *E. coli K12* DH1 infected with pHC79 vector cosmid were also set up.

6.5.1 SCREENING OF A pHC79/ *R. salmoninarum/Sau3A* GENE LIBRARY FOR HAEMOLYTIC ACTIVITY.

The library of recombinant pHC79-containing bacterial colonies, and associated control plates were screened for haemolytic activity using the erythrocyte/ agarose overlay method described in 6.3.1 (above), using 5 ml of overlay per plate of preformed colonies. The overlayed plates were incubated at 25°C for 24 h. prior to examination.

In total, the recombinant cosmid library was screened on four occasions, twice with horse erythrocytes and twice with trout erythrocytes. On the plates overlayed with horse erythrocytes, there were no visible signs of haemolytic activity. However, one of the plates overlayed with a trout erythrocyte suspension contained 3 colonies exhibiting haemolytic activity. The haemolytic colonies were removed by stabbing with a sterile Pasteur pipette, with the resultant plug of agar/bacteria being streaked on to an LB agar plate containing 50 μg/ml ampicillin prior to incubation at 37°C for 24h. After incubation, these plates were overlayed with trout erythrocyte/ agarose supension to confirm the haemolytic phenotype. Of the three cultures overlayed, only one retained haemolytic activity on subsequent passage.

As rainbow trout blood was difficult to obtain, and costly in terms of the number of animals that need to be sacrificed per overlaying experiment, the haemolytic clone was tested for activity against a more convenient source of erythrocytes from horse blood. The clone was found to be equally active against horse erythrocytes, which were subsequently used (unless otherwise stated) in the erythrocyte overlays performed in this research project. The haemolytic activity exhibited by the recombinant clone isolated from the pHC79/*R.salmoninarum/Sau 3A* gene library, can be seen in Figs. 6.2 and 6.3.

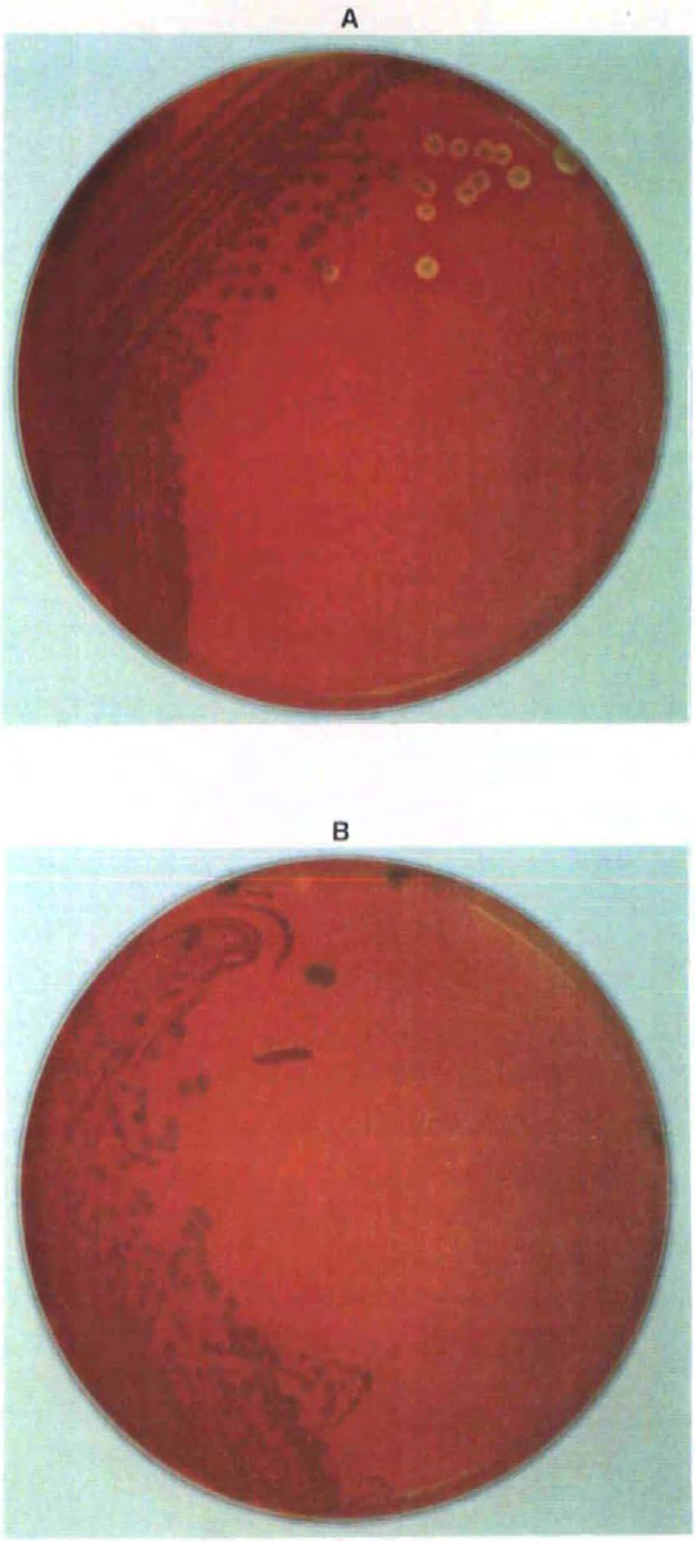


Figure 6.2 Haemolysis of rainbow trout erythrocytes by a recombinant clone REN/COS1 isolated from a pHC79/*R.salmoninarum/Sau 3A* gene library. Shown are zones of haemolysis in a PBSA/agarose/erythrocyte overlay after 24 h. incubation at 25°C. **A**= streak plate of REN/COS1, **B**= streak plate of *E. coli* DH1/pHC79 control.

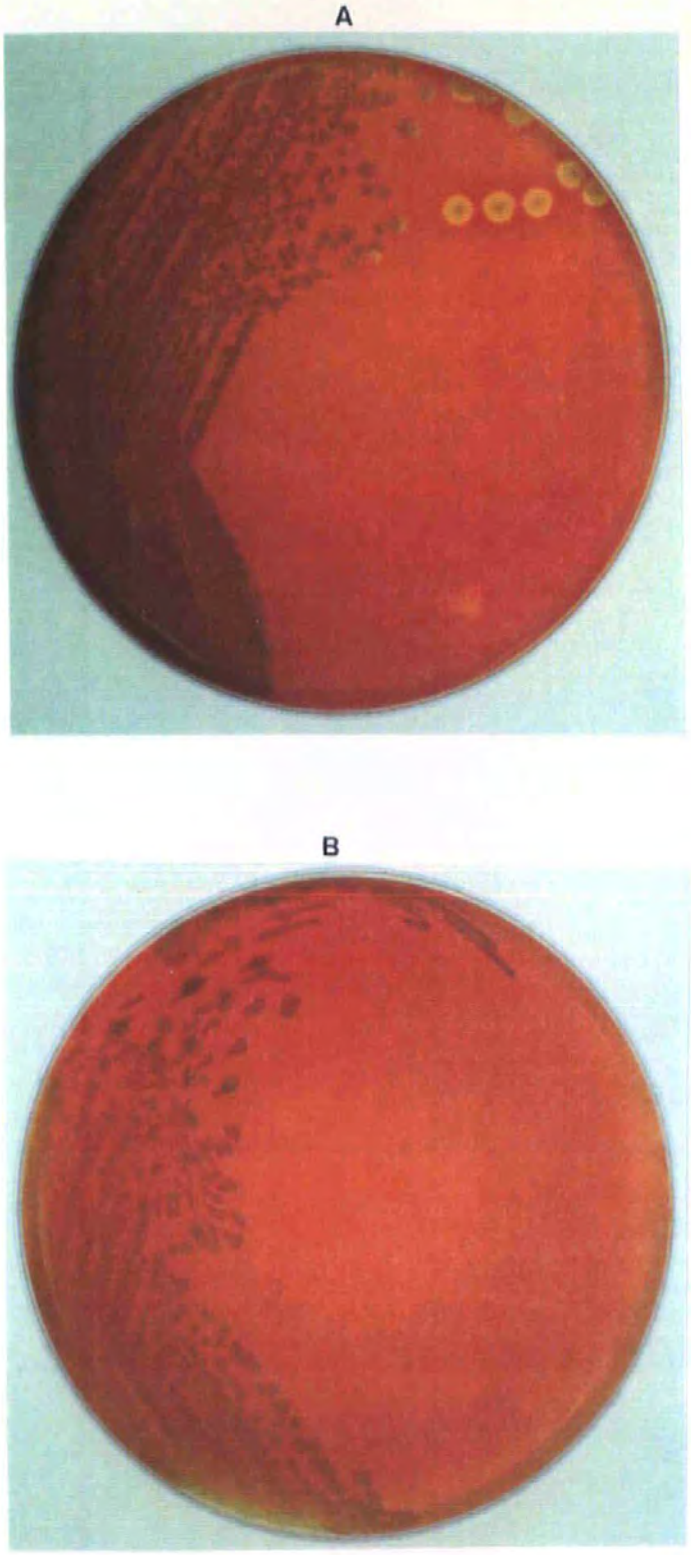


Figure 6.3 Haemolysis of horse erythrocytes by a recombinant clone REN/COS1 isolated from a pHC79/*R.salmoninarum/Sau 3A* gene library. Shown are zones of haemolysis in a PBSA/agarose/erythrocyte overlay after 24 h. incubation at 25°C. **A**= streak plate of REN/COS1, **B**= streak plate of *E. coli* DH1/pHC79 control.

The recombinant haemolytic clone was designated REN/COS1 and a culture of it was stored in glycerol at -20°C prior to further analyses.

6.5.2 SCREENING OF A pHC79/ *R.salmoninarum/Sau3A* GENE LIBRARY FOR PROTEOLYTIC ACTIVITY.

The recombinant pHC79/*R.salmoninarum/Sau3A* library was also screened for proteolytic activity. The method employed was identical to that described for the screening of plasmid gene libraries (6.4.1.2 and 6.4.2.2, above).

The Library and associated vector controls were overlayed with 5 ml of substrate/agarose mixture and incubated at a specified temperature for a specified time period. After incubation, the overlayed plates were examined for signs of proteolytic activity. Bacteria were incubated at both 20 and 37°C prior to overlaying with substrate and at the same two temperatures after overlays were in place. The library and controls were screened four times per substrate, but there were no indications of proteolytic activity on any of the plates examined.

6.5.3 IMMUNOSCREENING OF A pHC79/*R.salmoninarum/Sau3A* GENE LIBRARY.

The recombinant pHC79/ *R.salmoninarum/Sau3A* library and vector controls were also screened for the presence of *R.salmoninarum* antigens using both rabbit anti-*R.salmoninarum* ATCC33209 ECP and rabbit anti-*R.salmoninarum* ATCC33209 whole cell sera in a manner identical to that described for plasmid gene libraries (6.4.1.3 and 6.4.2.3, above). The recombinant library was screened a total of four times with anti-*R.salmoninarum* sera, however, none were found to contain immunopositive recombinant bacterial clones.

6.6 DISCUSSION.

The isolation of a given DNA sequence, once it has been inserted into a cloning vector and stably maintained as a recombinant bacterial or viral clone, can be both a frustrating

and time consuming endeavour. This fact is highlighted by the experimental work described in this chapter, where a considerable number of *R.salmoninarum* gene libraries were screened for the reward of just one recombinant clone suited for further study.

In addressing the general lack of success experienced in isolating clones of interest from *R.salmoninarum* gene libraries constructed in *E. coli K12* host/vector systems, it is worth considering once again, the factors that can influence the outcome of any gene library screening protocol. As intimated both in the preceding chapter of this thesis, and in the introduction to the present chapter these factors can be seen to fall into three groupings; those resulting from gene library construction, and those that result from inadequacies in the screening proceedure itself. A third factor that must also be considered, especially with regard to the present work, is the expression of any gene sequences contained in the recombinant library.

Although each step of the construction of gene libraries is usually carefully monitored, there may be deficiencies in the finished products with regard to the extent that they represent the whole of the target genome. The commonest contributor to unrepresentative libraries is the presence of a high proportion of non-recombinant DNA molecules as a result of inefficient DNA ligations. Under sub-optimal conditions, it is possible for the ligation reaction to result in a large number of vector-only ligations, and this can effect dramatically the success of any subsequent screening protocol. It can be seen that if a significant proportion of the putative recombinant clones in fact only contain the parent cloning vector, the number of individuals that need to be screened for a high probability of recovering a gene sequence of interest (the value N in Clarke's formulae)
would have to be higher than originally predicted. If this case was applied to the *R.salmoninarum* gene libraries, it could explain the lack of success experienced during the screening of these libraries for cloned *R.salmoninarum* macromolecules.

However it is unlikely that the *R.salmoninarum* libraries were deficient in this manner,

for the following reasons. Firstly and simply, considerably more recombinants than predicted by Clarke's formula were screened in each library. Secondly, the λ and cosmid libraries possessed their own built-in selection process with regard to vector-only ligation events. The λgt11 library was constructed utilizing vector arms, thus preventing the manifestation of vector-only molecules amongst the subsequent gene library as the vector/vector molecules would be too small to be packaged in to λ particles. The likelihood of vector only molecules appearing in the pHC79 cosmid-based library was also extremely small, as not only were the vector molecules dephosphorylated to suppress recircularization and self-ligation , the size of such a molecule would also be insufficient for packaging into λ particles. Vector-only molecules were selected out of the λ L47.1 library by the initial amplification on an *E. coli K12* P2 lysogen, and therefore exploiting the Spi phenotype of the parent vector DNA. The possibility of a high proportion of vector only DNA molecules was also considered when *R.salmoninarum* gene libraries were constructed in plasmid cloning vectors. All plasmid gene library construction protocols contained a vector molecule dephosphorylation step to suppress recircularization and self-ligation, in addition, libraries were also subjected to blue/white screening to ensure a high proportion of recombinants.

Whilst it can be stated, that with suitable preparation and planning, the construction of a representative gene library, *vis a vis* the fragmentation of target DNA, its ligation to a cloning vector, and the introduction of the resulting recombinant DNA molecules into a suitable host cell, is a relatively straightfoward procedure, the expression of gene sequences contained within recombinant molecules is another matter altogether. The non expression of cloned *R.salmoninarum* gene sequences would have seriously hampered the progress of this research project, and although a number of steps were taken in an attempt to prevent this from occurring, the lack of *R.salmoninarum* gene expression in *E. coli K12* host/vector cloning systems may be the major contributing factor to the general lack of

success experienced in screening the different gene libraries.

Prior to the construction and screening of *R.salmoninarum* gene libraries, it was recognised that the novelty of the experimental work with regard to the use of the bacterium in cloning experiments, may provide problems. As there were no published accounts of cloning *R.salmoninarum* DNA into *E. coli K12* host/vector systems, it was not known that if complete gene sequences could be cloned, whether these would be expressed. However, an assumption was made, taking into consideration the large number of genes from a wide range of bacterial taxa that have been successfully cloned and expressed in *E. coli K12* host vector systems, that if suitable precautions were taken with respect to the cloning vectors employed and the screening protocols adopted, it would be possible to clone and express *R.salmoninarum* gene sequences.

A wide range of cloning vectors were employed to construct gene libraries. Possible incompatibilities between the machinery of gene expression in *R.salmoninarum* and in the *E. coli K12* host were addressed by the use of cloning vectors with strong *E. coli* promoters, and vectors where lac-gene fusions could occur. High copy number vectors were used so as to circumvent low-level gene expression, whilst low copy number vectors were employed to reduce the gene dosage effect of any cloned *R.salmoninarum* component that may be toxic to the *E. coli K12* host.

The screening protocols adopted for *R.salmoninarum* gene libraries were also designed to overcome possible problems arising from gene expression. Taking into account that *R.salmoninarum* is a fish pathogen, clones were grown and screened at a range of temperatures so as to try and identify *R.salmoninarum* components that may be expressed at specific temperatures and to detect heat labile molecules.

A number of different enzyme substrates were used to screen libraries, as both the reported proteolytic and haemolytic activities of *R.salmoninarum* have yet to be characterized. Antisera raised to whole cells and the ECP of the bacterium were used to

try and detect previously uncharacterized *R.salmoninarum* antigens.

Two of the *R.salmoninarum* gene libraries yielded clones of interest. The λgt11/*R.salmoninarum/EcoRI* contained a number of immunopositive phage plaques, but on further analysis there was no effective and convenient means of discriminating between recombinant and control vector-only phage. Explanations for this could be that the antigen cloned from *R.salmoninarum* was antigenically homologous with an *E. coli K12* component, or that the immunopositive phage were mutants over-expressing an *E. coli K12* component recognised by antibodies in rabbit serum. The cosmid pHC79/ *R.salmoninarum/Sau3A* library yielded three haemolytic clones, one of which remained stable on sub-culture. The characterization and manipulation of this haemolytic cosmid clone form the subject of the rest of this thesis.

CHAPTER 7: MANIPULATION AND STRUCTURAL ANALYSIS OF A *R.salmoninarum* DNA SEQUENCE ENCODING HAEMOLYTIC ACTIVITY.

7.1 INTRODUCTION.

Once a clone exhibiting a desired genetic property is selected from a gene library, analysis can begin on the recombinant DNA molecule contained within. At first the recombinant DNA molecule is isolated and purified free from potentially interfering host cell constituents such as nucleic acids and proteins. This purified DNA can then be used to facilitate further studies (Old & Primrose, 1989; Singer & Berg, 1991).

After demonstrating the presence of inserted foreign DNA within a cloned recombinant molecule, the next step is to estimate the size of the insert. This can be readily performed by cleaving the recombinant DNA molecule using restriction endonucleases, liberating the insert and comparing its electrophoretic migration with molecular size markers. If however the DNA insert is large (>10 kbp), electrophoretic migration can be an unreliable determinant of molecular size as such large DNA molecules do not resolve well. It may also be the case, that the points of insert for the foreign DNA may not regenerate suitable sites for restriction endonuclease cleavage (this can be the case when vector DNA is cleaved with *Bam HI* and the target DNA has been fragmented with *Sau 3A*, in this case, there is a 1:16 chance that a suitable *Bam HI* site will regenerate).These problems however can be overcome by cleaving the recombinant DNA molecule with a variety of restriction endonucleases, to produce digests containing a number of smaller fragments whose size can be then readily determined by electrophoresis.

The genomic DNA fragments used to construct gene libraries, especially those libraries formed using bacteriophage and cosmid cloning vectors are often much larger than the genetic sequence of interest. Consequentially, not only will the cloned DNA fragment contain non-essential intergenic regions, it may also contain other functional gene sequences. Where the goal of cloning gene sequences from an organism is to isolate

and characterize specific encoded protein molecules, these non-essential regions of DNA should ideally be removed from the initial recombinant DNA molecule.

The removal of exogenous gene sequences, and the localization of a specific coding gene, is an important step in the genetic analysis of a recombinant DNA molecule and can be acheived by subcloning procedures (Singer & Berg, 1991; Rodriguez & Tait, 1983). The process of subcloning involves the isolation and cloning of different portions of the original recombinant DNA molecule in order to identify the smallest piece of DNA that contains the genetic sequence of interest. Subclones can be functionally or physically identified, utilizing the same method used to isolate the parent clone during the initial screening of the gene library from which it was originally isolated. In the most widely used and simplest form of subcloning, a DNA sequence known to contain a desired genetic region is cleaved with restriction endonucleases other than those used in the construction of the original recombinant molecule. The resulting fragments of the DNA can then be ligated to a cloning vector, usually a plasmid, transformed into a host cell and subsequent transformants screened for the desired property.

The process of subcloning can be repeated using different endonucleases and plasmid vectors to reduce the amount of extragenic DNA, and thus obtain a recombinant molecule with a suitably smaller insert, thus simplying further characterization of the desired sequence.

Once the subcloning proceedure has been completed, analysis of the inserted sequence of interest can begin with the construction of a restriction endonuclease map. A set of fragments produced by cleavage of a DNA molecule with a restriction endonuclease can provide a characteristic "fingerprint" of that DNA. By analysing the fragments produced by several different endonucleases alone and in combinations, one can construct a physical map, which not only provides valuable information for the further manipulation and analysis of a genetic sequence, but can, if detailed enough, define uniquely a genomic

segment.

After this initial structural analysis has taken place, it is advisable, before undertaking any further lengthy studies of a cloned DNA sequence, to establish the fidelity of the cloning process. Firstly, does the fragment originate from the genomic DNA of interest ?. Secondly, because sequence alterations due to recombination, deletion and insertion events may occur during replication in host/vector systems, does the fragment a true represent faithfully the sequence found on the parental genomic DNA ?.

The basic approach to the assessment of cloning fidelity is to use the cloned DNA sequence as a hybridization probe against the genomic DNA from which it was supposedly derived, and to demonstrate, that the cloned sequence anneals to a homologous genomic DNA segment with similarly spaced restriction endonuclease cleavage sites.

This chapter describes the initial structural analysis of a cloned DNA sequence, encoding haemolytic activity, obtained from the pHC79/*R. salmoninarum/Sau 3A* cosmid gene library. The size of the cloned fragment was determined and the haemolysin encoding region subcloned on a *Sal I* fragment of approximately 1.65 kbp. A physical map of this fragment was made using restriction endonuclease cleavage site analysis and the presence of a homologous DNA segment within the *R. salmoninarum* genome was demonstrated using Southern blot hybridization.

7.2 DETERMINATION OF INSERT SIZE FOR THE RECOMBINANT COSMID pRHLY1

7.2.1 INITIAL SIZE ESTIMATION OF COSMID DNA.

Cosmid DNA was isolated from 1.5 ml. of an overnight culture of the haemolytic *E. coli* clone by the miniprep method (see 3.5.2.1). The DNA was then subjected to agarose gel electrophoresis in a 0.3% gel in conjunction with 0.25 μg of bacteriophage lambda wild type DNA and 1.0 μg of lambda/*Hind III* DNA as molecular size standards. Although the DNA molecules were not strictly comparable, as the cosmid molecule was intact

supercoiled DNA whilst the standards were linear molecules, inspection of the gel after electrophoresis (Fig. 7.1) revealed a cosmid molecule of a size in the region of 25-30 kbp, this cosmid was designated pRHLY1.

A bulk preparation of purified pRHLY DNA was isolated by the triton lysis proceedure (3.5.1.1, method 1) followed by CsCl/EtBr gradient ultracentrifugation, and stored as a solution in TE buffer at 4°C.

7.2.2 SIZE ESTIMATION USING RESTRICTION ENDONUCLEASE CLEAVAGE.

The most widely used method for determining the size of large recombinant DNA molecules, and subsequently the size of the insert DNA, is to cleave the molecule with a number of different restriction endonucleases and to measure the size of the resultant fragments observed after agarose gel electrophoresis. This method was utilized to estimate the size of the DNA insert within the recombinant cosmid pRHLY1.

Approximately 0.5 μg amounts of pRHLY1 DNA were cleaved using the restriction endonucleases *Eco RV, Hind III,* and *Sal I* for 18 h. at 37° C. DNA fragments from cleaved cosmid molecules were subjected to agarose gel electrophoresis using a 0.5% gel in conjunction with a range of molecular size markers (Fig 7.2). All three restriction endonucleases cleaved pRHLY1 to produce fragments of DNA of varying sizes. However, the size of all of the fragments produced by *Eco RV* cleavage could not be readily estimated by comparison with the molecular size markers used. Therefore, the size of pRHLY1 was calculated using the DNA fragments produced by cleavage with *Hind III* and *Sal I* only.

Hind III cleavage of pRHLY1 produced 10 DNA fragments. The sizes of these fragments were estimated as 0.7 kbp.,1.1 kbp.,1.5 kbp.,2.25 kbp.,2.3 kbp.,4.0 kbp.,5.4 kbp.,6.4 kbp.,7.5 kbp. and 8.0 kbp., a total of 39.15 kbp. of DNA.

Sal I cleavage of pRHLY1 produced 8 DNA fragments. The sizes of these fragments were estimated as 1.65 kbp., 2.4 kbp., 4.0 kbp., 4.05 kbp., 5.4 kbp., 6.2 kbp., 7.5 kbp.

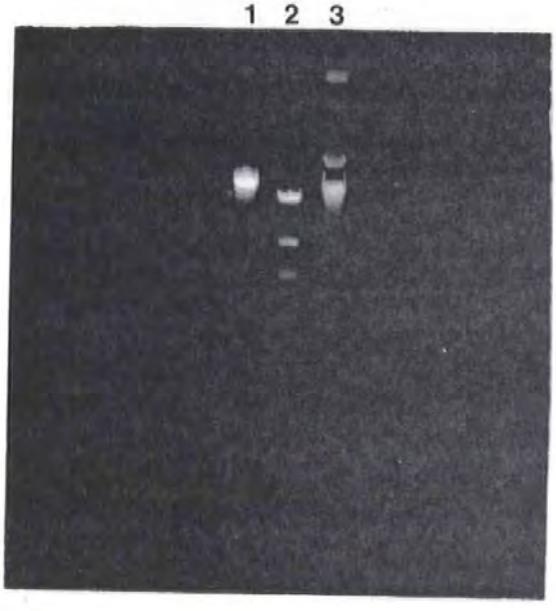


Figure.7.1 Agarose gel electrophoresis of the recombinant cosmid pRHLY1.
LANE 1: Lambda wild type DNA, 48 kbp. LANE 2: Lambda/*Hind III* markers (from top to bottom), 23.1 kbp., 9.4 kbp., 6.6 kbp., 4.36 kbp. LANE 3: pRHLY1 DNA (0.2 μg.).

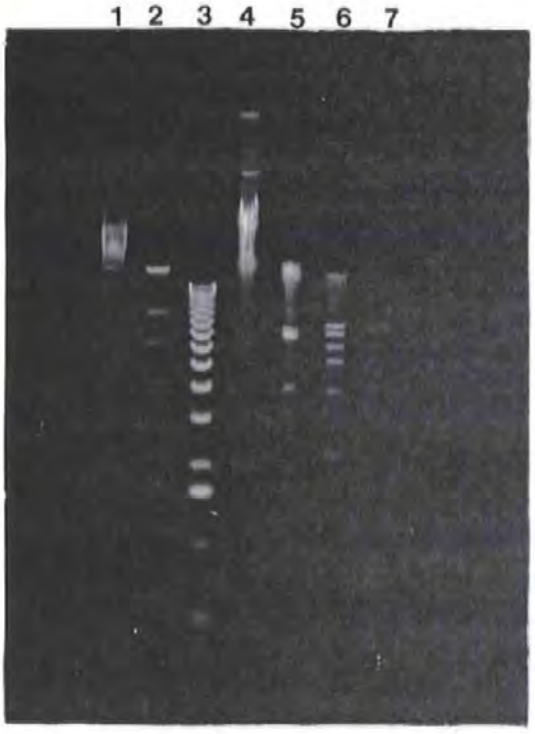


Figure 7.2. Agarose gel electrophoresis of restriction endonuclease-cleaved pRHLY1 DNA.

LANE 1: Lambda wild type DNA, 48 KBP. LANE 2: Lambda/*Hind III* markers (from top to bottom), 23.1 kbp., 9.4 kbp., 6.6 kbp., 4.36 kbp. LANE 3: 1 kbp. ladders (from top to bottom), 12 kbp., 11 kbp., 10 kbp., 9 kbp., 8 kbp., 7 kbp., 6 kbp., 5 kbp., 4 kbp., 3 kbp., 2 kbp., 1.6 kbp., 1 kbp., 0.5 kbp. LANE 4: pRHLY1 DNA. LANE 5: pRHLY1/*Eco RV* DNA. LANE 6: pRHLY1/*Hind III* DNA. LANE 7: pRHLY1/*Sal I* DNA.

and 8.0 kbp., a total of 39.2 kbp. of DNA. Using this data the size of pRHLY1 was estimated. Subtracting the 6.7 kbp of DNA which represents the pHC79 cloning vector, the DNA insert within pRHLY1 was of a size in the region of 32 kbp.

7.3 SUBCLONING OF pRHLY1 DNA INTO THE PLASMID VECTOR pBR328 USING *Hind III.*

The next stage in the analysis of the cloned insert was to subclone the haemolysis conferring DNA sequence into a plasmid vector. The plasmid chosen for this purpose was pBR328, Widely used in *E. coli* hosts,the plasmid possesses several unique sites for restriction endonuclease cleavage and the insertion of foreign DNA. The plasmid carries as selective markers the genes for resistance to the antibiotics ampicillin, chloramphenicol and tetracycline.

Restriction endonuclease cleavage of pRHLY11 (Fig 7.2) showed that digestion of the cosmid with *Hind III* produces a large number of DNA fragments of a size smaller than the maximum capacity of plasmid vectors (10 kbp), and as pBR328 posesses a unique cleavage site for this endonuclease it was decided that *Hind III* would be used for the subcloning DNA fragments from pRHLY1.

Initially, pRHLY1 DNA (2 μg) and pBR328 DNA (1μg) was cleaved using *Hind III* for 18 h. at 37°C and successful cleavage confirmed by subjecting a portion of the DNA/enzyme mixture to agarose gel electrophoresis in conjunction with molecular size markers.

Cleaved target DNA (pRHLY1) was then ligated to cleaved vector DNA (pBR328) using T4 DNA ligase for 18 h. at 15°C. The success of ligation was determined by agarose gel electrophoresis, and 0.5 μg of ligated DNA used to transform *E. coli* K12 strain DH1. Aliquots of 5 μl,10 μl, 20 μl, 50 μl and 100 μl from the transformation mixture were spread on to LB agar containing ampicillin (100 μg/ml) and Chloramphenicol (25 μg/ml). The agar plates were incubated at 37°C for 18 h. until bacterial colonies (1-2 mm diam.)

were visible. Those plates containing <300 colonies were overlayed with horse blood/agarose and incubated overnight at 20° C. Blood overlay of 622 ampicillin/chloramphenicol resistant bacterial colonies revealed 9 exhibiting a haemolytic phenotype. These colonies were removed and a pure culture obtained of each.

7.3.1 INITIAL ANALYSIS OF RECOMBINANT PLASMIDS pRHLY2 - pRHLY10.

Plasmid DNA was isolated from overnight cultures of the 9 haemolytic subclones by the miniprep method and subjected to agarose gel electrophoresis in a 0.5% gel in conjunction with miniprep isolated pBR328 DNA (Fig.7.3). Inspection of the gel revealed that all of the haemolytic subclones contained recombinant plasmid DNA molecules, since they were of a greater size than the pBR328 vector DNA, indicating the presence of inserted DNA fragments. These recombinant plasmids were designated pRHLY2 - pRHLY10 respectively.

7.3.2 ANALYSIS OF DNA INSERTS WITHIN RECOMBINANT PLASMIDS pRHLY2 - pRHLY10.

Recombinant subclones expressing a given phenotype and constructed in a similar manner i.e. identical target and vector DNA cleaved with an identical restriction endonuclease, should theoretically have common DNA fragment(s) containing the DNA sequence encoding the phenotype. Also, subclones constructed in a similar manner should contain a common DNA fragment corresponding to the cloning vector.

To confirm the presence of common DNA fragments within the recombinant subclone plasmids, miniprep isolated DNA of pRHLY2 - pRHLY10 and pBR328 were cleaved with *Hind III* for 18 h. at 37°C and the resultant DNA fragments subjected to agarose gel electrophoresis in a 0.5% gel. (Fig.7.4)

Although the gel ran at a slight angle, it was still possible to identify 2 common fragments within each recombinant plasmid, one corresponding to pBR328 DNA and the other (higher in the gel) to inserted DNA derived from cosmid pRHLY1.

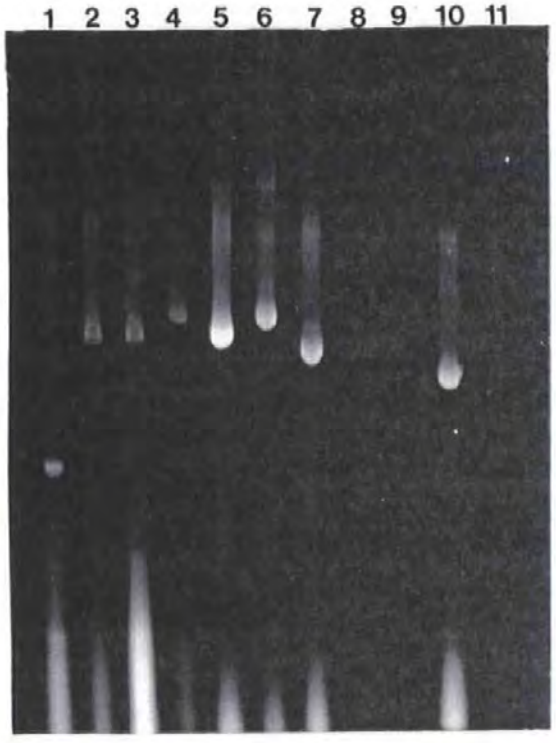


Figure 7.3. Agarose gel electrophoresis of recombinant plasmids pRHLY2-pRHLY10. LANE 1: pBR328 DNA. LANES 2-11: DNA from recombinant plasmids pRHLY2 - pRHLY10.

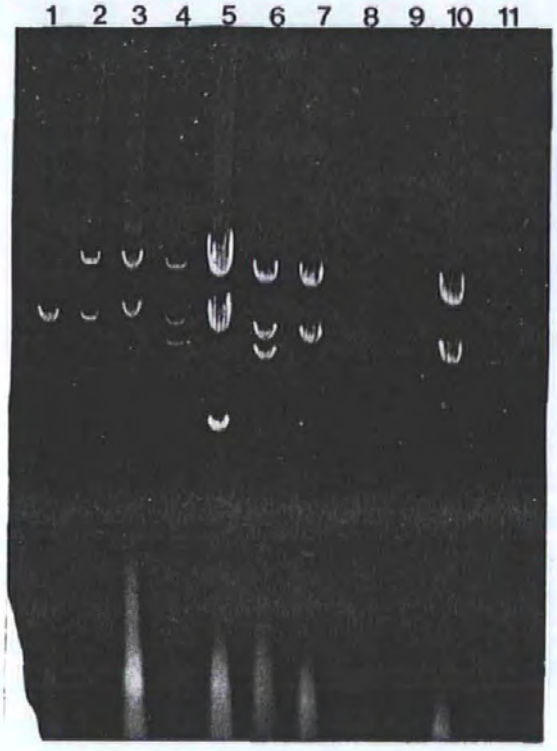


Figure 7.4. agarose gel electrophoresis of restriction endonuclease-cleaved DNA from the recombinant plasmids pRHLY2-pRHLY10.
LANE 1: pBR328/*Hind III* DNA. LANES 2-11: *Hind III* cleaved DNA from plasmids pRHLY2 - pRHLY10.

From the 9 recombinant plasmids, pRHLY2, containing a single *Hind III* generated fragment insert, was chosen for further analysis.

A bulk preparation of pRHLY2 DNA was made by maxiprep isolation and purified by CsCl/EtBr gradient ultracentrifugation. The purified DNA was stored as a solution in TE buffer at 4° C.

7.3.3 DETERMINATION OF THE SIZE OF THE INSERT WITHIN pRHLY2.

DNA (0.4 μg) was cleaved using *Hind III* for 18 h. at 37°C and subjected to agarose gel electrophoresis in a 0.5% gel in conjunction with molecular size markers (Fig.7.5).By comparison with the molecular size markers, the *Hind III* generated DNA fragment insert within pRHLY2 was estimated to be approximately 7.5 kbp.

7.4. SUBCLONING OF THE RECOMBINANT PLASMID pRHLY2 INTO THE PLASMID VECTOR pGD103.

Although the initial subcloning with *Hind III* greatly reduced the size of the cloned haemolysis conferring DNA sequence, it was decided to attempt to reduce the size of this sequence by yet another subcloning procedure.

The vector chosen for this purpose was the low copy number plasmid pGD103 (Fig 5.7, p 103). The plasmid possesses a multiple cloning site with several unique restriction endonuclease cleavage sites and carries as a selective marker a gene coding for resistance to the antibiotic kanamycin.

7.4.1 CHOICE OF RESTRICTION ENDONUCLEASE FOR THE SUBCLONING OF pRHLY2 DNA INTO pGD103.

About 0.4 μg amounts of pRHLY2 DNA were cleaved using the restriction endonucleases *Bam HI, Eco RI, Hinc II* and *Sal I* for 18 h. at 37°C, cleaved DNA was subjected to agarose gel electrophoresis in conjunction with molecular size markers (Fig.7.6). Using the information provided by this gel, it was decided to use *Sal I* for the next subcloning procedure.

Figure 7.5. Agarose gel electrophoresis of pRHLY2/*Hind III* DNA.
LANE 1: 1 kbp. ladders (from top to bottom), 12 kbp., 11 kbp., 10 kbp., 9 kbp., 8 kbp., 7 kbp., 6 kbp., 5 kbp., 4 kbp., 3 kbp., 2 kbp., 1.6 kbp., 1 kbp., 0.5 kbp. LANE 2: pRHLY/*Hind III* DNA.

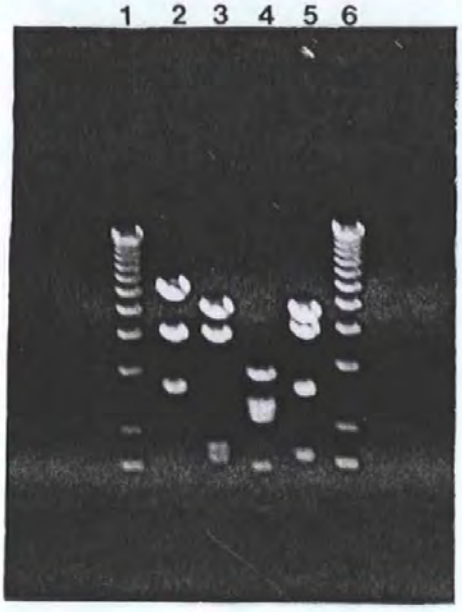


Figure 7.6. Agarose gel electrophoresis of pRHLY2 DNA cleaved with the restriction endonucleases *Bam HI, Eco RI, Hinc II,* and *Sal I.*
LANE 1: 1 kbp. ladders (from top to bottom), 12 kbp., 11 kbp., 10 kbp., 9 kbp., 8 kbp., 7 kbp., 6 kbp., 5 kbp., 4 kbp., 3 kbp., 2 kbp., 1.6 kbp., 1 kbp. LANE 2: pRHLY2/*Bam HI* DNA. LANE 3: pRHLY2/*Eco RI* DNA. LANE 4: pRHLY2/*Hinc II* DNA. LANE 5: pRHLY2/*Sal I* DNA. LANE 6: 1 kbp. ladders.

7.4.2 SUBCLONING OF pRHLY2 DNA INTO pGD103.

The subcloning proceedure was similar to that described in section 7.3 (above). Target (pRHLY2) DNA and vector (pGD103) DNA were cleaved using *Sal I* and the resultant DNA fragments ligated using T4 DNA ligase at a target:vector ratio of 2:1.

Ligated DNA (0.5 μg) was transformed into *E. coli* K12 strain DH1 and aliquots of the transformation mixture plated on to LB agar plates containing kanamycin (25 μg/ml).

Bacterial colonies produced after 18h. incubation at 37°C were overlayed with agarose containing horse erythrocytes and incubated for a further 18 h. at 20°C

Although the efficiency of transformation was poor (a total of 250 kanamycin resistant colonies), 2 bacterial colonies were found to exhibit a haemolytic phenotype. These colonies were removed and a pure culture of both prepared.

7.4.3 ANALYSIS OF THE RECOMBINANT PLASMIDS pRHLY20 AND pRHLY21.

Plasmid DNA was isolated from overnight cultures of the 2 haemolytic subclones by the miniprep method and analysed as in section 7.3.1 (above). Both subclones contained recombinant plasmid DNA which were designated pRHLY20 and pRHLY21 respectively.

Cleavage with the subcloning enzyme *Sal I* followed by agarose gel electrophoresis on a 0.5% gel in conjunction with molecular size markers, revealed a single common insert fragment within both pRHLY20 and pRHLY21 (Fig. 7.7). The size of the inserted *Sal I* fragment was estimated to be approxiamately 1.65 kbp.

7.5 PHYSICAL MAPPING OF RESTRICTION ENDONUCLEASE CLEAVAGE SITES OF A 1.65 kbp, *Sal I* GENERATED, HAEMOLYSIS-CONFERRING DNA FRAGMENT.

The mapping of restriction endonuclease sites within a cloned DNA insert is more easily acheived when an accurate restriction map of the cloning vector DNA is available. Knowing the positions of cleavage sites in a vector molecule simplifies the mapping of

Figure 7.7. Agarose gel electrophoresis of *Sal I* cleaved DNA from recombinant plasmids pHLY20 & pRHLY21.

LANE 1: 1 kbp. ladders (from top to bottom): 12 kbp., 11 kbp., 10 kbp., 9 kbp., 8 kbp., 7 kbp., 6 kbp., 5 kbp., 4 kbp., 3 kbp., 2 kbp., 1.6 kbp., 1 bkp., 0.5 kbp. LANE 2: pRHLY20/*Sal I* DNA. LANE 3: pRHLY21/*Sal I* DNA.

inserted DNA. This is because the position of sites in the insert can be mapped relative to the known vector sites after cleavage with restriction endonucleases either alone or in combination.

The vector used to construct the recombinant plasmid molecule pRHLY20, was the plasmid pGD103, a vector for which a detailed restriction map was not available. Therefore it was decided to remove the 1.65 kbp, *Sal I* generated, haemolysis conferring insert from pRHLY20 and use it to form a recombinant molecule with the well characterized plasmid vector pBR328.

The cloning of the *Sal I* insert within pRHLY20 into pBR328 was performed in a similar manner to the subcloning proceedures described in sections 7.3 and 7.4 (above). However, only pRHLY20 insert DNA was ligated to pBR328 as the insert fragment was excised from an agarose gel, away from pGD103 vector DNA, and isolated by electroelution into TE buffer. Target (*Sal I* insert) and vector (pBR328) were ligated at a 2:1 ratio and the ligated DNA molecules transformed into *E. coli* K12 strain DH1. *E. coli* clones obtained from the transformation were screened by blood agarose overlay, and those exhibiting a haemolytic phenotype were isolated, and pure cultures prepared.

Restriction endonuclease cleavage, and agarose gel electrophoresis, of recombinant plasmid DNA from these cultures was performed, and a recombinant haemolysis conferring plasmid isolated which contained a single 1.65 kbp *Sal I* generated insert. This plasmid was designated pRHLY11, prepared in bulk and used for restriction endonuclease mapping.

7.5.1 CHOICE OF RESTRICTION ENDONUCLEASES FOR THE MAPPING OF pRHLY11: SINGLE ENZYME DIGESTS.

The first part of the restriction mapping proceedure consisted of the cleavage of pRHLY11 with a number of single enzyme digests, so as to determine whether any sites for these enzymes occur on the cloned insert, and also to ascertain their value in

'combination' reactions. DNA from pRHLY11 (0.4 μg) was cleaved with 19 different restriction endonucleases at 37°C for 18 h. The enzymes chosen were either those known to cleave pBR328 vector DNA once or not at all. After enzyme digestion, the resulting DNA fragments were separated using agarose gel electrophoresis in a 0.8% gel in conjunction with molecular size markers (Fig. 7.8).

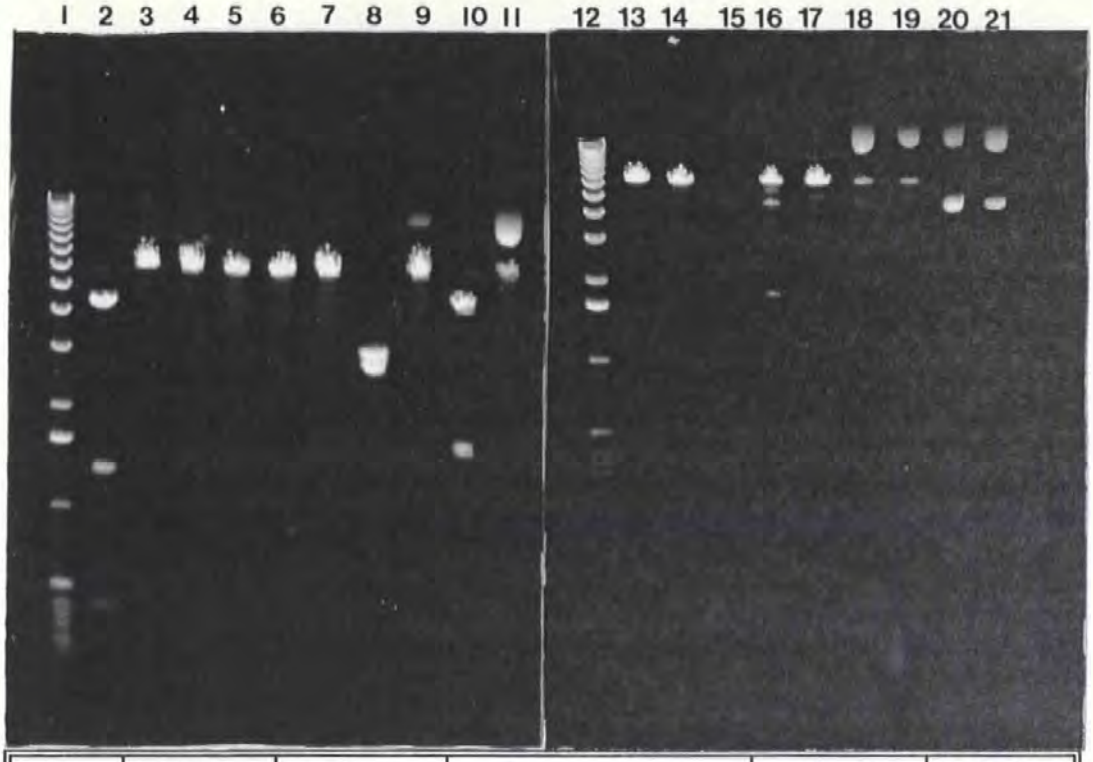
Cleavage with *Ava I* and *Pvu I*, produced 2 or more fragments, and therefore must cleave the DNA insert in pRHLY11 as there is only one site for these enzymes on the pBR328 vector molecule. It can also be seen that the enzymes *Hpa I* and *Kpn I* cleave pRHLY11, forming a linear molecule. These enzymes have no cleavage sites on the pBR328 vector DNA and therefore must cleave the 1.65 kbp DNA insert within pRHLY11.

7.5.2. RESTRICTION ENDONUCLEASE MAPPING OF pRHLY11 DNA: DOUBLE ENZYME DIGESTS.

Using the information provided in 7.5.1 (above), restriction endonuclease cleavage was performed using two enzymes in combination (double digests). Combinations were made using both a 'primary' enzyme, known to cleave the insert in pRHLY11, and a 'secondary' enzyme whose cleavage site was far enough from the insert to produce DNA fragments easily visualized after agarose gel electrophoresis.

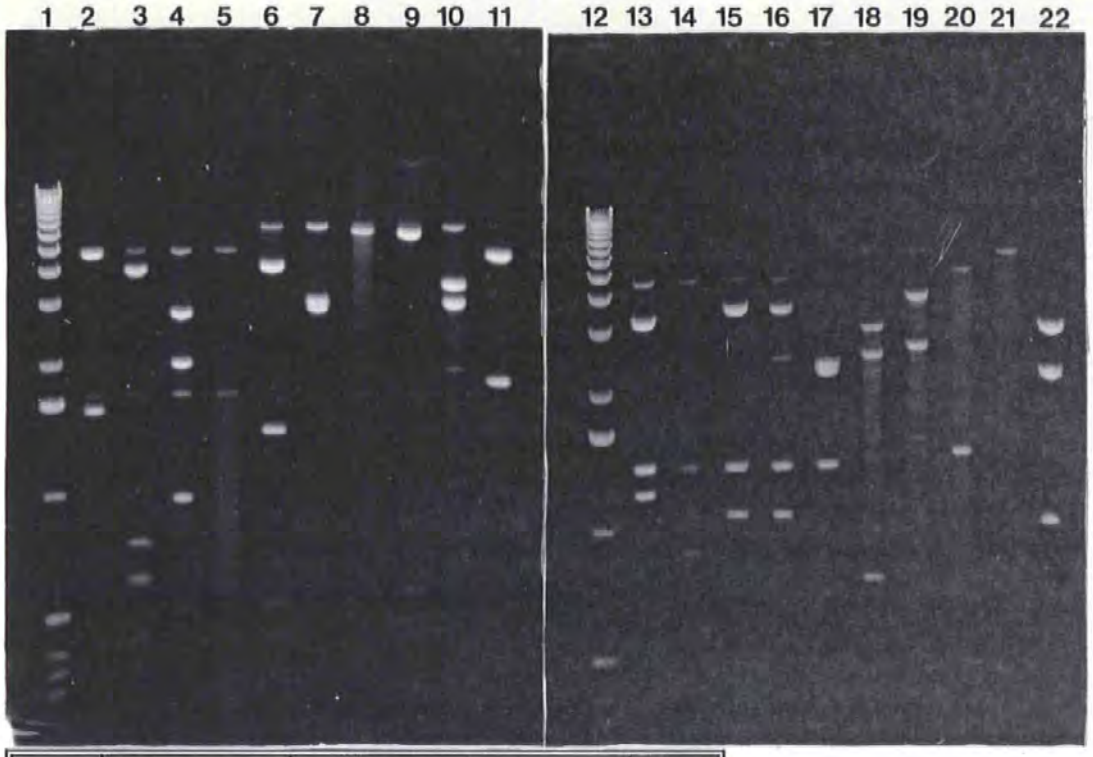
As with the single enzyme digestion, pRHLY11 DNA was cleaved at 37°C for 18 h. prior to agarose gel electrophoresis in conjunction with molecular size standards. The gels containing the resulting fragments from double digestion of pRHLY11 and the estimates of the size of fragments produced, can be seen in Fig 7.9.

The restriction endonuclease cleavage sites within the pRHLY11 insert were then mapped, the deduced maps are shown in figures 7.10 - 7.13.



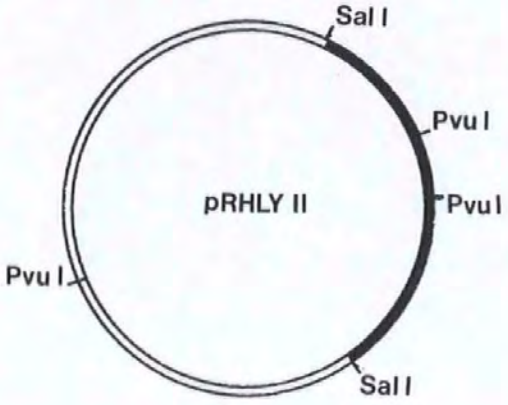
Lane	Enzyme	No. of fragments	No. of cleavage sites		Size of fragments (kbp.)	Total
			on vector	on insert		
1	Markers	–	–	–	–	–
2	*Ava I*	3	1	2	4.6, 1.4, 0.45	6.45
3	*Bam HI*	1	1	0	6.5	6.5
4	*Cla I*	1	1	0	6.5	6.5
5	*Eco RI*	1	1	0	6.5	6.5
6	*Hind III*	1	1	0	6.5	6.5
7	*Pst I*	1	1	0	6.5	6.5
8	*Pvu I*	3	1	2	3.25, 3.0, 0.25	6.5
9	*Pvu II*	1	1	0	6.5	6.5
10	*Sal I*	2	1	0	4.9, 1.6	6.5
11	*Eco RV*	1	1	0	6.5	6.5
12	Markers	-	-	-	6.5	-
13	*Sph I*	1	1	0	6.5	6.5
14	*Xmn I*	1	1	0	6.5	6.5
15	*Bgl II*	-	-	-	-	-
16	*Hpa I*	1	0	1	6.5	6.5
17	*Kpn I*	1	0	1	6.5	6.5
18	*Sac I*	0	0	0	0	-
19	*Sma I*	0	0	0	0	-
20	*Xba I*	0	0	0	0	-
21	*Xho I*	0	0	0	0	-

Figure 7.8. Single restriction endonuclease digests of the recombinant plasmid pRHLY11.



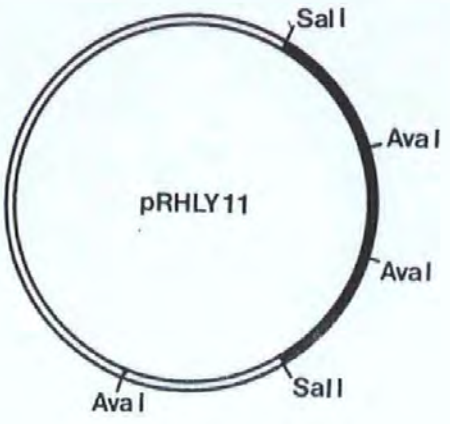
Lane	Endonuclease combination	Sizes of fragments produced (kbp.)	Total
1	*MARKERS*	-	-
2	*Sal I/Hpa I*	4.9, 1.55.	6.45
3	*Sal I/Ava I*	4.25, 0.8, 0.65, 0.625, 0.45.	6.75
4	*Sal I/Pvu I*	2.75, 2.05, 0.95, 0.55, 0.2.	6.5
5	*Sal I/Kpn I*	-	-
6	*Hpa I/ Ava I*	4.1, 1.4, 0.55, 0.45.	6.5
7	*Hpa I/Pvu I*	3.05, 3.00, 0.4, 0.2.	6.65
8	*Hpa I/ Kpn I*	-	-
9	*Hpa I/Hind III*	6.0, 0.6.	6.6
10	*Hpa I/Pst I*	3.6, 3.0.	6.6
11	*Hpa I/Eco RI*	4.7, 1.8.	6.5
12	*MARKERS*	-	-
13	*Ava I/Pvu I*	3.6, 1.4, 1.1, 0.45.	6.55
14	*Ava I/Kpn I*	3.7, 1.4, 1.0, 0.45.	6.55
15	*Ava I/Hind III*	3.5, 1.4, 1.2, 0.45.	6.55
16	*Ava I/Pst I*	3.2, 2.7, 0.8.	6.7
17	*Ava I/Eco RI*	3.95, 2.75.	6.7
18	*Kpn I/Pvu I*	5.25, 1.45.	6.7
19	*Kpn I/Pst I*	-	-
20	*Kpn I/Eco RI*	3.1, 2.4, 1.1.	6.7
21	*Kpn I/Hind III*	-	-
22	*Ava I/Eco RI*	-	-

Figure 7.9. Double restriction endonuclease digests of the recombinant plasmid pRHLY11.



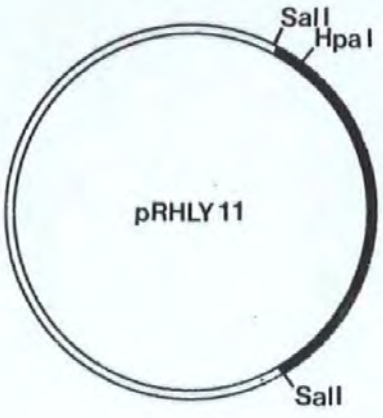
ENZYME COMBINATION	SIZE OF FRAGMENTS PRODUCED (kbp.)
Sal I/Pvu I	2.75, 2.05, 0.95, 0.55, 0.2 .
Hpa I/Pvu I	3.05, 3.00, 0.4, 0.2 .
Ava I/Pvu I	3.2, 1.4, 1.2, 0.35 .
Kpn I/Pvu I	3.2, 2.7, 0.8 .
Pvu I/Eco RI	3.1, 2.4, 1.1 .

Figure 7.10. Map of the cleavage sites for the restriction endonuclease *Pvu I* within the recombinant plasmid pRHLY11.



ENZYME COMBINATION	SIZE OF FRAGMENTS PRODUCED (kbp.)
Sal I/Ava I	2.75, 2.05, 0.95, 0.55, 0.2 .
Hpa I/ Ava I	3.05, 3.00, 0.4, 0.2 .
Ava I/Pvu I	3.2, 1.4, 1.2, 0.35 .
Ava I/Hind III	3.2, 2.7, 0.8 .
Ava I/Pst I	3.7, 2.4, 1.1 .
Ava I/ Eco RI	3.5, 1.4, 1.2, 0.4 .

Figure 7.11. Map of the cleavage sites for the restriction endonuclease *Ava I* within the recombinant plasmid pRHLY11.



ENZYME COMBINATION	SIZE OF FRAGMENTS PRODUCED (kbp.)
Sal I/Hpa I	4.9, 1.55 .
Hpa I/ Ava I	4.1, 1.4, 0.55, 0.45 .
Hpa I/Pvu I	3.05, 3.0, 0.4, 0.2 .
Hpa I/Kpn I	6.2, 0.35 .
Hpa I/Hind III	6.0, 0.6 .
Hpa I/Pst I	3.6, 3.0 .
Hpa I/ Eco RI	4.7, 1.8 .

Figure 7.12 Map of the cleavage sites for the restriction endonuclease *Hpa I* within the recombinant plasmid pRHLY11.

ENZYME COMBINATION	SIZE OF FRAGMENTS PRODUCED (kbp.)
Kpn I/Pvu I	3.2, 2.7, 0.8 .
Kpn I/Pst I	3.95, 2.7 .
Kpn I/Eco RI	5.25, 1.45 .

Figure 7.13. Map of the cleavage sites for the restriction endonuclease *Kpn I* within the recombinant plasmid pRHLY11.

Figure 7.10 shows the deduced map for the restriction endonuclease *Pvu I*. Cleavage of pRHLY11 with *Sal I* and *Pvu I* together gives a good idea of the sizes of fragments produced by *Pvu I* cleavage of the insert DNA, because *Sal I* liberates the insert and the fragments produced represent 2 parts of the vector molecule and 3 parts of insert DNA. Study of the fragments obtained from the other double digestions containing *Pvu I* confirm 2 cleavage sites on the insert as well as their position.

Figure 7.11 shows the deduced map for *Ava I*. Again, when used in conjunction with *Sal I*, the number of cleavage sites within the insert can be determined. The other *Ava I* containing digestions confirm the location of 2 cleavage sites within the insert, and the production of common fragments of 1.4 kbp and 0.45 kbp make the positioning of the cleavage sites straightforward.

The mapping of the cleavage sites for the enzymes *Hpa I* and *Kpn I* (Figs. 7.12, 7.13) could not be performed with the same ease as with *Pvu I* and *Ava I*, as only a rough estimate of the position of the sites could be made. The difficulties encountered however, do to some extent serve to demonstrate some of the problems of restriction mapping. Both *Hpa I* and *Kpn I* appear to cleave the DNA insert within pRHLY11 at sites extremely close to one end, so that the accurate positioning of cleavage sites depends on the ability to determine the size of small DNA fragments (< 0.1 kbp) or to measure the size of any DNA fragment within the limits of plus or minus 0.1 kbp The determination of DNA fragment size by electrophoretic mobility compared with molecular size markers, especially in an agarose gel of a concentration needed to resolve fragments of up to 15-20 kbp. is not an accurate method for determining the size of DNA molecules and small molecules may even run off the gel.

By carefully studying the pattern and size of fragments produced by cleavage of pRHLY11 with *Hpa I* and *Kpn I* (Fig. 7.9), it is possible to approximate the position of the cleavage sites for these two enzymes. Firstly, the position of the *Hpa I* cleavage site.

Lane 2 contains the digest with *Sal I* and *Hpa I*, which produces 3 fragments, 2 intensely fluorescing bands and one faint band. The faint band can be seen in all of the double digests made with *Sal I* and corresponds to the whole 1.65 kbp of DNA insert contained in pRHLY11. The presence of this band, is indicative of the partial cleavage of insert DNA. However the partial digestion observed in lane 2, does help us to deduce that *Hpa I* cleaves the insert DNA close (approx. 0.1 kbp) to one end. By studying the other *Hpa I* containing digests, especially in combination with *Hind III* (Lane 9) the position at the appropriate end of the insert can be deduced.

The cleavage site for *Kpn I* (Fig. 7.13) can again only be approximated . Study of Figure 7.9 reveals that the double digestions made with this enzyme appear to generate a large number of randomly sized DNA fragments, visualised as a 'smear' of fluorescence along the length of the gel. These randomly sized fragments are the result of 'star' activity by *Kpn I*. Star activity is a phenomenon exhibited by a number of restriction enzymes under non optimal conditions, and is the loss of sequence specificity (Kessler and Holtke, 1986). The presence of star activity. along with the apparent small distance between the *Kpn I* cleavage site and one end of the DNA insert make the mapping of the site particularly difficult, but using the information contained in the pRHLY11 cleavage maps for *Ava I*, *Pvu I* and *Hpa I* and the fragments produced by the *Kpn I/Pst I* double digest, the position of cleavage can be estimated.

Finally, a complete restriction map for the enzymes *Pvu I*, *Ava I*, *Hpa I* and *Kpn I* was constructed, and can be seen in Figure 7.14.

7.6 DETERMINATION OF CLONING FIDELITY: SOUTHERN BLOT HYBRIDIZATION OF *Sal I* CLEAVED *R.salmoninarum* GENOMIC DNA.

To establish the fidelity of the cloning proceedures used to produce the 1.65 kbp, *Sal I* generated fragment within the haemolysis conferring plasmid pRHLY11, a Southern hybridization experiment was performed. Southern-blot hybridization can determine

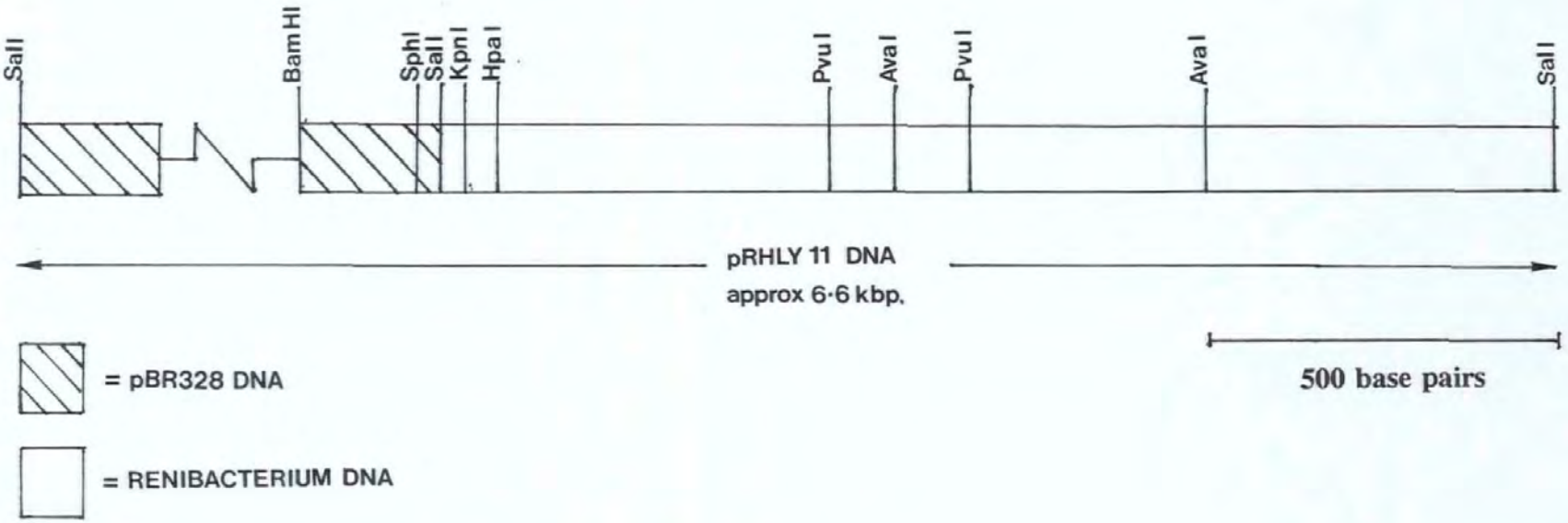


Figure 7.14. Restriction endonucleases cleavage map of the recombinant plasmid pRHLY11.

whether a cloned DNA fragment was derived from the appropriate organism (in this case *R.salmoninarum*) and, that the fragment is truly representative of the genomic DNA sequence. The method of Southern blot hybridization is described fully below, but basically consists of three main steps:

1. Cleavage of genomic DNA with the appropriate restriction endonuclease and separation of the resulting fragments by agarose gel electrophoresis.

2. Transfer of the DNA fragments to a membrane support by capillary action.

3. Hybridization of the immobilised DNA using the cloned DNA fragment (suitably labelled) as a probe, followed by the detection of probe DNA - genomic DNA hybrids.

7.6.1 PREPARATION OF DNA FOR SOUTHERN BLOT HYBRIDIZATION.

Approximately 10 μg of genomic DNA from *R.salmoninarum* strains PPD, Lea, MT444, DR143, NCIMB 1116 and the bacteria *Aeromonas salmonicida* strain CM30, *Staphylococcus aureus,* and *E.coli* K12 strain DH1 were cleaved for 18 h. at 37°C with *Sal I.* At the same time the haemolysis conferring 1.6 kbp. *Sal I* insert was isolated by enzyme cleavage and electrophoretic elution from the plasmid pRHLY11 (see section 7.5, above).

Insert DNA (0.2 μg) and the cleaved genomic DNA samples were subjected to agarose gel electrophoresis in a 0.8% gel in conjunction with molecular size markers (Fig. 7.15)

7.6.2 SOUTHERN TRANSFER OF DNA FRAGMENTS TO A NITROCELLULOSE MEMBRANE.

In the capillary transfer method of Southern (1975), fragments of DNA are carried from an agarose gel in a flow of liquid and deposited on the surface of a nitrocellulose membrane.

To aid the efficient transfer of DNA from gel to membrane, the agarose gel from 7.6.1 (above) was treated by soaking for 10 min. in 100 ml of 0.2 M HCl, 45 min. in 100 ml of 1.5 M NaCl/0.5 M NaOH, and 30 min. in 100 ml of 1.5 M NaCl/1 M Tris (pH 7.4).

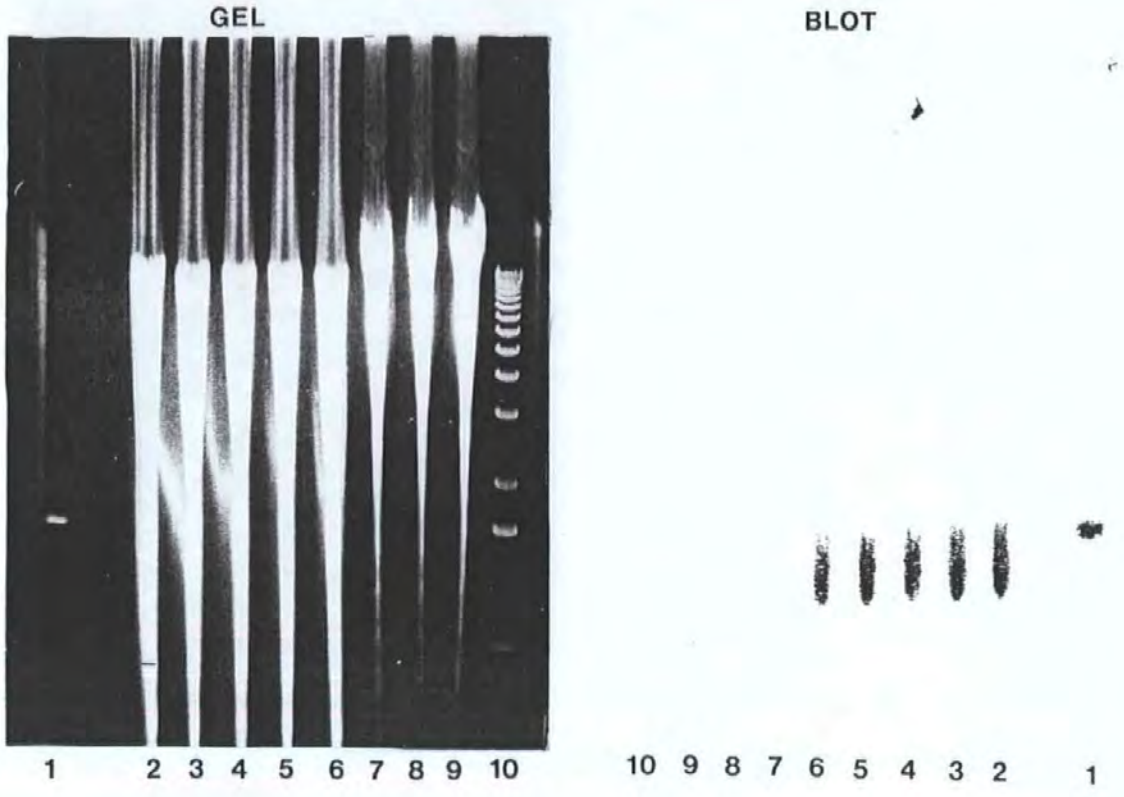


Figure 7.15. Southern blot hybridization of *Sal I* cleaved *R.salmoninarum* DNA. LANE 1: 1.6 kbp. *Sal I* fragment from pRHLY11 LANE 2: *R.salmoninarum* PPD/*Sal I* genomic DNA. LANE 3: *R.salmoninarum* Lea/*Sal I* genomic DNA. LANE 4: *R.salmoninarum* MT444/*Sal I* genomic DNA. LANE 5: *R.salmoninarum* DR143/*Sal I* genomic DNA. LANE 6: *R.salmoninarum* NCIMB1116/*Sal I* genomic DNA. LANE 7: *S.aureus* /*Sal I* genomic DNA. LANE 8: *A.salmonicida* CM30/*Sal I* genomic DNA. LANE 9: *E.coli* K12 (DH1)/*Sal I* genomic DNA.

The treatment with HCl results in partial depurination of the DNA, whilst exposure to NaOH hydrolyses the phosphodiester backbone of the DNA. The third treatment merely neutralises the strong alkali. The combined treatment results in single-stranded DNA fragments that are rapidly transferred from gel to membrane.

Whilst the gel was neutralizing, a piece of Whatman 3MM paper was wrapped around a glass staining dish to form a support both longer and wider than the agarose gel. The wrapped support was placed in a large plastic tray and the tray filled with transfer buffer (10x SSC) until the level of the buffer was half way up the support. The 3MM paper was then thoroughly wetted with transfer buffer.

A piece of nitrocellulose membrane (Schleicher & Schuell BA85) was cut to the same size as the agarose gel and thoroughly wetted in deionised water followed by soaking for 2 min. in transfer buffer.

After neutralization had taken place, the agarose gel was inverted and placed underside uppermost on to the gel support. A mask of aluminium foil was made so as to cover the top of the gel support but to leave the gel exposed. This mask acts as a barrier to prevent a short circuit i.e. liquid from flowing directly to the paper towels placed on top of the gel thus reducing the efficiency of DNA transfer.

The nitrocellulose membrane was carefully placed on to the gel avoiding the trapping of air bubbles. Two pieces of 3MM paper, previously soaked in 2x SSC wre then placed on top of the membrane and smoothed down with a clean glass rod. Paper towels, cut to the same size as the 3MM paper were stacked to a height of 10 cm on top of the gel/membrane/3MM sandwich and compressed with a weight of approx. 500 g. The transfer of DNA was then allowed to proceed for 18h. After this period, the membrane was dried between 2 pieces of 3MM paper at ambient temperature for 30 min. The DNA was then firmly attached to the membrane by baking at 80°C for 2 h. in a vacuum oven.

7.6.3 PREPARATION OF A DIGOXIGENIN-LABELLED DNA PROBE FROM THE 1.65 kbp *Sal I* FRAGMENT CONTAINED IN pRHLY11.

To detect probe DNA-target DNA hybrids after hybridization, the probe has to be labelled. Until recently, the majority of nucleic acid probe sequences were labelled with radioisotopes such as ^{32}P (Maitland *et al,* 1987) and hybrids detected by autoradiography. Over the past few years, the choice of labelling and detection methods has greatly increased and several non-radioactive enzyme-based systems have been developed. One of these systems was chosen for the labelling of the 1.65 kbp *Sal I* generated DNA probe, namely the Nonradioactive DNA labelling and Detection Kit (Boehringer Mannheim GmbH, Mannheim, Germany).

The labelling and detection proceedure using this kit, consists of 3 distinct parts.

1. The incorporation of a nucleotide analog (digoxigenin-11-dUTP) into DNA by the random primed labelling technique.

2. Hybridization of the digoxigenin-labelled probe to immobilised DNA.

3. Detection of the labelled probe with an antibody-enzyme conjugate, anti-digoxigenin-alkaline phosphatase. The location of the antigen-antibody conjugate is visualized by an enzyme-linked colour reaction.

For this experiment, 1 μg of the 1.65 kbp *Sal I* insert DNA from pRHLY11 was denatured by heating at 95°C for 10 min. and immediately chilling on ice. The DNA labelling mixture was made by adding the following to a 0.5 ml microfuge tube on ice: 1 μg of denatured probe DNA, 2 μl hexanucleotide mix (Boehringer), 2 μl dNTP labelling mix (Boehringer), 14 μl analytical grade water and 1 μl (2 units) of Klenow enzyme (Boehringer).

After a brief centrifugation, the mixture was incubated at 37°C for 20 h.. The labelling reaction was terminated by the addition of 2 μl of a 0.2 M EDTA solution (pH 8.0) and the DNA precipitated using 2.5 μl of 0.4 M LiCl and 75 μl of ice-cold ethanol.

Precipitation was performed at -70°C for 30 min. after which the DNA was recovered by centrifugation at 13,000 x g for 10 min. and dried under vacuum. The DNA was then dissolved at 37°C for 30 min. in 50 μl of TE buffer. The labelled DNA was stored frozen at -20°C until use.

7.6.4 DNA HYBRIDIZATION.

After baking in the vacuum oven, the DNA attached to the nitrocellulose membrane was hybridized with the digoxigenin-labelled probe in the following manner.

The membrane was at first prehybridized in a sealed plastic bag with 20 ml of hybridization solution (5x SSC, 0.1% N-lauroylsarcosine-Na salt, 0.02% SDS and 1% blocking reagent (Boehringer). At the same time 25 μl of the labelled DNA probe solution was denatured by heating at 95°C for 10 min followed by immediate chilling in ice.

After prehybridization, the prehybridization solution was discarded and replaced with 15 ml of fresh hybridization solution containing 25 μl of denatured digoxigenin-labelled DNA probe. The plastic bag was resealed and hybridization allowed to take place for 18 h. at 68°C. After hybridization and prior to the detection of hybridized probe DNA, the nitrocellulose membrane was washed for 2 x 5 min. with 50 ml of 2x SSC, 0.1% SDS, followed by 2 x 15 min. at 68°C with 50 ml of 0.1x SSC, 0.1% SDS.

7.6.5 ENZYME-LINKED ANTIBODY DETECTION OF PROBE DNA-TARGET DNA HYBRIDS.

The nitrocellulose membrane was washed for 2 min. in 50 ml of buffer 1 (100mM.Tris-HCl; 150mM. NaCl, pH 7.5) prior to antibody exposure. After washing the membrane was exposed at ambient temperature for 45 min. to anti-digoxigenin-alkaline phosphatase conjugate (Boehringer) diluted 1:5000 in 20 ml of buffer 1.

Unbound antibody was removed by washing for 2 x 15 min. in 100 ml of buffer 1, and the membrane equilibrated for 5 min. in 25 ml of buffer 3 (100mM Tris-HCl; 100mM NaCl; 50mM $MgCl_2$,pH 9.5). The equilibrated membrane was then exposed, in the dark,

to the colour developing solution (45 μl NBT solution (Boehringer), 35 μl X-phosphate solution (Boehringer) in 10 ml of buffer 3.

After 5 min. development, the probe-hybridized DNA bands were clearly visible and the colour reaction terminated by washing the nitrocellulose membrane in 50 ml of TE buffer. To record the result of the Southern hybridization, the membrane was photocopied (Fig. 7.15).

The hybridization experiment clearly shows that the labelled 1.6 kbp. insert DNA hybridised to a homologous sequence within the genomic DNA of all 5 strains of *R.salmoninarum* tested but with no genomic sequences for the control bacterial DNAs. This shows that the cloned insert within the haemolysis conferring plasmid pRHLY11 originated from the genome of *R.salmoninarum.*

Although the hybridized fragments show distortion as a result of uneven electrophoretic mobility, it can also be seen that the cloned insert is truly representative of a 1.6 kbp genomic DNA sequence bounded by cleavage sites for the restriction endonunuclease *SalI.*

7.7 DISCUSSION.

This chapter has described the manipulation and primary structural analysis of a portion of cloned DNA isolated from the pHC79/*R.salmoninarum/Sau 3A* cosmid gene library.

The recombinant haemolysis conferring cosmid pRHLY1 was shown to contain a DNA insert of approximately 32 kbp in length. Using the restriction endonucleases *Hind III* and *Sal I*, in conjunction with the plasmid cloning vectors pBR328 and pGD103 the original insert was reduced in size by subcloning to 7.5 kbp in the recombinant plasmid pRHLY2 and to 1.65 kbp in the recombinant plasmid pRHLY20. The 1.65 kbp. *Sal I* generated insert contained within pRHLY20 was also cloned into the plasmid vector pBR328 to form the recombinant plasmid pRHLY11. All of these plasmids conferred haemolytic properties to their *E.coli* hosts, showing that the haemolysis conferring sequence was present in the insert DNA. The subcloning proceedure had therefore

succeeded in removing a large portion of genetic information external to the haemolysis conferring DNA sequence, this exogenous DNA may have hindered the successful analysis of the haemolysis conferring sequence, so the subcloning prooceedure can be seen as beneficial. However, if at a later date it was discovered that important DNA sequences have been removed by the subcloning process, or indeed a portion of the gene coding for a haemolytic molecule was missing, the original cosmid and all of the recombinant cosmid and plasmid molecules constructed during subcloning were retained for future study.

The gross anatomy of the smallest haemolysis conferring DNA fragment was analysed by mapping the restriction endonuclease cleavage sites within the fragment, and a physical map of the cleavage sites for *Ava I, Pvu I, Hpa I* and *Kpn I* was constructed.

Finally, it was established by Southern blot hybridization, that the cloned haemolysis conferring DNA sequence is not only homologous to a sequence contained within the genome of the *R.salmoninarum* strain used to construct the original gene library, but the same sequence is common to strains of the bacteria isolated from different host salmonid species and various geographical locations.

Although it is possible that the cloned haemolysis conferring DNA contains a complete coding sequence for the expression of a haemolytic molecule, this is *not* possible to determine, using the information gained during the structural analysis experiments described above. However, if an intact open reading frame containing both initiating and terminating codons exists within the smallest cloned haemolysis conferring fragment, some idea of the size of the haemolytic molecule can be formed. One approximation made by molecular biologists, which is widely accepted, is that 1 kbp of prokaryotic (or cDNA) coding DNA will produce a protein molecule of approximately 3.7×10^4 Daltons. It can therefore be calculated that the maximum possible size of the haemolytic molecule produced by the cloned DNA sequence is in the region of 5.8×10^4 Daltons.

Other manipulations could have been performed on the cloned DNA, as many methods

exist for further structural study of genetic information within cloned sequences. For instance, methods exist for the determination of gene orientation within a cloned DNA fragment, also it is possible to determine the presence of promoter regions within cloned DNA as well as the position of a gene within a DNA fragment. All of these methods could have been applied to the study of the cloned haemolysis conferring DNA from *R.salmoninarum,* but their absence in this study would be negated by the determination of the full nucleotide sequence of the cloned DNA, which is reported later in this thesis.

CHAPTER 8: CHARACTERIZATION OF THE MOLECULAR DETERMINANT OF HAEMOLYTIC ACTIVITY ENCODED BY A CLONED FRAGMENT OF *R.salmoninarum* DNA.

8.1 INTRODUCTION.

The preceding chapters of this thesis describe the experimental work undertaken to clone a fragment of *R.salmoninarum* DNA that confers haemolytic activity on *E. coli K12* host strains. The next set of analyses to be performed, were designed so as to attempt an initial characterization of the molecular determinant of haemolytic activity itself, the putative *R.salmoninarum* haemolysin (RHLY).

Without prior knowledge of the true nature of a foreign gene product cloned into *E. coli*, the molecular biologist can be faced with a difficult task. Before any comprehensive biochemical characterization can be performed, the cloned product must be isolated and purified free of interfering *E. coli* components. However, the success of such isolation and purification procedures is dependent on the development and utilization of specific and efficient protocols for the detection of the cloned product (Finlay, 1992).

Sometimes, a cloned gene is highly expressed in a host bacterium, and its product can be readily detected by simple separation analysis techniques such as PAGE. However, it is usually the case that a specific and selective method has to be available for the detection of the cloned product amongst the large number of host cell components. One of the most common detection methods utilizes the biochemical properties of the cloned molecule such as any enzyme activity. However if the molecule is produced in small amounts, or a similar property is present in the host cell, this means of detection may be inappropriate. One of the most specific and selective detection protocols available, which allows for the simple and efficient detection of even the smallest amounts of gene product, utilizes specific antibodies raised against the product, in immunological assays (Sambrook *et al.*, 1989). When the biochemical properties of a cloned product are uncharacterized, or no

suitable immunologically-based method is available, an investigator may yet be able to detect a molecule for which a cloned coding sequence is available.

The products of cloned genes that are carried on plasmid or λ-based vectors may be specifically labelled and subsequently detected without the masking effect of the large number of molecules that result from the expression of the host cell genome (Dougan and Kehoe, 1984; Old and Primrose, 1989). A number of individual methods exist to study the products of cloned genes in this manner, and these can be conveniently placed in to two groups; those utilising *in vivo* transcription and translation processes, and those where the gene product is visualised as a result of an *in vitro* cell-free gene expression system (Pratt *et al.*, 1981). The *in vitro* cell-free transcription and translation systems available, rely on the presence of all the biochemical components of protein synthesis extracted from certain cell types, but without a functional genome. If a DNA replicon containing coding sequences is combined with these components in the presence of radioactively-labelled methionine, transcription and translation will occur, leading to the production of a radiolabelled polypeptide which may be readily detected by autoradiographic techniques. A variety of cell free transcription and translation systems are now available commercially for both prokaryotic and eukaryotic cloning systems (Old and Primrose, 1989). The most widely used *in vivo* systems also rely on the specific radiolabelling of a gene product synthesized in the absence of host cell genome expression, but this occurs inside *E. coli* cells. Perhaps the most popular method, is the minicell system (Dougan and Kehoe, 1984). Minicells are small (0.1μm), spherical, anucleate bodies produced at the ends of certain mutant strains of rod-shaped bacterium (Frazer and Curtiss, 1975) including *E. coli*. These bacterial mutants occasionally exhibit aberrant cell division in which a septum is produced close to one end of the cell, resulting in the formation of a minicell. Minicells contain very little or no chromosomal DNA, and can remain metabolically active for long periods, they are also for a limited time, able to transcribe and translate DNA molecules

that have been introduced into them. If minicells containing recombinant DNA are incubated in a medium containing radiolabelled methionine, the polypeptides coded for by this DNA will be specifically labelled.

The second *in vivo* method for specifically labelling cloned gene products, involves the irradiation of an *E. coli recA uvrA* mutant with UV light. Exposure of the mutant *E. coli* to UV light results in the cessation of protein synthesis and extensive degradation of chromosomal DNA over a period of several hours. This method has been named the maxicell method and was first described by Sancar *et al.* (1979). If maxicells have been transformed with a multicopy plasmid (e.g. pBR328), the plasmid molecules that have escaped UV 'hits' will continue to replicate for several hours within the cells. If radioactive methionine is added, the majority of gene products labelled, will be plasmid encoded.

Once the gene product of interest has been detected, the characterization process can begin. Ideally, as previously mentioned, the cloned product should be isolated and purified free of potentially interfering host cell components. However, if there is available a 'negative control ', e.g. a host cell containing vector DNA alone, it may be possible using comparative experiments to investigate a number of the properties of the cloned product.

Even if an investigator is able to isolate and purify a cloned product of interest, and is able to establish some of the biochemical characteristics of the molecule, the experimentation may prove meaningless if the cloned product is not a true representative of that which occurs in the organism from which the cloned DNA originates. Although precautions are taken throughout the cloning process, there are opportunities, commencing from the initial isolation of the target DNA, and continuing through subsequent subcloning procedures, for mutations to occur in the coding sequence of interest. It is therefore prudent in any gene cloning experiment, to establish the fidelity of the cloning procedure with respect to the native gene product.

The experimental work in this chapter, represents the attempted characterization of the unknown molecular determinant of haemolytic activity (RHLY) that is coded for by a cloned fragment of *R.salmoninarum* DNA. The detection of the RHLY protein in preparations of haemolytic *E. coli* clones was followed by the partial characterisation of its biochemical properties and an investigation into the basis of its haemolytic activity. The presence of an equivalent molecule amongst the protein components of *R.salmoninarum* was also investigated.

8.2 DETECTION OF THE MOLECULAR DETERMINANT OF HAEMOLYTIC ACTIVITY ENCODED ON THE RECOMBINANT PLASMID pRHLY11.

The first stage in the characterization of the cloned *R.salmoninarum* haemolysin, involved its detection in the *E. coli K12* DH1 clone containing the recombinant plasmid pRHLY11. To aid the detection of the unknown protein, a culture of the DH1 host containing pBR328 (the parent plasmid to pRHLY11) was used as a comparative control.

8.2.1 SDS-PAGE ANALYSIS OF THE HAEMOLYTIC *E. coli K12* CLONE.

Broth cultures (100ml) of DH1/pRHLY11 and DH1/pBR328 were grown overnight at 37°C in a shaking water bath. The bacterial cells were then harvested by centrifugation at 5,000 x g for 15 min. at 4°C. Harvested cells were washed twice in PBSA and finally resuspended in 5 ml of PBSA prior to 3 x 1 min. bursts of sonication. These sonic preparations were stored at -20°C until use. Extracellular products of the two bacterial cultures, were obtained by ammonium sulphate precipitation (90% saturation) of the original culture supernatant.

The protein concentration of both the sonic preparation and the ECP fraction was determined, and approximately 40μg of protein from each was separated by SDS-PAGE using a 10% gel under denaturing conditions. After electrophoresis, the gel was stained with coomassie blue to visualize the separated protein bands. The stained gel can be seen in Fig. 8.1. Examination of the gel reveals a large number of proteins in both fractions

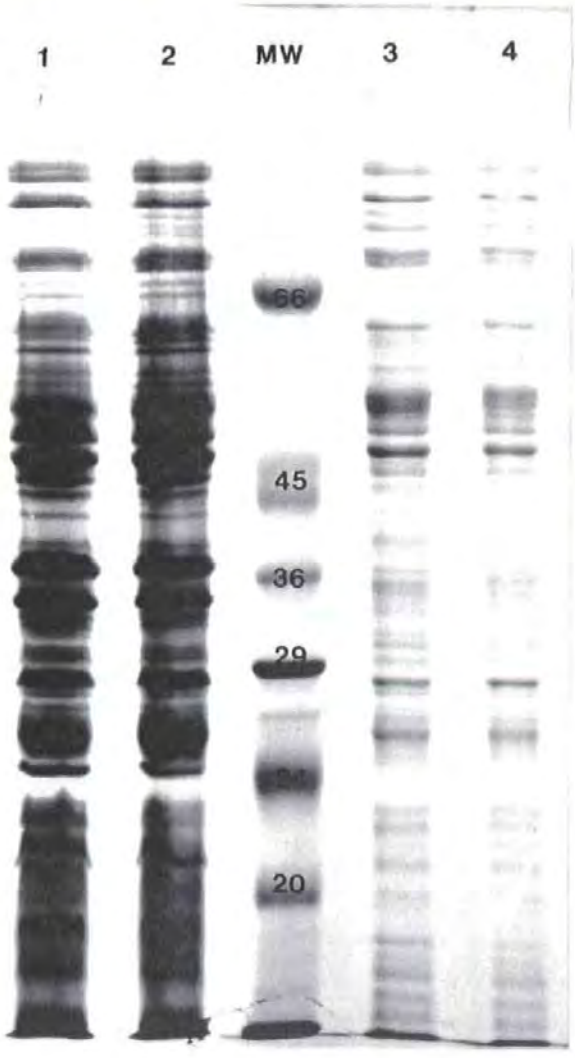


Figure 8.1 SDS-PAGE analysis of a haemolytic *E. coli* clone containing the recombinant plasmid pRHLY11. Showing the electrophoretic separation (10% gel) of a sonic preparation of whole cells (lanes 1 & 2) and the ECP fraction (lanes 3 & 4) of the haemolytic *E. coli* clone DHI/pRHLY11 and the comparative control clone DH1/pBR328. Lanes 1 & 3: DH1/pBR328. Lanes 2 & 4: DH1/pRHLY11. MW= protein molecular weight standards (sizes in kilodaltons).

of the *E. coli* clones, however, it was not possible to detect any obvious supplementary protein bands in either preparation of the haemolytic clone DH1/pRHLY11, which could have indicated the presence of the RHLY molecule.

8.2.2 IMMUNOBLOT ANALYSIS.

In a further attempt to detect the haemolytic determinant encoded by pRHLY11, the sonic preparations and concentrated ECP fractions prepared in 8.2.1 above were subjected to immunoblot analysis using both rabbit anti-*R.salmoninarum* ATCC33209 whole cells and rabbit anti-*R.salmoninarum* ATCC33209 ECPs.

Approximately 10μg of protein from each cell fraction was subjected in duplicate to SDS-PAGE under denaturing conditions, followed by electrotransfer to a nitrocellulose membrane. The protein containing membranes were then immunostained as described in 3.4.5 (above) using rabbit anti-*R.salmoninarum* ATCC33209 whole cell serum or rabbit anti-*R.salmoninarum* ATCC33209 ECP serum as the primary antibody, and peroxidase-conjugated swine anti-rabbit immunoglobulins (P217, Dako) as the secondary antibody. Antibody binding was detected by DAB/$NiCl_2$ colour development and the developed blot are shown in Fig. 8.2. Examining the developed immunoblots, it was seen that a number of antibody bound proteins were visible in the tracks corresponding to protein preparations from DH1/pRHLY11 and in those tracks corresponding to DH1/pBR328 protein preparations. It was also noted that antibodies in rabbit antiserum raised against *R.salmoninarum* ECP appear to bind to fewer proteins in the preparations of *E. coli* clones than those in serum raised against whole cells. In fact, visible antibody binding to the blot probed with anti-ECP serum, was confined to proteins of estimated sizes in the region of 45-60 kDa, and these proteins appear to be present only in the whole cell protein preparations of the *E. coli* clones. In contrast, antibodies present in rabbit antiserum raised against *R.salmoninarum* whole cells, bound to a large number of protein bands, both in protein preparations from the RHLY containing clone and the vector containing control

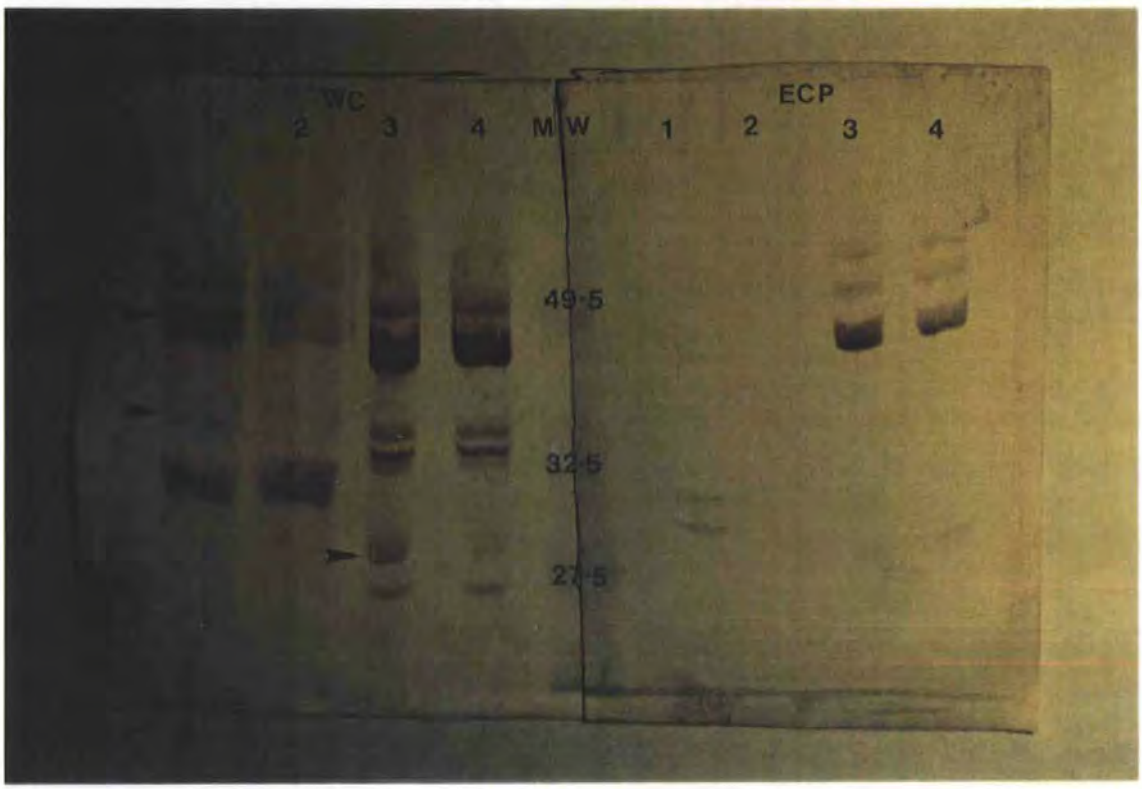


Figure 8.2 Immunoblot of protein preparations from the *E. coli* clones DH1/pRHLY11 and DH1/pBR328 using antibodies raised against *R.salmoninarum*. Shows antibody-bound proteins detected by rabbit anti-*R.salmoninarum* ATCC33209 whole cell (**WC**) and rabbit anti-*R.salmoninarum* ATCC33209 ECP (**ECP**) sera. Lanes indicated are comparable in both blots. Lane 1: DH1/pRHLY11 ECP, Lane 2: DHI/pBR328 ECP, Lane 3: DH1/pBR328 whole cells. Lane 4: DH1/pBR328 whole cells. Arrows indicate the location of antibody-bound proteins unique to the RHLY containing clone. **MW** = molecular weight markers (kDa).

clone. Antibody binding to proteins present in preparations of both *E. coli* clones, could be explained either by the presence of circulating antibodies to *E. coli* in the rabbit used to raise the antiserum, or antigenic homology between proteins found in *R.salmoninarum* and *E. coli.*

The immunoblot probed with anti-*R.salmoninarum* whole cells, also indicates the presence of antibody-bound protein bands in the preparation made from the RHLY-containing *E. coli* clone, that did not appear to be present in preparations of the vector containing *E. coli* control. Two supplementary antibody-bound proteins were visible in the ECP preparation of DHI/pRHLY11 and are of sizes in the region of 45-50 kDa and 35-40 kDa, whilst one supplementary band of approximately 28 kDa could be seen in the whole cell preparation of the clone. These antibody-bound proteins, may indicate the presence of a molecule or molecules corresponding to the molecular determinant(s) of haemolytic activity in *E. coli* DH1/pRHLY11.

8.2.3 ERYTHROCYTE/AGAROSE OVERLAY.

The next stage in the attempted detection of the cloned haemolytic determinant, was to overlay electrophoretically separated proteins from the different fractions of DH1/pRHLY11 and DH1/pBR328 with erythrocyte/agarose suspensions. About 60μg of protein from each *E. coli K12* fraction was subjected to SDS page under denaturing conditions in a 10% gel. After electrophoresis, the SDS was removed by washing for 15 min. in PBSA + 10% (v/v) triton X100, followed by 2 x 5 min washes in PBSA alone. The surface of the gel was gently blotted dry with filter paper, followed by overlaying with 10 ml of molten erythrocyte/agarose suspension. After the agarose had set the overlayed gel was incubated for 24 h. at 25°C in a sealed plastic lunchbox containing damp tissue. After incubation, the overlayed gel was examined for signs of haemolytic activity.

Two polyacrylamide gels were treated in this manner, one overlayed with

agarose/rainbow trout erythrocytes, and the other with agarose/horse erythrocytes, however, no signs of haemolytic activity were detected.

In an attempt to circumvent any denaturation of the haemolytic component by either exposure to SDS or the mercaptoethanol present during electrophoresis, fractions of the *E. coli* clones were also subjected to native PAGE in 5% gels. These gels were overlayed with erythrocyte/agarose suspensions and incubated in the same manner as before, however none were found to show signs of haemolytic activity.

8.3 MINICELL ANALYSIS OF THE RECOMBINANT PLASMID pRHLY11.

As demonstrated by the set of experiments performed in 8.2 (above), one of the major problems in detecting and characterizing plasmid encoded products in bacteria, is that they can readily be obscured by the large amount of proteins encoded by the host genome. As the cloned haemolytic molecule did not appear to be produced in large amounts, and could not be conclusively detected by immunoblotting with anti-*R.salmoninarum* serum or visualized by using erythrocyte overlay, it was decided to specifically label the product(s) of the coding sequence(s) present on the cloned *R.salmoninarum* DNA in the absence of significant expression from the *E. coli* host chromosome. The method chosen was an *in vivo* transcription and translation method utilizing *E. coli* DS410 minicells.

Instead of using the pBR328 based recombinant plasmid pRHLY11 as a DNA template for minicell analysis, it was decided to use the recombinant plasmid pRHLY20. This plasmid contains the same 1.65kbp *Sal I* fragment of *R.salmoninarum* DNA as pRHLY11, but it is contained in the low copy number vector pGD103. By using a low copy number vector, it was hoped that any toxic affects of the RHLY molecule on the metabolic activity of the *E. coli* cell would be decreased. A comparative negative control template (pGD103 DNA) was also used.

The method used to study the recombinant plasmid pRHLY20 using *E. coli* minicells, was a variation of the method of Dougan and Kehoe (1984).

8.3.1 INTRODUCTION OF PLASMID DNA INTO THE *E. coli K12* MINICELL STRAIN DS410.

A broth culture (10 ml) of the minicell producing strain *E. coli K12* DS410 was grown with vigorous shaking overnight at 37°C in brain-heart infusion medium (BHI). Two 1.5ml amounts of the overnight culture were removed and the bacterial cells present transformed separately with 0.25μg amounts of pRHLY20 DNA and pGD103 DNA. The transformation mixtures were plated out on to BHI agar containing kanamycin (25μg/ml) as the selective antibiotic and incubated overnight at 37°C. Bacteria containing pGD103 were selected by their ability to grow in the presence of kanamycin, whilst bacteria containing pRHLY20 were selected by their ability to grow on kanamycin-containing medium and to produce haemolysis in horse erythrocyte/agarose overlays. The presence of the appropriate plasmid DNA within DS410 clones was confirmed by miniprep isolation and agarose gel electrophoresis.

8.3.2 ISOLATION OF MINICELLS.

Broth cultures (200ml) of DH1/pRHLY20 and DH1/pGD103 were grown with shaking overnight at 37°C in BHI medium containing kanamycin (25μg/ml). Each culture was initially centrifuged at 2,000 x g for 5 min. at 4°C, after which, the minicell-containing supernatants were removed and placed separately into fresh centrifuge tubes and re-centrifuged at 15,000 xg for 10 min. (at 4°C) to pellet the minicells and remaining normal bacteria. The minicells were then purified as follows.

Minicell pellets were resuspended in 2 ml of PGS (0.85% w/v NaCl, 0.03% w/v KH_2PO_4, 0.06% w/v Na_2HPO_4, 100μg/ml gelatin) and vortexed vigorously to disrupt any cell aggregates. The resuspended minicells were carefully layered on to 25 ml sucrose gradients and centrifuged at 4,000 x g for 20 min. Sucrose gradients were constructed by dispensing 25 ml of 20% (w/v) sucrose in PGS into 30 ml plastic universal bottles, freezing at -20°C and thawing slowly overnight at 4°C. After gradient centrifugation

normal cells formed a band low down in the gradient. The minicells formed a broad band in the top half of the gradient, this band was removed and the minicells pelleted by centrifugation (15,000 x g for 10 min. at 4°C). The pellet was again resuspended in 2 ml of PGS and this suspension layered on to another sucrose gradient. The gradient centrifugation procedure was repeated on two more occasions until only a single minicell-containing band was present. The purified minicells were then washed in 10 ml of PSG.

8.3.3 RADIOLABELLING OF PLASMID ENCODED PRODUCTS.

The washed pellets of purified minicells were resuspended in a 5 ml volume of methionine assay medium (Difco) supplemented with thiamine (0.5μg/ml) and incubated with shaking at 37°C for 20 min, to allow them to reach maximum metabolic activity. Then, 20μCi of trans-^{35}S-label methionine and cysteine (ICN Flow) was added so as to facilitate the labelling of plasmid encoded polypeptides, and incubation continued for a further 15 min. Labelled minicells were recovered by centrifugation at 15,000 x g for 10 min.

8.3.4 DETECTION OF LABELLED PLASMID ENCODED POLYPEPTIDES.

Labelled minicells were suspended in 100μl of SDS-PAGE sample buffer and subjected to SDS-PAGE in a 10% gel. Electrophoretically separated proteins were transferred by electroblotting on to a nitrocellulose filter. The protein containing filter was allowed to dry, before an autoradiograph was set up by placing the filter in contact with X-OMAT radiographic film (Kodak). The film was exposed in darkness for 36 h. prior to development according to the manufacturers instructions, in GBX developing solutions (Kodak). The developed autoradiograph is shown in Fig. 8.3. Examination of the autoradiograph revealed the presence of a number of labelled proteins, several of which were present only in the electrophoretically separated minicell preparation of DS410/pRHLY20. These proteins were presumably the products of a coding region or regions present on the cloned *R.salmoninarum* DNA contained within pRHLY20. Either

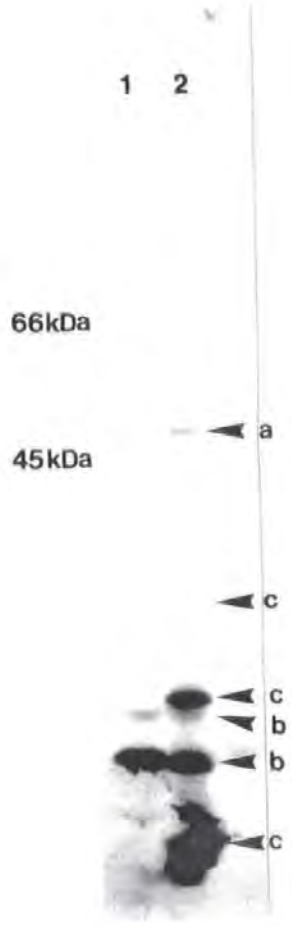


Figure 8.3 Autoradiograph of specifically labelled proteins obtained by the minicell analysis of the haemolysis-conferring plasmid pRHLY20. Showing the electrophoretic separation (10% gel) of ^{35}S-labelled proteins produced minicell analysis of the haemolytic *E. coli* DS410/pRHLY20 (lane 2) and the comparative control clone DS410/pGD103 (lane 1). Indicated (**a**) is the putative full sized RHLY protein, and possible breakdown products (**c**), along with a common pGD103 encoded protein (**b**). The molecular weights shown were estimated from the markers used in the original separation gel.

the labelled proteins represent the separate products of expression from a number of coding sequences that together form the molecular basis of haemolytic activity, or more likely, are breakdown products of the largest visible labelled protein, estimated to be approximately 48-50 kDa in size. It is interesting to note, that the labelled proteins encoded by the plasmid pRHLY20 were of roughly equivalent sizes to three of the antibody-bound protein bands visualised in immunoblots of proteins from the RHLY-containing clone *E. coli* DH1/pRHLY11 (see 8.2.2, above).

8.4 ATTEMPTS TO DEVELOPE A CELL-FREE ASSAY SYSTEM FOR THE CLONED HAEMOLYSIN.

Prior to any attempts to analyse the biochemical properties of the cloned haemolytic molecule, it was considered vital that a simple but effective cell-free assay system was developed. Such a system would not only be of use for the characterization of the haemolysin, but could be used at a later date to trace the molecule during any purification protocol. As it had been shown that the haemolysin could not be specifically and selectively detected using antibodies raised against *R.salmoninarum* proteins, it was decided to try and detect the molecule by virtue of its haemolytic activity.

8.4.1 MICROTITRE PLATE HAEMOLYTIC ASSAY.

Sonic extracts and ECP fractions of the *E. coli K12* clones DH1/pRHLY11 and DH1/pBR328 were prepared as described in 8.2.1 (above). The protein preparations were diluted in PBSA at 4°C to give a final concentration of 60μg of protein per 100μl of solution. Each of the separate protein preparations were then added to a separate well in the first column of a 96 well plastic microtitre plate. Each protein preparation was then subjected to doubling serial dilutions in 50μl of PBSA across the twelve wells in its respective row. To these wells, 50μl of a washed erythrocyte suspension (1% v/v in PBSA) was added and the contents of the well mixed. A control well consisting of 50μl of PBSA and 50μl of erythrocyte suspension was set up for each assay performed.

One assay was performed using horse erythrocytes and a second using rainbow trout erythrocytes. The assay plates were incubated at 20°C, and examined at regular intervals for signs of haemolytic activity until 48 hours had elapsed. No sign of haemolytic activity was observed in any of the wells.

The experiment was repeated using initial protein concentrations of 200μg, 100μg, 50μg, 25μg and 10μg, and incubation temperatures of 5°C, 10°C, 15°C and 30°C. However, no sign of haemolytic activity was observed in any of the wells.

8.5 INITIAL STUDY OF THE BIOCHEMICAL CHARACTERISTICS OF CLONED *R.salmoninarum* HAEMOLYSIN.

The difficulties encountered in detecting the RHLY molecule, prevented the purification of the protein for use in further characterization. In consultation with Dr. A.E Ellis of the S.O.A.F.D laboratory at Aberdeen, who has had many years experience in isolating and purifying macromolecules of bacterial origin, the merits of a possible 'blind' purification protocol were discussed. So called blind purification protocols would have involved the comparative chromatographic separation of the protein components of both *E. coli* DH1/RHLY11 and the control bacteria, with detection of the RHLY protein dependent on the absence of a similarly-sized molecule in preparations of the control. This method of purification could possibly have succeeded, however, the lengthy process of comparative purification was not thought to be possible when the time constraints on the research project were considered.

The failure of attempts to isolate and purify the RHLY protein, mean that a full characterization of its biochemical properties and mode of action was not possible. However, by using the *E. coli* clone DH1/pRHLY11 and the comparative negative control bacterium *E. coli* DH1/pBR328, it was possible to undertake a qualitative study of the properties of RHLY in substrate/agarose overlay experiments.

8.5.1 ACTIVITY OF RHLY-CONTAINING *E. coli* CLONES EXPOSED TO ERYTHROCYTES FROM DIFFERENT ANIMAL SPECIES.

It was already known that RHLY confers upon its host *E. coli* cell, the ability to lyse both trout and horse erythrocytes. Experiments were also undertaken to assess the spectrum of this haemolytic activity with respect to erythrocytes from other animal species.

Cultures of DH1/pRHLY11 and DH1/pBR328 were set up by streak plating on to LBA containing both ampicillin (50μg/ml) and chloramphenicol (25μg/ml) and incubated at 37°C until colonies (1-2 mm diam.) were visible (18-24 h.). After incubation, the colony-containing plates were overlayed with erythrocytes suspended in molten PBSA/agarose as described previously (Chapter 6) followed by a further 48 h. incubation at 25°C. The overlayed plates were examined at regular intervals for the presence of haemolytic activity.

Using this method, erythrocytes from horse, sheep, rabbit, chicken, guinea pig, rat, hamster, mouse and rainbow trout were tested as substrates for haemolytic activity by cultures of the RHLY-containing *E. coli* clone and the *E. coli* DH1/pBR328 negative control. The RHLY-containing *E. coli* clone was found to exhibit haemolytic activity against all of the erythrocyte types tested, except for those from rat. Haemolytic activity was seen to be absent in cultures of the negative control *E. coli.*

8.5.2 THE EFFECT OF TEMPERATURE ON THE HAEMOLYTIC ACTIVITY OF RHLY-CONTAINING *E. coli* CLONES.

The effect of temperature on the haemolytic activity of RHLY-containing clones was also studied. Cultures of DH1/pRHLY11 and DH1/pBR328 were overlayed with horse erythrocyte/agarose suspensions as previously described, the overlayed plates were then incubated for 72 h. or until haemolysis was observed. The incubation temperatures used in this experiment were 4, 10, 15, 20, 25, 30 and 37°C. Haemolytic activity was observed

in all overlayed cultures of DH1/pRHLY11. Haemolysis occurred most rapidly at 37°C, taking 15 h. to develop. As the incubation temperature decreased, the time taken for haemolytic activity to occur increased. RHLY-containing clones took 18 h. to lyse erythrocytes at 30°C, approximately 20 h. at both 20°C and 25°C, 36 h. at 15°C, 48 h. at 10°C and approximately 70 h. at 4°C.

8.5.3 THE EFFECT OF OXYGEN AVAILABILITY ON THE HAEMOLYTIC ACTIVITY OF RHLY-CONTAINING *E. coli* CLONES.

The *E. coli* clone DH1/pRHLY11 and the comparative negative control DH1/pBR328 overlayed with horse erythrocytes, were also incubated in oxygen-rich and anaerobic conditions, to assess any visible effect on the RHLY-mediated haemolytic activity caused by varying conditions of oxygen availability. Overlaid cultures of the RHLY-containing *E. coli* clone and comparative negative controls were incubated in sealed containers, either under anaerobic conditions facilitated by a CO_2-generating Gaspak system (BBL) or in an oxygen-rich environment created by the addition of oxygen from a storage cylinder. The plates were then incubated at 25°C. for 24 h. prior to examination of haemolytic activity.

Haemolytic activity from the RHLY-containing *E. coli,* was observed in the erythrocyte overlays of plates incubated in both aerobic and anaerobic conditions, and the zones produced were of an equivalent size. However, in the anaerobically incubated plates the erythrocyte overlays had darkened considerably, possibly due to the deoxygenation of haemoglobin.

8.5.4 THE EFFECT OF pH ON THE HAEMOLYTIC ACTIVITY OF RHLY-CONTAINING *E. coli* CLONES.

The effect of pH on the generation of haemolysis by RHLY-containing *E. coli* was also studied. Cultures of DH1/pRHLY11 and DH1/pBR328 were overlayed with horse erythrocytes suspended in an agarose solution as before, but the pH of the erythrocyte suspension was varied by dissolving the agarose in buffered saline solutions. Approximate

pH values of 4, 5, and 6 were provided by 0.1 M citrate buffer (Hudson and Hay, 1989) plus 0.85% w/v NaCl, whilst pH values of 7, 8 and 9 were provided by 0.05 M tris-HCl buffer.

Plates were overlayed in the normal way and incubated for 24 h. at 25°C, prior to examination. RHLY-mediated haemolysis was observed at all pH values, with resultant haemolysis zones being of equivalent size.

8.6 CHARACTERIZATION OF THE MODE OF ACTION OF THE CLONED HAEMOLYSIN.

The activity of RHLY against a wide range of erythrocyte types, is an indication that its function *in vivo* may be not that of a specific lytic agent for salmonid red blood cells or salmonid cells in general. It possibly reflects the actions of a membrane-active cytolytic molecule, able to disrupt the membranes of a wide range of eukaryotic cells. Cytolytic molecules vary in their mode of action, but a large number of those originating from bacterial pathogens, tend to damage membranes by the degradation of either the protein or lipid components (Stephens and Pietrowski, 1986).

Once again, due to time constraints, a full characterization of the mode of action of the cloned RHLY protein was not possible. However the RHLY-containing *E. coli* clone DH1/pRHLY11 and the vector only control were tested for proteolytic and lipolytic properties using substrate/agarose overlays and by culture on substrate-containining LB agar.

Substrate overlays were perfomed on 24 h. cultures of DH1/pRHLY11 and DH1/pBR328 using PBSA/agarose containing 10% v/v egg yolk suspension (LabM) for the detection of lipolytic/phospholipolytic activities, and PBSA/agarose containing 1% w/v casein for the detection of proteolytic activity. Overlayed plates were incubated for 24 h. at 25°C prior to examination. No indications of lipolytic or proteolytic activity were found.

The *E. coli* clones were also cultured on LB agar containing either 10% v/v egg yolk

suspension or 1% w/v casein, these plates were incubated at 25°C for 48 h. prior to examination. No indications of either proteolytic or lipolytic activity were observed.

8.7 DETECTION OF A PROTEIN ANTIGEN HOMOLOGOUS TO RHLY IN A WHOLE CELL PREPARATION OF *R.salmoninarum.*

As a specific assay for the RHLY protein was not available, and time constraints prevented further lengthy experimentation, the isolation and purification of the molecule was not attempted. Isolated and purified RHLY could have been used to raise antibodies that would be used in experiments to determine the presence of a similar molecule in *R.salmoninarum* preparations.

However, some time after the main bulk of the practical phase of this research project was terminated, an antiserum raised to a β-galactosidase/RHLY fusion protein became available. The possible high value in using a specific antiserum against RHLY to detect any similar proteins in *R.salmoninarum*, warranted one further experiment.

Using the nucleotide sequence data detailed in the next chapter of this thesis, a β-galactosidase/RHLY fusion protein was constructed by a co-worker in the laboratory, Dr T.H. Grayson and a rat antiserum raised against it.

The construction of the fusion protein, and the raising of rat antiserum is described in detail in the work of Grayson (1993), whose research project focused on the use of cloned *R.salmoninarum* proteins for BKD vaccine design.

Immunoblots performed by Dr. Grayson, using the antiserum raised against the β-galactosidase/RHLY fusion protein, indicated that protein antigens present in whole cell preparations (but not ECP) of *R.salmoninarum* isolates, are recognized.

In an attempt to further validate the application of gene cloning procedures for further studies of *R.salmoninarum*, it was decided to use the rat anti-β-galactosidase/RHLY fusion serum in an immunoblot of a *R.salmoninarum* whole cell protein preparation.

Approximately 20 μg of a sonicated preparation of *R.salmoninarum* MT444 whole cells

was subjected to SDS-PAGE in a 10% polyacrylamide gel under denaturing conditions. The electrophoresed proteins were then transferred to a nitrocellulose membrane where they were probed using rat anti-β-galactosidase/RHLY serum as the primary antibody (1:250) and peroxidase conjugated rabbit anti rat immunoglobulins (P450 Dako) as the secondary antibody (1:1000). Bound antibody was visualised by DAB/$NiCl_2$ colour development.

The developed immunoblot can be seen in fig 8.4. The most distinct antibody-bound protein was estimated to be approximately 49 kDa in size, whilst less distinct bands were seen to occur at approx. 80 kDa, 37 kDa and 29 kDa. Apart for the diffuse band at approx. 80 kDa, these proteins are of a similar size to proteins detected in RHLY-containing *E. coli* clones using anti-*R.salmoninarum* whole cell serum, and similar to specifically labelled proteins detected by minicell analysis of the RHLY encoding plasmid pRHLY11. The immunoblotting experiments of Grayson (1993), using the same antiserum raised to the β-galactosidase/RHLY fusion protein, revealed antibody binding to proteins of similar size in a number of whole cell preparations of different *R.salmoninarum* isolates.

8.8 DISCUSSION.

The experimental work described in this chapter, represents the attempts made to detect, isolate and characterize the molecular determinant of haemolytic activity encoded on the 1.65 kbp fragment of DNA cloned from *R.salmoninarum*. Although it was not possible due to the time constraints placed on this research project to isolate and purify the RHLY protein, the results obtained, do however answer some of the many questions regarding the cloned *R.salmoninarum* haemolysin, and provide impetus for further study.

The first experiments undertaken involved the analysis of protein preparations from the haemolytic *E. coli* clone DH1/pRHLY11 and the control *E. coli* DH1/pBR328 bacteria for the presence of protein molecules that represent the cloned haemolysin. SDS-PAGE

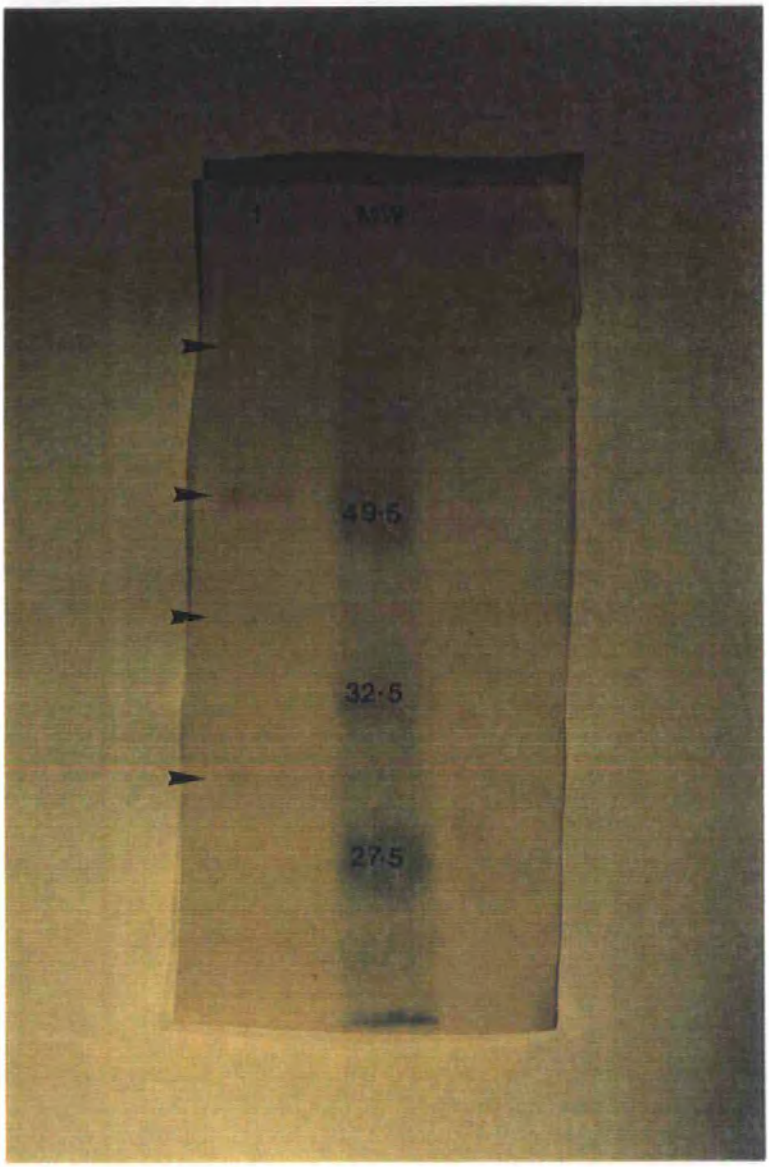


Figure 8.4 Immunoblot of *R.salmoninarum* MT444 whole cell protein preparation using antibodies raised to a β-galactosidase/RHLY fusion protein. Lane 1: *R.salmoninarum* MT444 whole cell proteins. **MW=** molecular weight markers (kDa). Arrows indicate antibody-bound proteins.

analysis was unsuccessful, as no extra protein bands were found in either whole cell or ECP preparations of the DH1/pRHLY11 clone. The RHLY protein was either present in such a small amount that it was not detectable with coomassie blue staining, or that its presence was masked by the large number of host cell proteins visible.

Immunoblot analysis of the protein preparations made from the above bacteria, was undertaken as it was thought it might be a more sensitive and selective method for the detection of the RHLY protein, utilising antibodies raised to *R.salmoninarum* to probe for proteins of renibacterial origin. Apart from binding to a number of proteins present in both preparations, antibodies from the anti-*R.salmoninarum* whole cell sera bound to 3 supplementary proteins of DH1/pRHLY11 of sizes in the region of 45-50 kDa, 35-40 kDa, and approximately 28 kDa. This was the first indication of the presence of putative RHLY protein, but as the cloned *R.salmoninarum* DNA fragment was only 1.65 kbp in length, it is unlikely that 3 separate protein molecules of these sizes could be encoded by pRHLY11. The maximum size protein that could be encoded by the cloned DNA is approximately 61 kDa, considerably smaller than the 100-110 kDa total size of the 3 proteins detected by immunoblotting. An explanation of the presence of 3 antibody-bound protein bands, is that they represented truncated products of the full size RHLY protein which was possibly represented by the largest antibody-bound protein of a size in the region of 45-50 kDa. The degradation of this protein may have occurred as a result of the activity of proteolytic enzymes within *E. coli*, or it may have reflected properties of the native *R.salmoninarum* protein. It is interesting to note that the putative RHLY bands were detected using antiserum raised to a whole cell preparation of an *R.salmoninarum* isolate, this may either have reflected inadequacies in the anti-*R.salmoninarum* ECP serum, or indicate that RHLY is cell-associated. Further experimentation needs to be carried out to attempt to determine why considerable antibody binding to *E. coli* proteins occurred in the immunoblots made utilising anti-*R.salmoninarum* sera. This may have been the result

of a high titre of circulating antibodies to *E. coli* components, in the rabbits used to raise the antisera, or an indication of possible antigenic homology. Future studies of this phenomenon, could involve the preabsorption of these antisera using an *E. coli* lysate (Harlow and Lane, 1988) or the use of specific anti-*E. coli* sera to probe preparations of *R.salmoninarum* for homologous antigens.

The next analysis performed on the pair of *E.coli* bacteria was a zymogram for haemolytic activity using electrophoretically separated protein preparations from the bacteria. Erythrocyte/agarose overlays of both SDS denaturing, and native PAGE, failed to detect haemolytic activity. This possibly reflected insufficient RHLY protein to facilitate haemolysis, or that the haemolytic activity was prevented by the processes utilised in the preparation of protein samples, or by some aspect of the electrophoretic separation technique.

Minicell analysis of the cloned *R.salmoninarum* DNA, was successful in establishing that the DNA does encode a protein molecule, and supported the findings of the previously prepared immunoblot. Four radiolabelled protein bands were found to be present in minicells containing the plasmid-borne *R.salmoninarum* DNA. Although no radiolabelled molecular weight standards were included for size determination, it was possible using markers included in the original electrophoresis gel, to determine that three of these protein bands were of sizes roughly equivalent to those detected in immunoblot analysis. Once again, it was concluded that the pRHLY11 encoded protein was of a size in the region of 48-50 kDa, and the other bands present represented truncated products of this molecule.

Due to the failure of the zymogram-mediated detection of RHLY, and the apparent non-discriminatory nature of the anti-*R.salmoninarum* sera, a simple specific assay for RHLY was sill not available for employment in any purification protocols.

A crude cell-free haemolytic assay method was attempted, using protein preparations

from the two *E. coli* bacteria combined with erythrocyte suspensions in microtitre plates. Despite varying the initial protein concentrations and the temperature of incubation, no haemolytic activity was detected. Failure of the assay could be attributed to many possible factors, and obviously further experimentation is needed before such an assay is disregarded. RHLY, may require defined conditions before haemolysis can occur, the variation of the assay conditions with regard to factors such as pH, oxygen availability, the presence of certain metallic ions, may prove successful. This assay may have failed because of the inherent nature of the haemolytic activity conferred by RHLY. The molecule may require chemical modification or activation and may rely on an as yet unidentified co-factor of bacterial origin. It is also possible that the putative cell-associated nature of RHLY, prevents its activity in cell free assay systems. The use of a concentrated preparation of the cell membranes of the pair of *E. coli* bacteria may resolve this.

Without isolation and purification of the RHLY protein, a comprehensive characterization of its biochemical properties and mode of action were not possible. However, using erythrocyte overlays of *E. coli* DH1/pRHLY11 and *E. coli* DH1/pBR328, a cursory qualitative study of these properties was undertaken.

The lysis of erythrocytes mediated by bacterial pathogens, rarely reflects the action of molecules specific to this cell type. Haemolysis is usually a reflection of the action of molecules, whose cytolytic activities are targeted to eukaryotic membranes in general. Erythrocytes represent a simple and efficient substrate for membrane-active cytolysins, in as much as damage to their membranes results in the readily detectable release of coloured cell contents (Bernheimer, 1988).

It had been established prior to this chapter, that RHLY was active against both trout and horse erythrocytes, but experiments in this chapter have shown that the cloned haemolytic component is active against at least nine mammalian, avian and piscine erythrocyte types. Therefore, this probably indicates an activity directed against cell membranes in general.

Future experimentation should include other membrane damage assay systems including the cytopathic effect observed in different cell lines, which may support the hypothesis that RHLY attacks membranes in general, and not solely erythrocytes.

The effect of temperature on RHLY-mediated haemolytic activity was also studied. Haemolysis was shown to occur at a wide range of temperatures from 4°C to 37°C. The observation that the time taken for visible haemolysis to occur increases, as the incubation temperature for overlaid plates decreases, either reflects temperature effects on an enzyme-mediated process, or on the growth rate of the *E. coli* host with the resultant influence on plasmid-borne gene expression.

A phemomenon noted to occur with all types of erythrocyte overlay of RHLY-containing *E. coli,* was the restriction of haemolytic activity to well separated bacterial colonies. This phenomenon could reflect the nature of RHLY with respect to its biochemical properties, or the inactivation of the molecule by increased amounts of *E. coli* components found in areas of dense bacterial growth. Two possible factors that may affect the activity of RHLY in areas of dense growth, are the accompanying changes in pH and oxygen availability. A brief study of the effects of both pH and oxygen availability however, failed to indicate either positive or negative effects on RHLY activity. High density growth of *E. coli,* may deplete the surrounding medium of vital nutrient molecules or metallic ions, or may result in the accumulation of *E. coli* products that suppress RHLY-mediated haemolytic activity.

Attempts at determining the mode of action of RHLY by investigating possible proteolytic and/or lipolytic activities were also inconclusive. Neither lipolytic/phospholipolytic activities against egg yolk, or proteolytic activity against casein were detected in RHLY-containing *E. coli.* It must be said that these experiments, maybe more than any others performed in this chapter, did in no way represent the full range of assays available. Future studies would be better served with a wider range of substrates for specific enzyme

activities, including substrates based on individual membrane components.

The final experiment described in this chapter, was performed some time after the practical phase of this research project had ended, but the possible high value of its results, warranted its undertaking.

The availability of a semi-specific antiserum to the cloned RHLY protein, meant that it was finally possible to attempt to confirm that a similar molecule occurs in preparations of *R.salmoninarum*. If this was proved, it would validate this entire research project in so much that gene cloning techniques applied to *R.salmoninarum* would be shown to produce a source of macromolecules originating from the pathogen for further characterization, and that these macromolecules need not be previously isolated from the bacterium.

Immunoblotting of *R.salmoninarum* MT444 whole cells was performed using the rat anti-β-galactosidase/RHLY serum, and a number of antibody-bound bands were detected. The most visible band corresponded to a protein of approximately 49.5 kDa, whilst other bands were present at 80 kDa, 37 kDa and 29 kDa. Three of these bands are equivalent to putative RHLY protein bands detected by immunoblot of RHLY-containing *E. coli* and minicell analysis of a plasmid containing the cloned RHLY encoding *R.salmoninarum* DNA fragment.

Immunoblots of *R.salmoninarum* isolates performed by Grayson (1993) using the same antiserum, revealed antibody-bound protein in whole cell preparations of the bacterium, but not in the ECP fraction. This would tend to support the findings in this chapter that antibodies in rabbit anti-*R.salmoninarum* whole cell serum can bind to the putative cloned RHLY molecule, whilst antibodies in antiserum raised to ECP of the organism do not. The major bands detected by the immunoblots of *R.salmoninarum* isolates performed by Grayson, were a protein antigen doublet at approximately 80 kDa. These bands are probably represented in the immunoblot performed in this chapter by a diffuse region of

antibody binding at an equivalent position. One possible explanation for an antigen homologous to RHLY at this position, is that it represents the RHLY bound strongly to another *R.salmoninarum* component.

Further experimentation is required to determine whether the antiserum raised to the β-galactosidase/RHLY fusion protein can be used in a selective and specific assay for RHLY in future studies of the protein.

This concludes the work performed on the attempted biochemical characterization of the cloned RHLY molecule. Although little has been determined regarding its structure and function, it has been shown that an equivalent protein occurs in *R.salmoninarum* and therefore the validity of the cloning proceedures used in this research project has been established.

Further characterization of the structure function aspects of the cloned RHLY protein is continued in the following, final experimental chapter of this thesis, in which the nucleotide sequence of the *rsh* gene, the gene encoding RHLY, and its derived amino acid sequence, are determined and analyzed.

CHAPTER 9. DETERMINATION AND ANALYSIS OF THE NUCLEOTIDE SEQUENCE OF A CLONED *R.salmoninarum* DNA FRAGMENT ENCODING THE RHLY PROTEIN.

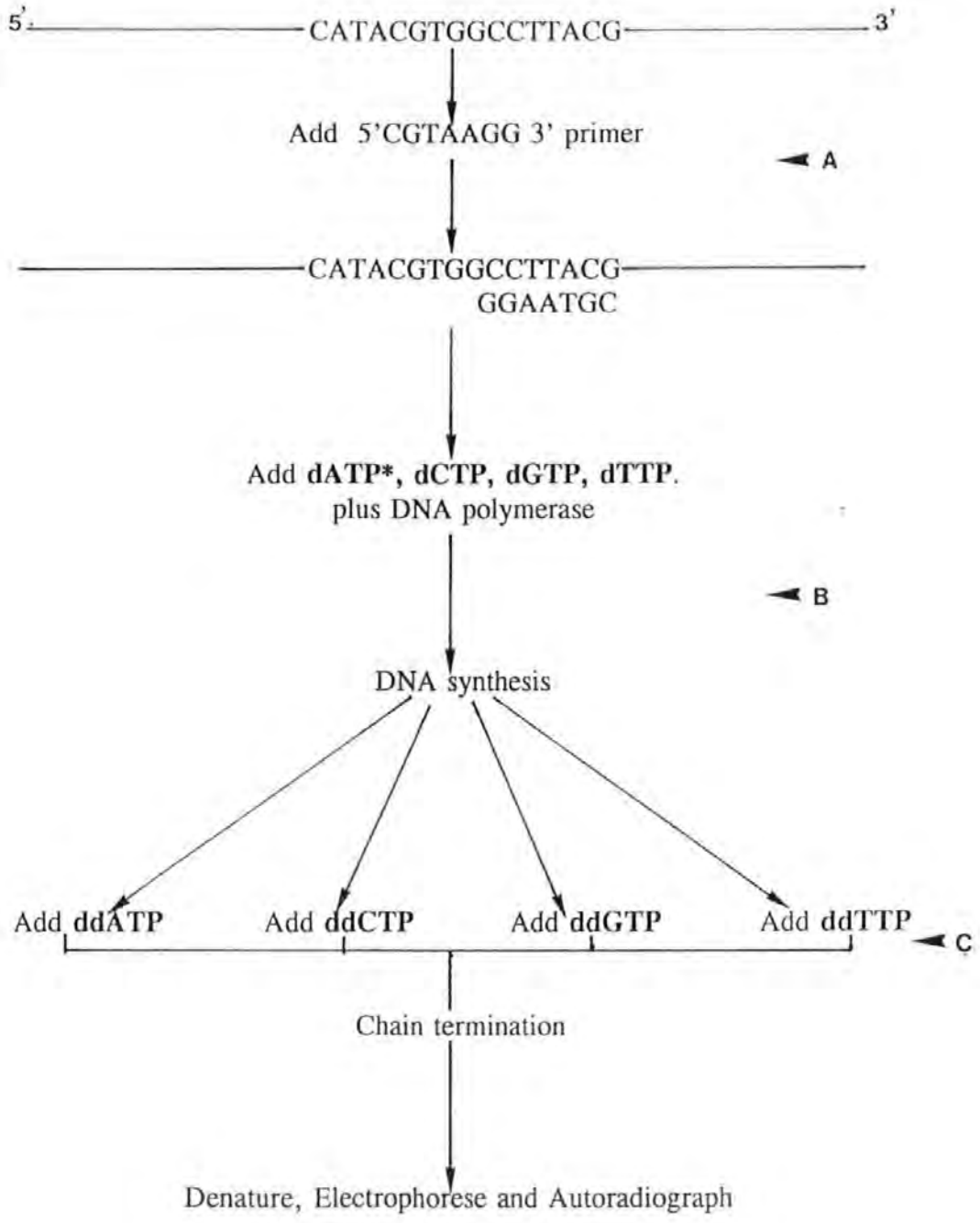
9.1 INTRODUCTION.

By determining the nucleotide sequence of a cloned DNA fragment, it is possible to accumulate a wealth of knowledge about its structure, function and even its evolutionary history. Rapid accurate methods for the determination of DNA sequences were developed shortly after recombinant DNA techniques were introduced, and in principal, there is now no limit to the length of DNA that can be sequenced.

Two main classes of DNA sequencing techniques exist, and are best represented by the enzymic, chain-termination method of Sanger *et al* (1977) and the chemical method of Maxam and Gilbert (1977). The latter has somewhat been superceded by the former, therefore it is this technique that was used in this study and is described below.

The chain-termination method of DNA sequencing (Fig. 9.1), involves the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single-stranded DNA template. Synthesis is initiated at only one site where a complementary oligonucleotide primer anneals to the template. The synthesis reaction is terminated by the incorporation of a nucleotide analog that will not support continued DNA elongation (hence the name chain-termination). The chain-terminating analogs are the 2',3'-dideoxynucleoside 5' triphosphates (ddNTPs). These lack the 3'hydroxyl group necessary for DNA chain elongation. When correct mixtures of deoxynucleoside triphosphates (dNTPs) and one of the four ddNTPs are used, enzyme-catalyzed synthesis will be terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated. Four separate reactions, each with a different ddNTP give complete sequence information. A radioactively labelled nucleotide is also included in the synthesis, so the labelled chains of various length can be visualized by autoradiography

Figure 9.1 DNA sequencing by the chain termination method (Sanger *et al.*, 1977). The diagram indicates the main stages of the method: A. A synthetic oligonucleotide primer is annealed adjacent to the sequence of interest, B. A mixture of deoxynucleotides including radiolabelled dATP is added to the annealed primer/template and DNA synthesis initiated by the addition of DNA polymerase. The DNA synthesis reaction mixture is split into four separate tubes and a different chain terminating dideoxynucleotide added to each, C. The newly-synthesized polynucleotides are then separated by electrophoresis and an autoradiograph prepared D.


5'
CATACGTGGCCTTACG
3'
Add 5'CGTAAGG 3' primer
A
CATACGTGGCCTTACG
GGAATGC
Add dATP*, dCTP, dGTP, dTTP.
plus DNA polymerase
B
DNA synthesis
Add ddATP
Add ddCTP
Add ddGTP
Add ddTTP
C
Chain termination
Denature, Electrophorese and Autoradiograph
G A T C
D

after separation by high resolution denaturing polyacrylamide-gel electrophoresis.

For a DNA fragment to be sequenced, it has first to be attached adjacent to a characterized 'priming' sequence. It is to this sequence that the complementary oligonucleotide primer binds to the template DNA strand so as to initiate the directed synthesis of DNA . The initial priming sequence is found in a specialized cloning vector, so once a DNA fragment of interest has been inserted into such a vector, it is possible to make bulk preparations of sequencing template for regular use.

The DNA fragment to be sequenced is often larger than the insert capacity of the sequencing vector,and also, larger than the relatively small fragments yielding the most efficient, reliable and accurate sequence information (100-800 bp.). It is therefore, often desirable to fragment the DNA, and ligate readily identifiable fragments to vector DNA. This is most readily achieved by using a sequencing vector containing a number of restriction endonuclease cleavage sites and ligating to it, fragments of the DNA of interest generated by one or a combination of these enzymes.

The most popular DNA sequencing vectors to date are those derived from the filamentous bacteriophage M13 (Messing and Vieira, 1982; Sambrook *et al.* 1990.), these vectors not only contain a multiple cloning region formed by a number of unique restriction endonuclease cleavage sites, but also, (with those vectors posessing the replication origin from M13 phage) can rapidly and easily produce single stranded DNA for use in sequencing reactions.

However, over the past few years, the requirement for single-strand DNA producing sequencing vectors has been overcome by the development of techniques that utilize double-stranded template (Chen and Seeburg, 1985). Prior to performing the sequencing reactions, double stranded template, usually in the form of a plasmid, is denatured by alkaline treatment to produce single stranded DNA. The ability to prepare sequencing templates in this manner, has enabled a wider range of sequencing vectors to be used,

including the popular pUC and pBR series of plasmids (Messing, 1987).

Once a template has been constructed, produced in bulk and purified, it can be used in the sequencing reactions. The first step in these reactions is to anneal the synthetic oligonucleotide primer that directs DNA synthesis along the segment of DNA of interest. The rapid development of improved sequencing protocols has resulted in a large variety of commercially available primers. Primers exist not only for a wide range of sequencing vectors, but also for the priming of DNA synthesis in both directions, i.e. once a DNA fragment has been inserted in to a vector, it is possible to sequence from both ends. This not only makes sequencing a DNA fragment quicker, but allows for larger fragments to be used.

Once primer-annealing has been performed, the two-stage DNA synthesis reaction can take place. In the first step, the primer is extended using limiting concentrations of the dNTPs, including radioactively labelled dATP. This step continues to complete incorporation of labelled nucleotide into DNA chains distributed randomly in length from several nucleotides to hundreds of nucleotides. In the second step, the concentration of all the dNTPs is increased and a ddNTP is added. Processive DNA synthesis occurs until all growing chains are terminated by a ddNTP.

The next stage of the DNA sequencing protocol involves the halting of the sequencing reactions with EDTA and formamide, the denaturing of the newly synthesized DNA by heating, and the running of the reaction products on polyacrylamide gels. After electrophoresis, an autoradiograph is produced by placing the gel in contact with x-ray film. After development, sequence information can then be read from the autoradiograph.

Once the nucleotide base sequence from the DNA fragment of interest has been compiled, there is a wealth of information that can be gathered. The processing of sequence information by eye is a major task and significant structural and functional features may evade detection. Fortunately, highly developed computer analysis methods are readily

available and programs in several standard computer languages are available for routine use.These programs can be run on both large computers as well as mini- and microcomputers, some have much greater capabilities than others, but broadly speaking they have the same function, to analyse both the structural and biological features of DNA sequences. The use of these computer packages enables the investigator to gain valuable information from a cloned DNA fragment. The base sequence can be analyzed for structural and physical features such as restriction endonuclease cleavage sites, base composition (including base pairing ratios) and repeating sequence motifs, but of more importance to the worker interested in molecular cloning, the biological significance of a segment of recombinant DNA can be investigated. Invariably, it is this data that interests a person who has by gene cloning been able to isolate a portion of DNA coding for the expression of a macromolecule that requires further characterization. By computer analysis the machinery of gene expression can be detected. Not only can the base sequence be analysed for the presence of an open reading frame, that once transcribed and translated would lead to the production of a complete polypeptide, but also accessory features such as ribosome binding sites, promoter and terminator regions may be detected.

It is also possible by computer analysis, to study the structure and composition of the protein molecule encoded by the nucleic acid sequence of interest. Once translated into an amino acid sequence regions of potential secondary structure such as disulphide bridges and alpha helices can be detected as well as functional properties such as hydrophobicity, antigenicity and enzyme active sites.

Another extremely useful analysis that can routinely be performed with both DNA and translated amino acid sequences using computer facilities is the comparison of a newly derived sequence with other sequences deposited in large databases. Many databases have been constructed containing thousands of nucleic acid and protein sequences from an equally large number of animal and plant species. It is now possible using powerful

computing systems to compare all or part of ones own sequence with those sequences lodged in the database. The ability to find and retrieve similar sequences has greatly enhanced our ability to characterize nucleic acid and protein molecules both structurally and functionally.

This chapter describes both the determination of the nucleotide sequence of the cloned haemolysis-conferring *R. salmoninarum* DNA fragment contained within the recombinant plasmid pRHLY11, and the subsequent analysis of this sequence using computer methods.

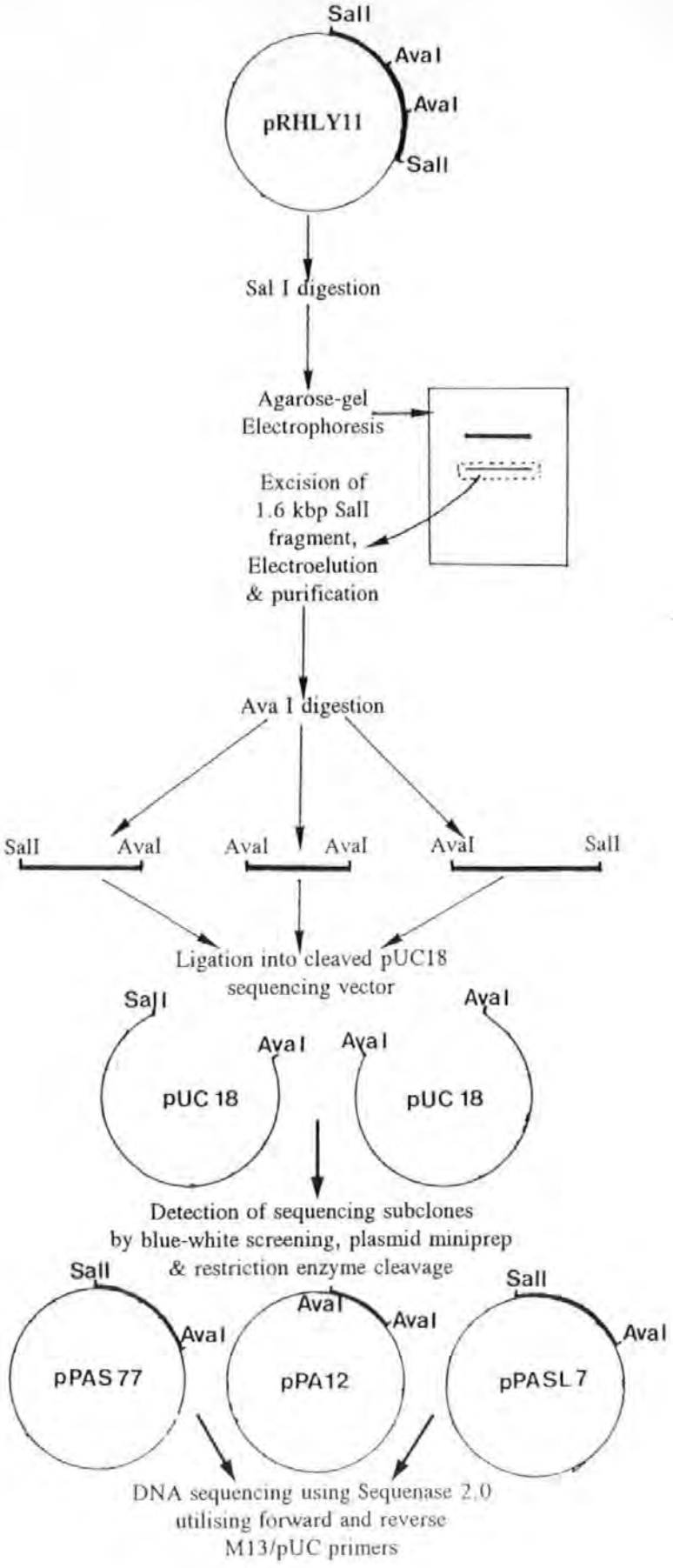
9.2 SEQUENCING OF THE 1.6 kbp. *Sal I* INSERT OF THE RECOMBINANT PLASMID pRHLY11.

The sequencing strategy used in this chapter is outlined in Fig.9.2. Briefly, sequencing templates were constructed in the plasmid vector pUC18 by subcloning DNA fragments generated by *Ava I* and *Sal I* restriction endonuclease cleavage of the 1.65 kbp. *Sal I* insert contained in the recombinant plasmid pRHLY11. These templates were then used in sequencing reactions using both forward and reverse M13/pUC sequencing primers in conjunction with a commercially available DNA sequencing kit (Sequenase version 2, U.S.B Biochemicals, Cleveland, Ohio, U.S.A.). The subsequent polyacrylamide-gel electrophoresis was performed using an LKB 2010 Macrophor Sequencing System (Pharmacia/LKB Biotechnology, Milton Keynes, U.K.).

9.2.1 CONSTRUCTION OF SEQUENCING TEMPLATES IN THE PLASMID VECTOR pUC18.

At a size of approximately 1.65 kbp, the *R. salmoninarum* DNA fragment contained within the recombinant plasmid pRHLY11 was considered to be too large for sequencing in one piece. It was therefore decided to subdivide the fragment and construct a number of subclones for sequencing. The sequencing vector chosen to accept these fragments was pUC18, a vector which contains a large number of unique restriction endonuclease cleavage sites in a 'multiple cloning region'(Fig.5.6,p.101). By consulting the physical

Figure 9.2 Strategy adopted for the DNA sequencing of the 1.6kbp *Sal I*-generated *R.salmoninarum* fragment in the recombinant plasmid pRHLY11.


SalI
AvaI
AvaI
pRHLY11
SalI
Sal I digestion
Agarose-gel
Electrophoresis
Excision of
1.6 kbp SalI
fragment,
Electroelution
& purification
Ava I digestion
SalI
AvaI
AvaI
AvaI
AvaI
SalI
Ligation into cleaved pUC18
sequencing vector
SalI
AvaI
AvaI
AvaI
pUC 18
pUC 18
Detection of sequencing subclones
by blue-white screening, plasmid miniprep
& restriction enzyme cleavage
SalI
AvaI
pPAS 77
AvaI
AvaI
pPA12
SalI
AvaI
pPASL 7
DNA sequencing using Sequenase 2.0
utilising forward and reverse
M13/pUC primers

map of restriction endonuclease cleavage sites on pRHLY11 (Fig.7.14,p.162), it was decided to liberate the *R. salmoninarum* DNA insert using the endonuclease *Sal I*, and to further subdivide the insert using the endonuclease *Ava I*. This cleavage would result in three restriction fragments of roughly equal size for subsequent subcloning procedures.

9.2.1.1 SUBDIVISION OF CLONED *R. salmoninarum* DNA FOR USE AS SEQUENCING TEMPLATES.

Approxiamately 10μg of pRHLY11 DNA was subjected to *Sal I* digestion overnight at 37°C, after which the cleavage products were seperated agarose gel electrophoresis (0.5% gel) and compared with molecular size markers. A band of approx. 1.6kbp, corresponding to the liberated *R. salmoninarum* insert DNA was excised from the gel and this fragment recovered by electroelution. After purification by phenol extraction and ethanol precipitation, this fragment was subsequently cleaved with *Ava I* overnight at 37°C. The products of this digest were subjected to agarose gel electrophoresis (1% gel) in conjunction with molecular size markers. Three DNA bands with sizes of approximately 0.65, 0.6 and 0.45kbp were excised from the gel, electroeluted and purified.

9.2.1.2 GENERATION OF SEQUENCING SUBCLONES IN pUC18.

Two 0.5μg. amounts of pUC18 were subjected to endonuclease cleavage in the following manner. Both portions were cleaved overnight at 37°C., one using *Sal I* and *Ava I* simultaneously to facilitate the insertion separately of the 0.65 and 0.6kbp. *Ava I/Sal I, R. salmoninarum* DNA fragments, and the other *Ava I* only to facilitate the insertion of the 0.45kbp., *Ava I* generated fragment.

To these cleaved vectors, the three fragments of *R. salmoninarum* DNA were added and ligated at a target to vector ratio of 3:1.

The ligated DNA was used to transform the *E. coli* K12 strain XL1-blue and the transformation mixture plated on to LB agar plates containing both ampicillin and tetracycline for the selection of pUC18-containing colonies as well as X-gal and IPTG so

as to allow blue/white screening. After 24h. incubation at 37°C, white colonies were picked, and cultured overnight at 37°C. as 10 ml. cultures in LB broth containing ampicillin and tetracycline. The plasmids contained in these recombinants were isolated by the miniprep procedure, and the presence of a single DNA insert of the desired size confirmed by cleavage with either *Ava I* or both *Ava I* and *Sal I* as appropriate.

Three suitable sequencing subclones were constructed using this proceedure :

1. Plasmid pPASL7: pUC18 containing a 0.65kbp. approx. *Ava I/ Sal I* fragment of *R. salmoninarum* DNA.

2. Plasmid pPAS77: pUC18 containing a 0.6kbp. approx. *Ava I /Sal I* fragment of *R. salmoninarum* DNA.

3. Plasmid pPA12: pUC18 containing a 0.45kbp. approx *Ava I* fragment of *R. salmoninarum* DNA.

These plasmids were isolated in bulk by maxiprep proceedure and purified for use in sequencing reactions by CsCl/EtBr density gradient ultracentrifugation.

9.2.2 DNA SEQUENCING USING THE SEQUENASE 2.0 KIT.

Since the introduction of the chain-termination method of DNA sequencing, rapid improvement of the protocol has occurred. This improvement, in part, has been due to the development of commercially available sequencing kits. One of the most popular DNA sequencing kits, is the Sequenase 2.0 kit supplied by United States Biochemicals. This kit utilises the enzyme Sequenase, a genetically engineered form of bacteriophage T7 DNA polymerase. The kit is supplied with all the components used in chain-termination sequencing reactions apart from radiolabelled dATP and specialised sequencing primers.

The standard protocol for performing sequencing reactions using the Sequenase 2.0 kit is outlined below.

9.2.2.1 PREPARATION OF DOUBLE-STANDED PLASMID TEMPLATE FOR DNA SEQUENCING.

Prior to their use in DNA sequencing reactions each plasmid template was alkali denatured to produce single stranded DNA. For each separate sequencing reaction template was prepared in the following manner.

Approximately 4μg. of plasmid DNA was dissolved in a total of 18μl. of Analar water, to which 2μl of 2M NaOH/2mM EDTA was added. After mixing, the solution was placed at 37°C. for 30 min to ensure complete denaturation. Denatured DNA was precipitated by the addition of 2μl of 3M sodium actetate and 75μl. of ice-cold ethanol followed by 15 min. at -70°C. The DNA was pelleted by centrifugation at 13,000 xg for 10 min, dried for 30 min. at 37°C and finally dissolved in 7 μl Analar water.

9.2.2.2 ANNEALING OF SEQUENCING PRIMERS.

For each set of four sequencing lanes, a single annealing reaction was used. The following were combined in a 0.5 ml. microcentrifuge tube :

pUC sequencing primer (0.5 pmol/μl)	1μl
reaction buffer	2μl
template DNA	7μl

The tube was capped and warmed to 65°C in a heating block. The block was then switched off to allow the tube to cool slowly over a period of 30 min. Once the temperature was below 35°C, annealing was taken to be complete. The tube was then placed on ice prior to its use in the labelling reaction.

9.2.2.3 DNA LABELLING REACTION.

For the standard labelling proceedure, both the dGTP labelling mix(7.5μM dGTP; 7.5μM dCTP; 7.5μM dTTP) and the SEQUENASE enzyme need to be diluted. The labelling mix was diluted 5 fold in Analar water, whilst the Sequenase 2.0 was diluted 1:8 in ice-cold enzyme dilution buffer (10mM TrisHCl pH 7.5; 5mM DTT; 0.5mg/ml BSA). The labelling mixture was as follows:

template-primer	10μl
DTT 0.1M	1μl
diluted labelling mix	2μl
[α-^{35}S] dATP (5μCi,1000-1500Ci/mmol, ICN Flow)	0.5μl
diluted Sequenase 2.0	2μl

After thorough mixing, the mixture was incubated for 3 min. at 20°C.

9.2.2.4 TERMINATION REACTIONS.

The chain termination proceedure, was performed immediately after labelling, using the termination mixes supplied with the Sequenase 2.0 kit. The composition of each mix was as follows:

ddG Termination mix = 80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddGTP, 50mM NaCl.

ddA Termination mix = 80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddATP, 50mM NaCl.

ddT Termination mix =80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddTTP, 50mM NaCl.

ddC Termination mix =80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddCTP, 50mM NaCl.

Four 0.5 ml centrifuge tubes were labelled G, A, T and C. Into each tube was dispensed 2.5μl of the corresponding termination mixture, the tubes were placed at 37°C for 2 min. prior to the end of the DNA labelling reaction.

When the labelling reaction was complete, 3.5μl of the reaction mixture was removed and transferred to each termination mixture. Each tube was incubated at 37°C for a further 3 min.

The termination reactions were halted by the addition of 4μl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF.), and after

thorough mixing, were stored at -20°C prior to polyacrylamide gel electrophoresis.

9.2.3 DENATURING POLYACRYLAMIDE-GEL ELECTROPHORESIS.

The quality of the electrophoretic separation of the oligonucleotides produced by the chain-termination sequencing reactions is often the factor which limits the extent of sequence information that can be determined in a single sequencing experiment. Dedicated sequencing gel systems have been developed, one such system being the LKB 2010 Macrophor sequencing system. Using this system polyacrylamide gels ranging from 0.1 to 0.4mm thick and up to 53 cm in length can be cast easily and quickly. Large numbers of samples can be electrophoresed by using either 26 or 36 well combs and up to 250 bases can be read from each track. Separation times using this system can be extremely fast,(below 2 hours). The handling of these thin gels is made easier because they are bound to one of the gel plates so as to allow quick and easy washing and drying prior to autoradiography. A thermostatic backing plate, through which water at a constant temperature is passed, prevents gel 'smiling', thus minimizing ambiguous sequence information.

9.2.3.1 PREPARATION OF POLYACRYLAMIDE GELS FOR USE IN LKB MACROPHOR DNA SEQUENCING SYSTEM.

Prior to gel casting, both of the glass plates were washed thoroughly in warm water and detergent, after rinsing and drying the plates were wiped with 70% ethanol to ensure a grease-free surface. For ease of gel handling, the gel is bound to the notched glass support plate, this is facilitated by treating this plate with γ-methacryloxypropyltrimethoxysilane (Sigma) (0.0025% in dH_2O pH 3.5) prior to gel casting. At the same time the gel is repelled from the thermostatic backing plate by treating this plate with the siliconising reagent Repelcote (B.D.H. Poole, U.K.).

Once treated, and dried, excess binding and repelling reagents were removed by wiping the glass plates with a lint-free tissue soaked in 70% ethanol.

Sequencing gels were cast according to the manufacturers instructions, at a thickness of

0.2mm. Gels of either 4% or 6% Acrylamide were cast, using the recipes shown in Appendix I of this thesis. Once polymerized (after approx. 5 mins), the gel sandwich was placed in the Macrophor apparatus, the buffer reservoirs were filled with TBE buffer (0.1M Tris., 0.083M Boric acid, 1mM EDTA), and the thermostatic plate connected to the water circulation system. Water at a constant temperature of 50°C was circulated through the plate and the gel was pre-electrophoresed for 30 min. at 1500 volts prior to sample loading.

9.2.3.2 ELECTROPHORESIS OF SEQUENCING REACTION OLIGONUCLEOTIDES.

Prior to electrophoresis, the terminated sequencing reactions were denatured by heating at 75°C for 4 mins. Immediately after denaturation, 2.5μl of each reaction mix was loaded on to the electrophoresis gel in a set of 4 lanes using the order G A T C reading left to right.

The first stage of electrophoresis consisted of a 5 min. period at 1500 V, after which the sample loading comb was removed and the electrophoretic run continued at 2500 V for the desired time period.

After electrophoresis, the gel sandwich was disassembled and the thermostatic backing plate removed. The gel, now attached to the notched glass plate, was immersed in 15% methanol. 10% acetic acid solution for 10 min. to facilitate the removal of urea from the gel. The gel was then carefully dried to the glass plate for 10 mins using a hairdryer.

9.2.3.3 AUTORADIOGRAPHY OF SEQUENCING GELS.

An autoradiograph of the sequencing gel was produced in the following manner. In a photographic darkroom, a 50 cm. length of X-OMAT AR autoradiography film (Eastman-Kodak, Rochester Co., N.Y., U.S.A.) was cut from a roll and laid on top of the dried sequencing gel. A clean glass plate, identical to that bearing the gel, was laid on top of the film and the gel/film sandwich held together with bulldog clips. This sandwich was

then placed in the Macrophor exposure box and the lid secured.

After 40 h. exposure, the sandwich was disassembled and the film developed in GBX developing chemicals (Eastman-Kodak Co., Rochester, N.Y., U.S.A.), according to manafacturers instructions.

9.2.3.4 SEQUENCING OF THE DNA INSERTS WITHIN THE PLASMIDS pASL7, pPAS77 AND pPA12.

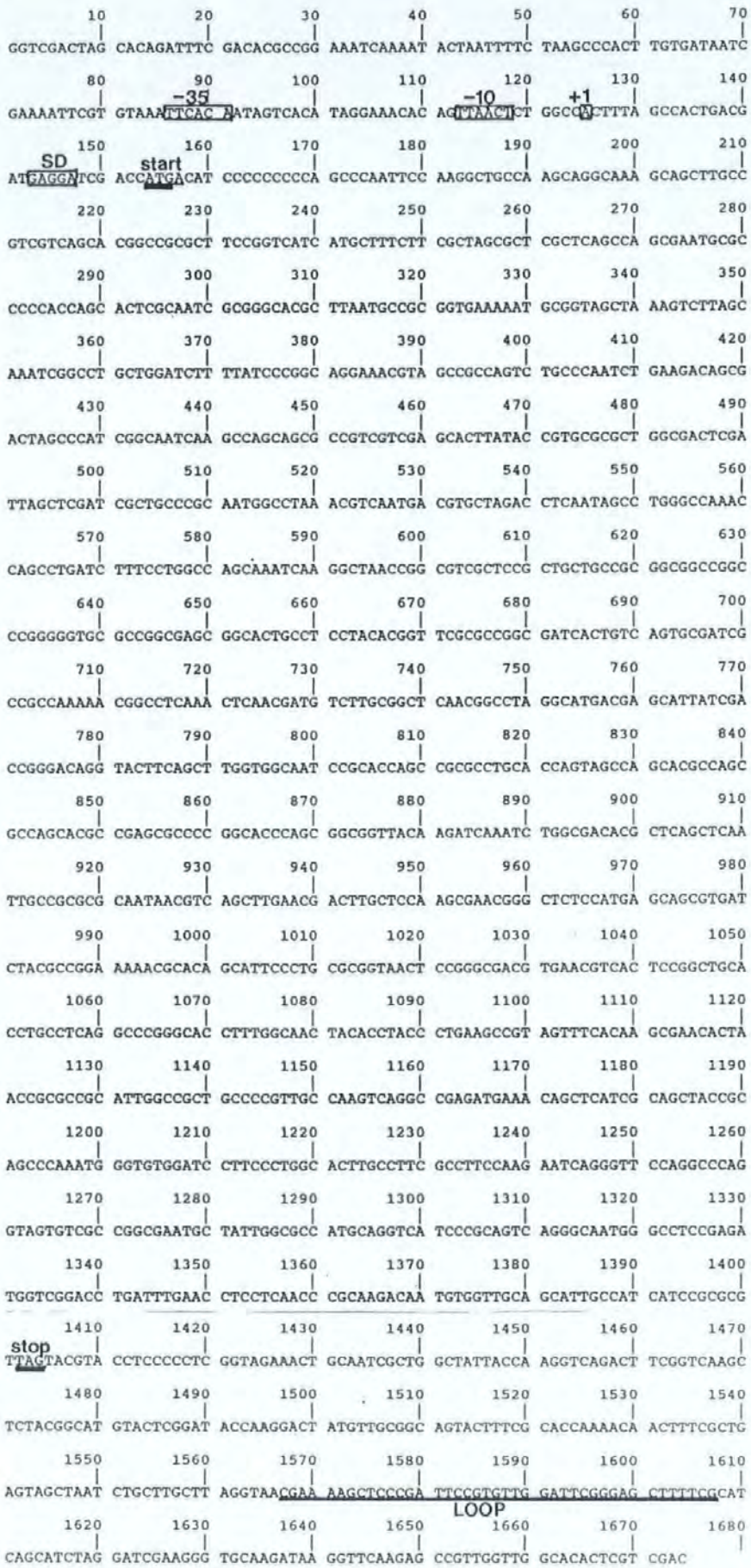
Full sequence data for the three sequencing subclones, pASL7, pAS77 and pA12 was obtained by varying both sequencing gel concentration and the time of each electrophoretic run. Each gel contained the oligonucleotide products of sequencing reactions performed using both forward and reverse sequencing primers. Four gels were run for each plasmid. 2 gels of 6% concentration, run for 1.5 and 3h. and 2 gels of 4% concentration run for 2 and 4h.

Using this procedure, it was possible resolve up to approximately 750 nucleotide bases per template, allowing at least a 50 base overlap from synthesis primed by both forward and reverse oligonucleotides.

9.2.4 COMPILATION OF THE COMPLETE NUCLEOTIDE SEQUENCE OF THE CLONED *R.salmoninarum* DNA FRAGMENT.

The complete nucleotide sequence of the cloned *R. salmoninarum* DNA fragment was compiled by comparing and joining at regions of overlap, the base sequences obtained from the three sequencing subclones. Using the restriction endonuclease cleavage map shown in Fig. 7.14 (p.162) it was possible to place fragments in the correct order and orientation occurring in the *Sal I* insert contained within pRHLY11. The complete nucleotide sequence was found to be 1674 nucleotides in length and is shown Fig. 9.3.

Figure 9.3 The complete nucleotide sequence of a 1.674 kbp *Sal I*-generated *R.salmoninarum* DNA fragment contained within the recombinant plasmid pRHLY11. The putative open reading frame coding for the haemolytic RHLY protein commences at the ATG initiation codon at nucleotide 154 (start) and finishes at the TAG termination codon at nucleotide 1402 (stop). Also shown are the putative -10 and -35 sequences, the transcriptional start nucleotide (+1), the putative Shine-Delgarno sequence (SD) and a possible transcriptional termination loop (LOOP).


-35
-10
+1
SD
start
stop
LOOP

9.3 NUCLEOTIDE SEQUENCE ANALYSIS.

Once compiled, the nucleotide sequence of the cloned *R. salmoninarum* DNA was entered as a data file into a personal computer previously installed with the Recombinant Toolkit version 4.0 sequence analysis software by Softshu (Biosoft, Cambridge, U.K.). This software consists of an easy to use, cursor controlled, menu-driven suite of programs for the analysis of nucleotide and amino acid sequences.

9.3.1 RESTRICTION ENDONUCLEASE CLEAVAGE SITE ANALYSIS.

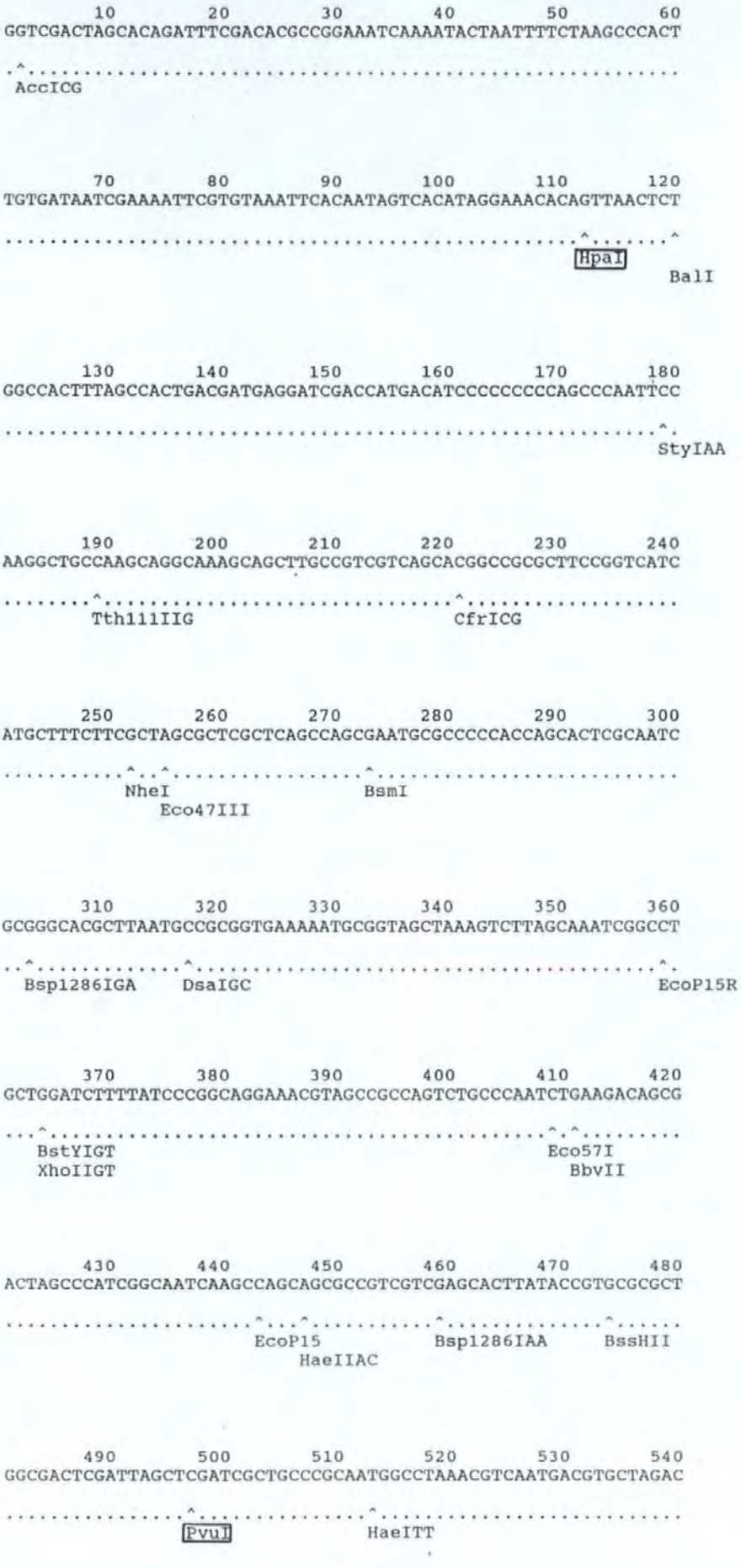
The first analysis to be performed on the nucleotide sequence was the production of a more comprehensive restriction endonuclease cleavage map. By determining which commercially available restriction enzymes could be used to cleave the cloned DNA fragment, any further manipulations may be more readily performed. The restriction program within Recombinant Toolkit, contains recognition site data for over 200 enzymes, and up to 32 kbp of sequence can be processed. The *R. salmoninarum* DNA sequence was analysed using this program and the results displayed on a printed output. The name near site restriction endonuclease cleavage map is shown in Fig 9.4.

9.3.2 OPEN READING FRAME ANALYSIS.

The next part of the analysis performed on the nucleotide sequence determined for the cloned *R.salmoninarum* DNA fragment was a computer-aided search for an open reading frame of nucleotide bases, that once transcribed into mRNA, would act as a template for the synthesis of the RHLY protein.

Open reading frame (ORF) analysis performed by the Recombinant Toolkit software, consists of a search of the nucleotide sequence of interest, in the six possible reading frames of base triplets, for the translation initiation sequence ATG followed by a search 'in frame' for the presence of one the three possible termination sequences TAA, TAG and TGA.

Figure 9.4. Computer-prepared restriction endonuclease cleavage map for the 1.674 kbp *Sal I*-generated *R.salmoninarum* DNA fragment contained in the recombinant plasmid pRHLY11. The map shows cleavage sites for restriction endonucleases having hexanucleotide recognition sites. Enzymes used for the construction of the estimated restriction map (Fig. 7.14, p.162) are boxed.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AccICG
70 80 90 100 110 120
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HpaI
BalI
130 140 150 160 170 180
GGCCACTTTAGCCACTGACGATGAGGATCGACCATGACATCCCCCCCCCAGCCCAATTCC
StyIAA
190 200 210 220 230 240
AAGGCTGCCAAGCAGGCAAAGCAGCTTGCCGTCGTCAGCACGGCCGCGCTTCCGGTCATC
Tth111IIG
CfrICG
250 260 270 280 290 300
ATGCTTTCTTCGCTAGCGCTCGCTCAGCCAGCGAATGCGCCCCCACCAGCACTCGCAATC
NheI
Eco47III
BsmI
310 320 330 340 350 360
GCGGGCACGCTTAATGCCGCGGTGAAAAATGCGGTAGCTAAAGTCTTAGCAAATCGGCCT
Bsp1286IGA
DsaIGC
EcoP15R
370 380 390 400 410 420
GCTGGATCTTTTATCCCGGCAGGAAACGTAGCCGCCAGTCTGCCCAATCTGAAGACAGCG
BstYIGT
XhoIIGT
Eco57I
BbvII
430 440 450 460 470 480
ACTAGCCCATCGGCAATCAAGCCAGCAGCGCCGTCGTCGAGCACTTATACCGTGCGCGCT
EcoP15
HaeIIAC
Bsp1286IAA
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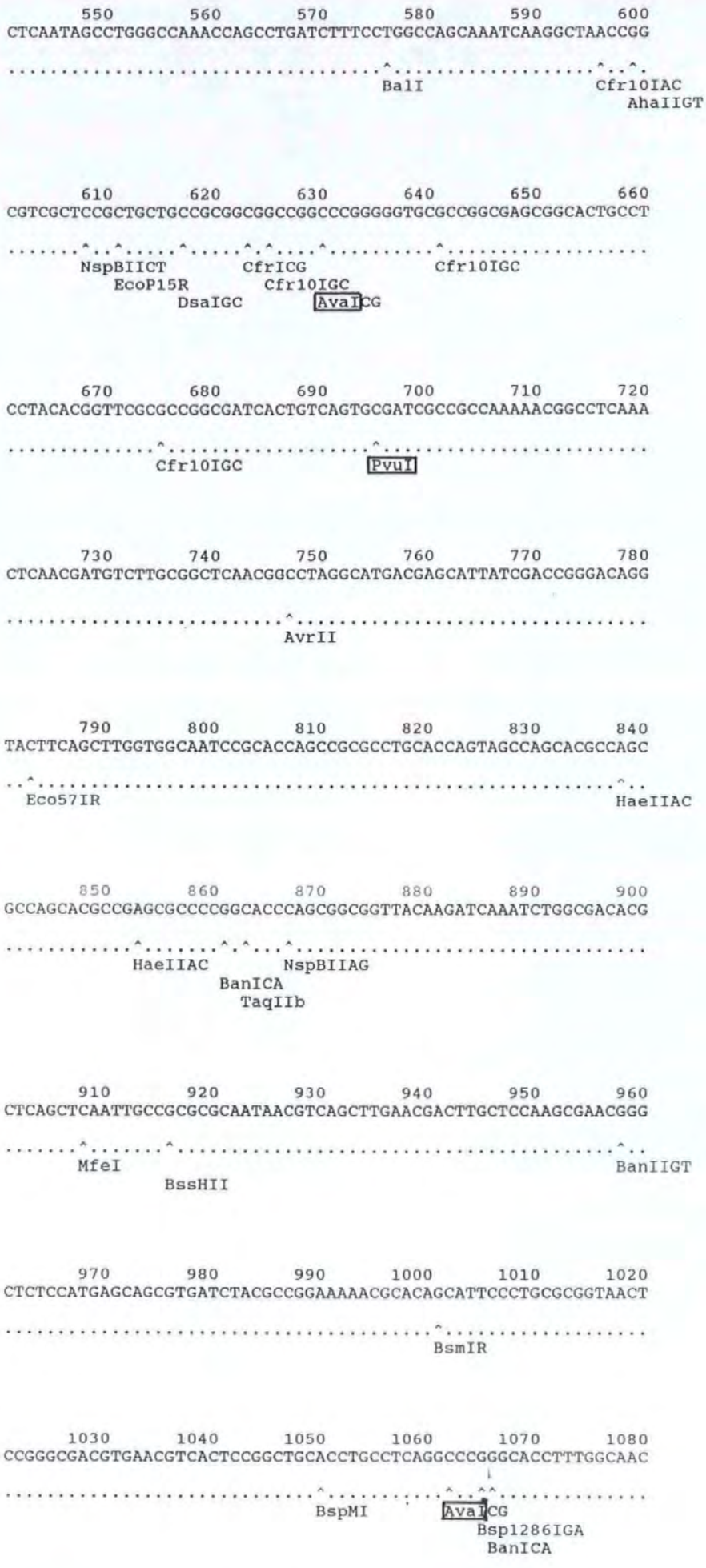
PvuI
HaeITT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BalI
Cfr10IAC
AhaIIGT
610 620 630 640 650 660
CGTCGCTCCGCTGCTGCCGCGGCGGCCGGCCCGGGGGTGCGCCGGCGAGCGGCACTGCCT
NspBIICT
CfrICG
Cfr10IGC
EcoP15R
Cfr10IGC
DsaIGC
AvaICG
670 680 690 700 710 720
CCTACACGGTTCGCGCCGGCGATCACTGTCAGTGCGATCGCCGCCAAAAACGGCCTCAAA
Cfr10IGC
PvuI
730 740 750 760 770 780
CTCAACGATGTCTTGCGGCTCAACGGCCTAGGCATGACGAGCATTATCGACCGGGACAGG
AvrII
790 800 810 820 830 840
TACTTCAGCTTGGTGGCAATCCGCACCAGCCGCGCCTGCACCAGTAGCCAGCACGCCAGC
Eco57IR
HaeIIAC
850 860 870 880 890 900
GCCAGCACGCCGAGCGCCCCGGCACCCAGCGGCGGTTACAAGATCAAATCTGGCGACACG
HaeIIAC
NspBIIAG
BanICA
TaqIIb
910 920 930 940 950 960
CTCAGCTCAATTGCCGCGCGCAATAACGTCAGCTTGAACGACTTGCTCCAAGCGAACGGG
MfeI
BanIIGT
BssHII
970 980 990 1000 1010 1020
CTCTCCATGAGCAGCGTGATCTACGCCGGAAAAACGCACAGCATTCCCTGCGCGGTAACT
BsmIR
1030 1040 1050 1060 1070 1080
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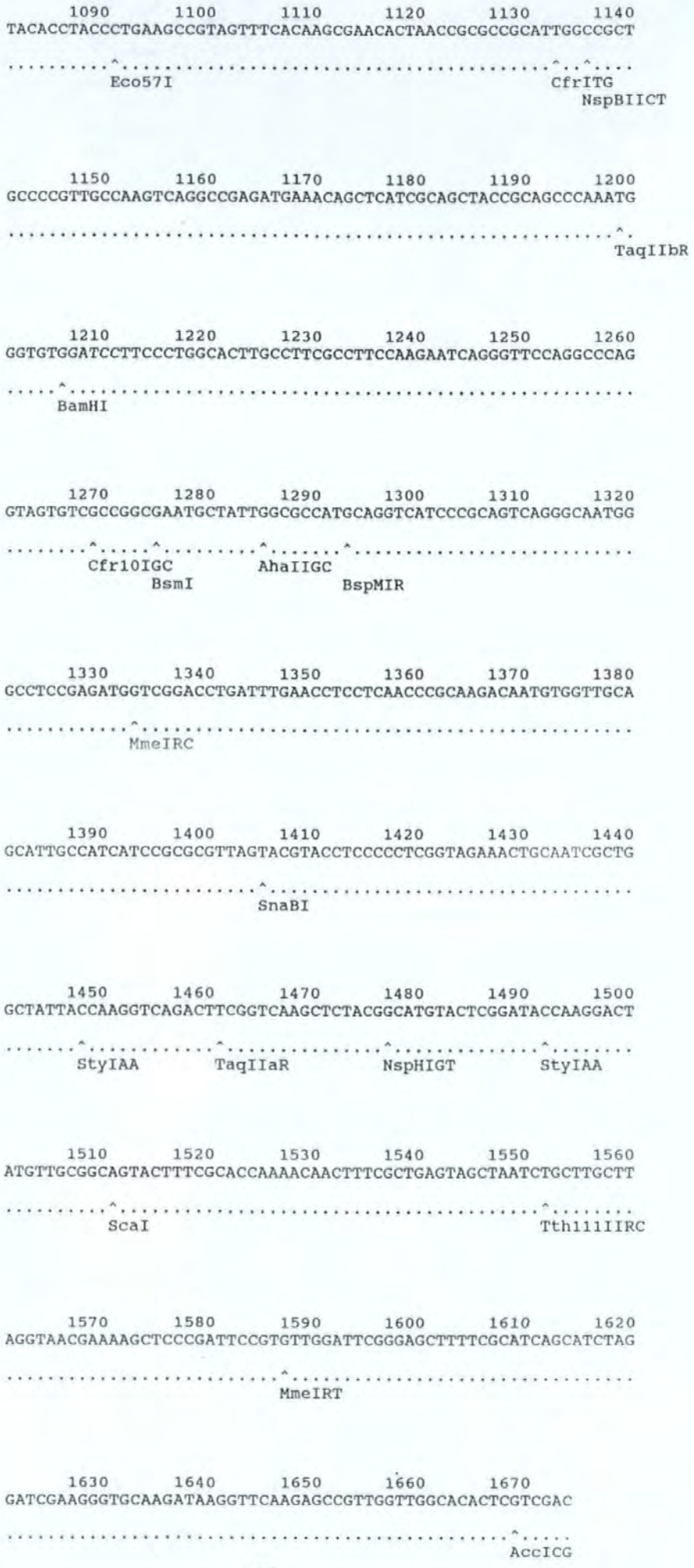
BspMI
AvaICG
Bsp1286IGA
BanICA


1090 1100 1110 1120 1130 1140
TACACCTACCCTGAAGCCGTAGTTTCACAAGCGAACACTAACCGCGCCGCATTGGCCGCT
Eco57I
CfrITG
NspBIICT
1150 1160 1170 1180 1190 1200
GCCCCGTTGCCAAGTCAGGCCGAGATGAAACAGCTCATCGCAGCTACCGCAGCCCAAATG
TaqIIbR
1210 1220 1230 1240 1250 1260
GGTGTGGATCCTTCCCTGGCACTTGCCTTCGCCTTCCAAGAATCAGGGTTCCAGGCCCAG
BamHI
1270 1280 1290 1300 1310 1320
GTAGTGTCGCCGGCGAATGCTATTGGCGCCATGCAGGTCATCCCGCAGTCAGGGCAATGG
Cfr10IGC
AhaIIGC
BsmI
BspMIR
1330 1340 1350 1360 1370 1380
GCCTCCGAGATGGTCGGACCTGATTTGAACCTCCTCAACCCGCAAGACAATGTGGTTGCA
MmeIRC
1390 1400 1410 1420 1430 1440
GCATTGCCATCATCCGCGCGTTAGTACGTACCTCCCCCTCGGTAGAAACTGCAATCGCTG
SnaBI
1450 1460 1470 1480 1490 1500
GCTATTACCAAGGTCAGACTTCGGTCAAGCTCTACGGCATGTACTCGGATACCAAGGACT
StyIAA
TaqIIaR
NspHIGT
StyIAA
1510 1520 1530 1540 1550 1560
ATGTTGCGGCAGTACTTTCGCACCAAAACAACTTTCGCTGAGTAGCTAATCTGCTTGCTT
ScaI
Tth111IIRC
1570 1580 1590 1600 1610 1620
AGGTAACGAAAAGCTCCCGATTCCGTGTTGGATTCGGGAGCTTTTCGCATCAGCATCTAG
MmeIRT
1630 1640 1650 1660 1670
GATCGAAGGGTGCAAGATAAGGTTCAAGAGCCGTTGGTTGGCACACTCGTCGAC
AccICG

Prior to nucleotide sequencing, there was no information available for the location of the ORF encoding the RHLY protein within the cloned *R.salmoninarum* DNA. However, information obtained during the attempted characterization of RHLY (see Chapter 8, above), proved useful in its detection. The mini-cell translational analysis of the cloned *R. salmoninarum* DNA fragment (8.3, above) indicates the encoded RHLY protein has a molecular weight in the range 48-50 kDa. Using the guideline that 1kbp of DNA can code for a protein of approx. 37 kDa, the ORF in the cloned *R. salmoninarum* sequence would be in the region of 1.3 to 1.35 kbp in length. Only one of the ORFs detected within the *R.salmoninarum* DNA sequence was of this magnitude, a 1248bp sequence spanning the region between the putative initiation sequence (ATG) at nucleotide 154 to a putative termination sequence (TAG) at nucleotide 1402. This ORF is indicated on the complete nucleotide sequence shownin Fig. 9.3

9.3.3 DETECTION OF TRANSCRIPTIONAL/TRANSLATIONAL CONTROL REGIONS ASSOCIATED WITH THE PUTATIVE *R.salmoninarum* OPEN READING FRAME .

All prior studies of the cloned *R.salmoninarum* DNA fragment had indicated that the production of the RHLY protein is governed by endogenous transcriptional and translational control, without aid from similar elements present on the cloning vector. It therefore follows, that in addition to the open reading frame, indications of some or all of these transcriptional and translational control regions, the so called 'machinery of gene expression' (Singer and Berg, 1991) should be present in the nucleotide base sequence determined for the *R.salmoninarum* DNA fragment.

The transcription of a given coding DNA sequence is initiated by the formation of a stable complex between the RNA polymerase enzyme and a characteristic control sequence called the promoter, present at the beginning of all transcription units. Nucleotide sequence and mutational analysis of a large number of prokaryotic promoter regions has

been performed (Hawley and Mc Clure, 1983) and revealed two regions that were consistently similar. One of the important sequences contains 6 or 7 bases and occurs about 10 bases upstream (i.e.,5') of the nucleotide at which transcription commences (+1 nucleotide). This sequence is commonly referred to as the -10 sequence or the 'Pribnow box' after its discoverer. A second region, typically 9 nucleotides long and clustered about 35 nucleotides upstream of the RNA initiation site is known as the -35 sequence. The -35 sequence has been implicated in the binding of RNA polymerase, which precedes the positioning of the enzyme at the Pribnow box (Singer and Berg, 1991).

Apart from transcriptional initiation regions, DNA sequences may also possess transcription terminators, consisting of inverted extended repeat sequences distal to the translation termination codon. These sequences induce the 3' ends of RNA transcripts to form 'stem-loop' structures which are thought to interfere with RNA polymerase activity and facilitate the disassociation of the synthesized mRNA chain from the template (Adhya and Gottesman, 1978).

The regulation of translation in prokaryotes can occur at one of the three stages, either in initiation, elongation or termination. Apart from the initiation and termination sequences detected during ORF analysis, there is also another sequence a 5-8 purine rich region known as the Shine-Delgarno (SD) sequence or commonly as the ribosome-binding site. The SD sequence is usually located approximately 5-10 nucleotides distal to the initiation codon and is where, as its name suggests, the 30S ribosome subunit binds to the mRNA prior to polypeptide synthesis.

The cloned *R. salmoninarum* DNA sequence was examined for possible regions of transcriptional and translational control, the putative -10 and -35 and +1 sequences along with a SD sequence and possible transcription termination loop are indicated on the complete nucleotide sequence of the cloned *R.salmoninarum* DNA displayed in Fig. 9.3.

9.4. DERIVATION OF A PRIMARY STRUCTURE FOR THE *R.salmoninarum* RHLY PROTEIN - AMINO ACID SEQUENCE.

The next proceedure performed on the nucleotide sequence of the cloned *R. salmoninarum* DNA fragment was to use the Recombinant Toolkit translation program to derive a primary amino acid sequence from the putative ORF of the RHLY protein. By deriving the amino acid sequence of the RHLY protein, it was hoped that further analysis of the structure and function of the haemolytic molecule could be undertaken.

Beginning at the initiation codon, the translation utilises the genetic code (see Appendix II) to convert base triplets to their corresponding amino acids until a termination codon is reached. The software outputs the information as a series of base triplets, each assigned with the appropriate 3 letter amino acid abbreviation. This output can be seen in fig 9.5

9.5. AMINO ACID SEQUENCE ANALYSIS USING THE DNASTAR SUITE OF COMPUTER PROGRAMS.

Once determined, the primary amino acid sequence coded for in a given ORF can be analyzed for clues to the biological properties of the polypeptide molecule produced. As many aspects of the structure and function of the cloned RHLY were not known, it was decided to analyze the amino acid sequence derived from the ORF present in the cloned *R.salmoninarum* DNA.

Once again the analysis of the sequence information was performed with the aid of an integrated suite of computer programs. This task was beyond the capabilities of the RT package, therefore a more powerful computer system was utilized. The DNASTAR sequence analysis system (DNASTAR Inc., Madison, WI., U.S.A.) was employed. This system installed on a high memory personal computer and interfaced with a CD-ROM data storage facility is capable of a large number of nucleotide and amino acid sequence manipulations.

Figure 9.5. The primary amino acid sequence of the haemolytic RHLY protein. The sequence was computer-derived from the 1248 bp ORF detected in the nucleotide sequence of the cloned *Sal I*-generated *R.salmoninarum* DNA fragment in the recombinant plasmid pRHLY11.

```
                                                  162                 171                 180
                                        Met  Thr  Ser  Pro  Pro  Gln  Pro  Asn  Ser
                                        ATG  ACA  TCC  CCC  CCC  CAG  CCC  AAT  TCC

            189                 198                 207                 216                 225
Lys  Ala  Ala  Lys  Gln  Ala  Lys  Gln  Leu  Ala  Val  Val  Ser  Thr  Ala
AAG  GCT  GCC  AAG  CAG  GCA  AAG  CAG  CTT  GCC  GTC  GTC  AGC  ACG  GCC

            234                 243                 252                 261                 270
Ala  Leu  Pro  Val  Ile  Met  Leu  Ser  Ser  Leu  Ala  Leu  Ala  Gln  Pro
GCG  CTT  CCG  GTC  ATC  ATG  CTT  TCT  TCG  CTA  GCG  CTC  GCT  CAG  CCA

            279                 288                 297                 306                 315
Ala  Asn  Ala  Pro  Pro  Pro  Ala  Leu  Ala  Ile  Ala  Gly  Thr  Leu  Asn
GCG  AAT  GCG  CCC  CCA  CCA  GCA  CTC  GCA  ATC  GCG  GGC  ACG  CTT  AAT

            324                 333                 342                 351                 360
Ala  Ala  Val  Lys  Asn  Ala  Val  Ala  Lys  Val  Leu  Ala  Asn  Arg  Pro
GCC  GCG  GTG  AAA  AAT  GCG  GTA  GCT  AAA  GTC  TTA  GCA  AAT  CGG  CCT

            369                 378                 387                 396                 405
Ala  Gly  Ser  Phe  Ile  Pro  Ala  Gly  Asn  Val  Ala  Ala  Ser  Leu  Pro
GCT  GGA  TCT  TTT  ATC  CCG  GCA  GGA  AAC  GTA  GCC  GCC  AGT  CTG  CCC

            414                 423                 432                 441                 450
Asn  Leu  Lys  Thr  Ala  Thr  Ser  Pro  Ser  Ala  Ile  Lys  Pro  Ala  Ala
AAT  CTG  AAG  ACA  GCG  ACT  AGC  CCA  TCG  GCA  ATC  AAG  CCA  GCA  GCG

            459                 468                 477                 486                 495
Pro  Ser  Ser  Ser  Thr  Tyr  Thr  Val  Arg  Ala  Gly  Asp  Ser  Ile  Ser
CCG  TCG  TCG  AGC  ACT  TAT  ACC  GTG  CGC  GCT  GGC  GAC  TCG  ATT  AGC

            504                 513                 522                 531                 540
Ser  Ile  Ala  Ala  Arg  Asn  Gly  Leu  Asn  Val  Asn  Asp  Val  Leu  Asp
TCG  ATC  GCT  GCC  CGC  AAT  GGC  CTA  AAC  GTC  AAT  GAC  GTG  CTA  GAC

            549                 558                 567                 576                 585
Leu  Asn  Ser  Leu  Gly  Gln  Thr  Ser  Leu  Ile  Phe  Pro  Gly  Gln  Gln
CTC  AAT  AGC  CTG  GGC  CAA  ACC  AGC  CTG  ATC  TTT  CCT  GGC  CAG  CAA

            594                 603                 612                 621                 630
Ile  Lys  Ala  Asn  Arg  Arg  Arg  Ser  Ala  Ala  Ala  Ala  Ala  Ala  Gly
ATC  AAG  GCT  AAC  CGG  CGT  CGC  TCC  GCT  GCT  GCC  GCG  GCG  GCC  GGC

            639                 648                 657                 666                 675
Pro  Gly  Val  Arg  Arg  Arg  Ala  Ala  Leu  Pro  Pro  Thr  Arg  Phe  Ala
CCG  GGG  GTG  CGC  CGG  CGA  GCG  GCA  CTG  CCT  CCT  ACA  CGG  TTC  GCG

            684                 693                 702                 711                 720
Pro  Ala  Ile  Thr  Val  Ser  Ala  Ile  Ala  Ala  Lys  Asn  Gly  Leu  Lys
CCG  GCG  ATC  ACT  GTC  AGT  GCG  ATC  GCC  GCC  AAA  AAC  GGC  CTC  AAA

            729                 738                 747                 756                 765
Leu  Asn  Asp  Val  Leu  Arg  Leu  Asn  Gly  Leu  Gly  Met  Thr  Ser  Ile
CTC  AAC  GAT  GTC  TTG  CGG  CTC  AAC  GGC  CTA  GGC  ATG  ACG  AGC  ATT

            774                 783                 792                 801                 810
Ile  Asp  Arg  Asp  Arg  Tyr  Phe  Ser  Leu  Val  Ala  Ile  Arg  Thr  Ser
ATC  GAC  CGG  GAC  AGG  TAC  TTC  AGC  TTG  GTG  GCA  ATC  CGC  ACC  AGC

            819                 828                 837                 846                 855
Arg  Ala  Cys  Thr  Ser  Ser  Gln  His  Ala  Ser  Ala  Ser  Thr  Pro  Ser
CGC  GCC  TGC  ACC  AGT  AGC  CAG  CAC  GCC  AGC  GCC  AGC  ACG  CCG  AGC

            864                 873                 882                 891                 900
Ala  Pro  Ala  Pro  Ser  Gly  Gly  Tyr  Lys  Ile  Lys  Ser  Gly  Asp  Thr
GCC  CCG  GCA  CCC  AGC  GGC  GGT  TAC  AAG  ATC  AAA  TCT  GGC  GAC  ACG

            909                 918                 927                 936                 945
Leu  Ser  Ser  Ile  Ala  Ala  Arg  Asn  Asn  Val  Ser  Leu  Asn  Asp  Leu
CTC  AGC  TCA  ATT  GCC  GCG  CGC  AAT  AAC  GTC  AGC  TTG  AAC  GAC  TTG

            954                 963                 972                 981                 990
Leu  Gln  Ala  Asn  Gly  Leu  Ser  Met  Ser  Ser  Val  Ile  Tyr  Ala  Gly
CTC  CAA  GCG  AAC  GGG  CTC  TCC  ATG  AGC  AGC  GTG  ATC  TAC  GCC  GGA

            999                1008                1017                1026                1035
Lys  Thr  His  Ser  Ile  Pro  Cys  Ala  Val  Thr  Pro  Gly  Asp  Val  Asn
AAA  ACG  CAC  AGC  ATT  CCC  TGC  GCG  GTA  ACT  CCG  GGC  GAC  GTG  AAC

           1044                1053                1062                1071                1080
Val  Thr  Pro  Ala  Ala  Pro  Ala  Ser  Gly  Pro  Gly  Thr  Phe  Gly  Asn
GTC  ACT  CCG  GCT  GCA  CCT  GCC  TCA  GGC  CCG  GGC  ACC  TTT  GGC  AAC

           1089                1098                1107                1116                1125
Tyr  Thr  Tyr  Pro  Glu  Ala  Val  Val  Ser  Gln  Ala  Asn  Thr  Asn  Arg
TAC  ACC  TAC  CCT  GAA  GCC  GTA  GTT  TCA  CAA  GCG  AAC  ACT  AAC  CGC

           1134                1143                1152                1161                1170
Ala  Ala  Leu  Ala  Ala  Ala  Pro  Leu  Pro  Ser  Gln  Ala  Glu  Met  Lys
GCC  GCA  TTG  GCC  GCT  GCC  CCG  TTG  CCA  AGT  CAG  GCC  GAG  ATG  AAA

           1179                1188                1197                1206                1215
Gln  Leu  Ile  Ala  Ala  Thr  Ala  Ala  Gln  Met  Gly  Val  Asp  Pro  Ser
CAG  CTC  ATC  GCA  GCT  ACC  GCA  GCC  CAA  ATG  GGT  GTG  GAT  CCT  TCC

           1224                1233                1242                1251                1260
Leu  Ala  Leu  Ala  Phe  Ala  Phe  Gln  Glu  Ser  Gly  Phe  Gln  Ala  Gln
CTG  GCA  CTT  GCC  TTC  GCC  TTC  CAA  GAA  TCA  GGG  TTC  CAG  GCC  CAG

           1269                1278                1287                1296                1305
Val  Val  Ser  Pro  Ala  Asn  Ala  Ile  Gly  Ala  Met  Gln  Val  Ile  Pro
GTA  GTG  TCG  CCG  GCG  AAT  GCT  ATT  GGC  GCC  ATG  CAG  GTC  ATC  CCG

           1314                1323                1332                1341                1350
Gln  Ser  Gly  Gln  Trp  Ala  Ser  Glu  Met  Val  Gly  Pro  Asp  Leu  Asn
CAG  TCA  GGG  CAA  TGG  GCC  TCC  GAG  ATG  GTC  GGA  CCT  GAT  TTG  AAC

           1359                1368                1377                1386                1395
Leu  Leu  Asn  Pro  Gln  Asp  Asn  Val  Val  Ala  Ala  Leu  Pro  Ser  Ser
CTC  CTC  AAC  CCG  CAA  GAC  AAT  GTG  GTT  GCA  GCA  TTG  CCA  TCA  TCC

           1404
Ala  Arg   *
GCG  CGT  TAG
```

CODON	AMINO ACID	No. IN SEQUENCE	%
GCA	Alanine	17	4.08
GCC	Alanine	29	6.97
GCG	Alanine	21	5.04
GCT	Alanine	13	3.12
AGA	Arginine	0	0
AGG	Arginine	1	0.24
CGA	Arginine	1	0.24
CGC	Arginine	8	1.92
CGG	Arginine	6	1.44
CGT	Arginine	2	0.48
AAC	Asparagine	15	3.60
AAT	Asparagine	12	2.88
GAC	Aspartic acid	9	2.16
GAT	Aspartic acid	3	0.72
TGC	Cysteine	2	0.48
TGT	Cysteine	0	0
CAA	Glutamine	8	1.92
CAG	Glutamine	12	2.88
GAA	Glutamic acid	2	0.48
GAG	Glutamic acid	2	0.48
GGA	Glycine	4	0.96
GGC	Glycine	16	3.84
GGG	Glycine	4	0.96
GGT	Glycine	2	0.48
CAC	Histidine	2	0.48
CAT	Histidine	0	0
ATA	Isoleucine	0	0
ATC	Isoleucine	15	3.60
ATT	Isoleucine	5	1.20
CTA	Leucine	4	0.96
CTC	Leucine	12	2.88
CTG	Leucine	6	1.44
CTT	Leucine	5	1.20
TTA	Leucine	1	0.24
TTG	Leucine	8	1.92
AAA	Lysine	7	1.68
AAG	Lysine	7	1.68
ATG	Methionine	8	1.92
TTC	Phenylalanine	5	1.20
TTT	Phenylalanine	3	0.72
CCA	Proline	7	1.68
CCC	Proline	7	1.68
CCG	Proline	14	3.36
CCT	Proline	8	1.92
AGC	Serine	19	4.56
AGT	Serine	4	0.96
TCA	Serine	6	1.44
TCC	Serine	7	1.68
TCG	Serine	7	1.68
TCT	Serine	3	0.72
ACA	Threonine	3	0.72
ACC	Threonine	7	1.68
ACG	Threonine	6	1.44
ACT	Threonine	6	1.44
TGG	Tryptophan	1	0.24
TAC	Tyrosine	5	1.20
TAT	Tyrosine	1	0.24
GTA	Valine	5	1.20
GTC	Valine	11	2.64
GTG	Valine	10	2.4
GTT	Valine	2	0.48

Figure 9.6 Codon usage for the amino acid sequence of the haemolytic RHLY protein.

The tasks to be undertaken with the DNASTAR system were firstly to analyse the amino acid sequence of the RHLY protein and to predict a number of aspects of its biochemistry, and secondly, to possibly gain further information about the structure/function aspects of the molecule by comparing the RHLY amino acid sequence with the large number of sequences lodged in the Program Identification Resources (PIR) protein database.

9.5.1 PREDICTION OF THE BIOCHEMICAL NATURE OF THE RHLY MOLECULE BY DNASTAR PROTEIN ANALYSIS.

The nucleotide sequence of the ORF detected in section 9.3.2 (above) was entered into the DNASTAR sytem where it was translated into an amino acid sequence. The DNASTAR-mediated translation confirmed the analysis performed earlier by the Recombinant Toolkit system, deriving an amino acid sequence of 416 residues in length identical to that shown in Figure 9.5 (above). The codon usage for the sequence was also calculated and found to be identical to that presented in Fig 5.6. The computer also calculated a molecular weight for the polypeptide of 42050 g/mol and a predicted isoelectric point (pI) of pH 10.28

9.5.1.1 SECONDARY STRUCTURE PREDICTION.

By analyzing the distribution of the various amino acid residues within a protein, the DNASTAR system can make a number of predictions on its secondary structure. These predictions, are based on empirical schemes derived from the distribution of the individual amino acids in proteins whose three-dimensional structures are known (Chou and Fasman, 1974). A number of secondary structure prediction schemes are available for use on protein sequence analysis software, each having its own merits, (Doolitle, 1986, 1990), those available on the DNASTAR system are those described by Garnier, 1978) and Chou and Fasman,(1974). Each prediction scheme utilizes a different algorithm to calculate the probability that a given segment of the amino acid sequence will be an α-helix, β-sheet structure or a turn (Fig 9.7).

9.5.1.2 HYDROPATHY PREDICTION.

Another analysis program available for amino acid sequences, and contained in the DNASTAR suite of programs, calculates the hydropathy of a given protein. The hydropathy of a given protein relates to the water relationship i.e. the hydrophilicity and hydrophobicity of different portions of the molecule. Hydropathy plots are calculated by the evaluation of overlapping segments of a given amino acid sequence, with the average score of each segment being plotted at its mid-point (Doolittle, 1986).

Hydropathy plots are useful when making judgements about which portion of a sequence are likely to be on the outside (or inside) of the protein, and may be useful in determining the presence of certain features such as membrane-spanning regions. DNASTAR uses the method of Hopp and Woods (1981) and Kyte and Doolittle (1982) to calculate hydropathy (Fig 9.7).

9.5.1.3 SURFACE INTERACTION AND ANTIGENIC INDEX.

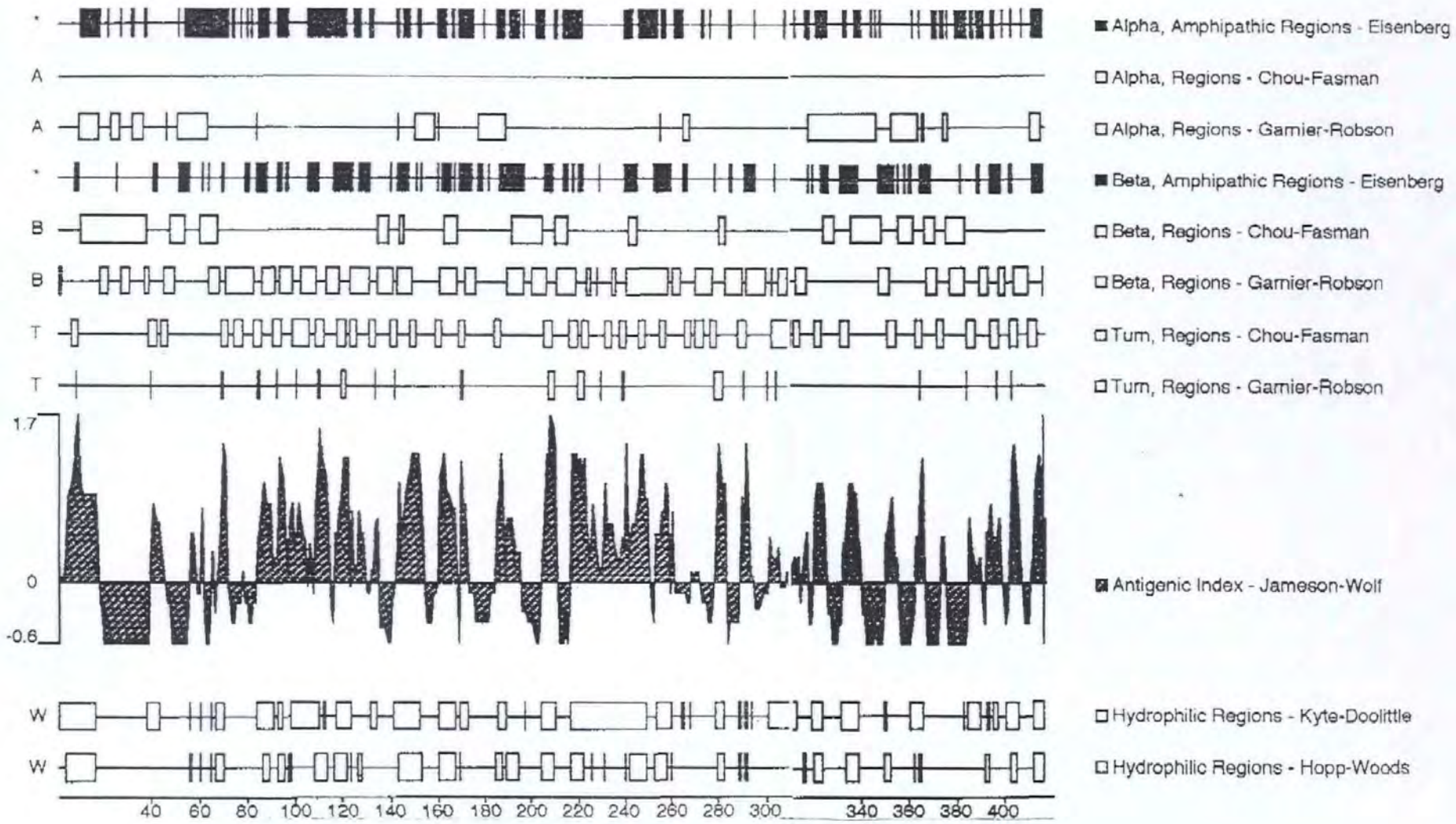
Two other predictions are made about an amino acid sequence using the DNASTAR protein analysis system, but their value in an initial study of the characteristics of a given protein is questionable. One algorithm, based on the work of Eisenberg *et al.* (1984) calculates the ampithatic nature of the various portions of the protein, indicating surface-active or membrane-active regions. The second algorithm, developed by Jameson and Wolf (1988), takes into account the predicted secondary structure of a protein to predict its antigenic index.

The computer-generated output resulting from the analysis of the RHLY protein sequence can be seen in Figure 9.7.

9.5.2 SEARCH OF THE PIR DATABASE FOR AMINO ACID SEQUENCES SHOWING HOMOLOGY WITH THE RHLY PROTEIN.

The DNASTAR PROSCAN program, is used to search the PIR protein database for amino acid sequences that show significant homology to another specified amino acid

Figure 9.7 Computer-generated output of DNASTAR secondary strucure prediction for the RHLY amino acid sequence. Shown are plots for α-helix, β-sheeting and turn regions for the protein, along with hydropathy, Eisenberg surface interactions and antigenic index as described in section 9.5 of the text.


Alpha, Amphipathic Regions - Eisenberg
Alpha, Regions - Chou-Fasman
Alpha, Regions - Garnier-Robson
Beta, Amphipathic Regions - Eisenberg
Beta, Regions - Chou-Fasman
Beta, Regions - Garnier-Robson
Turn, Regions - Chou-Fasman
Turn, Regions - Garnier-Robson
1.7
0
-0.6
Antigenic Index - Jameson-Wolf
Hydrophilic Regions - Kyte-Doolittle
Hydrophilic Regions - Hopp-Woods
40 60 80 100 120 140 160 180 200 220 240 260 280 300 340 360 380 400

sequence contained in a protein (.PRO) data file. By using this facility, it was hoped that homologous amino acid sequences could be found, that may aid the further characterization of the cloned *R. salmoninarum* RHLY protein.

The computer derived amino acid sequence of the RHLY protein (Fig 9.5, above) was entered into the DNASTAR system as a data file (RHLY.PRO), using the 1 letter amino acid abbreviations (see Appendix II). The contents of RHLY.PRO, can be seen in Fig. 9.8 .

and the PROSCAN program instructed to search the entire PIR database for sequence homology. Each of the 23,321 amino acid sequences contained within the database were compared with the RHLY.PRO data file in a search taking approxiamately 48 hours. After this time, the results of the database search were obtained as a printed results table containing the 200 best sequence matches.

The score for sequence homology obtained using the PROSCAN program corresponds to the total number of identical amino acid residues over the whole sequence compared, but unfortunately does not necessarily indicate areas of homology within a given sequence. As the initial purpose of the PROSCAN search was to attempt to find protein sequences which would aid further characterization of the haemolytic protein from *R. salmoninarum*, certain criteria would have to be applied in the choice of sequences for further homology analysis. The general approach taken by persons who regularly use the PROSCAN program as an aid to the characterization of derived novel amino acid sequences, is to first examine the matched sequences for proteins from similar organisms or proteins possessing similar properties followed by those amino acid sequences with a similarity score of over 50 (K.Martin, pers. comm) as calculated by the FASTA rapid sequence comparison method (Pearson, 1990). The sequences chosen for further analysis can be seen in fig 9.9.

MTSPPQPNSKAAKQAKQLAVVSTAALPVIMLSSLALAQPANAPPPALAIAGTLNA
AVKNAVAKVLANRPAGSFIPAGNVAASLPNLKTATSPSAIKPAAPSSSTYTVRAG
DSISSIAARNGLNVNDVLDLNSLGQTSLIFPGQQIKANRRRSAAAAAAGPGVRRR
AALPPTRFAPAITVSAIAAKNGLKLNDVLRLNGLGMTSIIDRDRYFSLVAIRTSRA
CTSSQHASASTPSAPAPSGGYKIKSGDTLSSIAARNNVSLNDLLQANGLSMSSVIY
AGKTHSIPCAVTPGDVNVTPAAPASGPGTFGNYTYPEAVVSQANTNRAALAAAP
LPSQAEMKQLIAATAAQMGVDPSLALAFAFQESGFQAQVVSPANAIGAMQVIPQ
SGQWASEMVGPDLNLLNPQDNVVAALPSSAR

Figure 9.8. The computer-derived amino acid sequence of the cloned *R.salmoninarum* RHLY protein using one-letter abbreviations. This form of the RHLY amino acid sequence was used as a data file (RHLY.PRO) for sequence analysis using the DNASTAR sequence analysis software.

Accession code	Match Sequence Title
A29605	protein a precursor - *Staphylococcus*
JS0516	dnir protein-*Escherichia coli*
WMBPP9	lysozyme- phage phi-29
A30309	isocitrate dehydrogenase (nad+)
A41487	protein p60 precursor- *Listeria monocytogenes*
S16815	snp1 protein - yeast
B36429	integrin alpha-6 chain precursor
SXAD41	hexon-associated protein (viii)
S20688	l4 pvii protein, c-terminal
S19850	pol polyprotein homolog
A27672	hypothetical gag polyprotein homolog
A36674	transcription factor hnf-3a
TNBE70	70.5k alpha trans-inducing protein
DWECL	l-serine dehydratase -*Escherichia coli*
R5KT3	ribosomal protein l3 -*Cyanophora*
A05275	insulin receptor protein
A33880	heparan sulfate proteoglycan
S06918	dna-binding protein phi-0
DWPSUP	urocanate hydratase - *Pseudomonas*
A42342	fatty acid-binding protein
S11153	amif protein - *Streptococcus pneumoniae*
JQ0530	microbial metalloproteinase precursor
S15940	ilvh protein - *Salmonella typhimurium*
A30578	hydroxymethylglutaryl-coa lyase
XMECF2	flaaii protein - *Escherichia coli*
VZBRGP	virulence protein - *Bordetella purtussis*
DEECG3	glyceraldehyde-3-phosphate dehydrogenase
A32207	arylsulfatase a precursor
TDRT	thy-1 membrane glycoprotein precursor
DJVZZW	dna-directed dna polymerase
RTHUG	hypoxanthine phosphoribosyltransferase
B23701	olfactory receptor protein f5
A35847	fos-related antigen- fruit fly
S11561	eba-175 protein- *Plasmodium falciparum*
MMRTS	s-laminin precursor - rat
BVECCT	cet protein - *Escherichia coli*
S04940	acrosin precursor - pig
JQ1118	protein kinase ssp31 - yeast
B42519	a18r protein - *Vaccinia* virus
TVHUB1	transforming protein (bcl-2-beta)
A28470	histone h1 - mouse
CGHU6C	collagen alpha 1 (ii) chain precursor
S18135	calcium release channel - pig
GRYCS7	sulphate permease - *Synechococcus*

Figure 9.9 Protein sequences in the PIR database showing homology to the RHLY protein. Sequences were detected using the PROSCAN software on the DNASTAR sequence analysis system. Those sequences shown are those with a homology score of above 50 as calculated by the FASTA method (Pearson, 1990).

9.5.3 ALIGNMENT OF SIMILAR SEQUENCES USING THE AALIGN PROGRAM.

Once similar protein sequences have been identified in the PIR database, the best regions of homology can be analyzed using the AALIGN program contained in the DNASTAR package. This program aligns two protein sequences using the algorithms of both Needleman and Wunch (1970) and Lipman and Pearson (1985).

Using the AALIGN program, the sequences shown in Fig 9.9 were compared with the derived amino acid sequence of the RHLY protein (RHLY.PRO). Only one of the PROSCAN-generated amino acid sequences was found to possess a region of strong homology with the RHLY protein, the *dniR* (dissimilatory nitrite reductase gene regulatory protein) of *Escherichia coli.* This region of homology comparison of *R.salmoninarum* RHLY and *E. coli dniR* is shown in Fig 9.10.

9.6 ANALYSIS OF THE AMINO ACID SEQUENCE HOMOLOGY BETWEEN *R.salmoninarum* RHLY PROTEIN AND THE *dniR* PROTEIN OF *E. coli.*

The complete amino acid sequence of *E. coli dniR* was obtained from the publication of Kajie *et al.* (1991) and transformed into the 1 letter abbreviation format. This sequence was then compared by eye with the *R.salmoninarum* RHLY amino sequence presented in the same format. It was found that the initial region of homology between the two sequences, approximately 30 residues in length was repeated in both proteins, once in the *E. coli* protein and twice in the *R.salmoninarum* protein. The two amino acid sequences, and the position and composition of the conserved repeats are shown in Fig. 9.11.

The presence of conserved amino acid repeat regions, may present some clues to the function of the RHLY protein, especially if the functional role of the repeats in the *dniR* protein have been determined. However, the authors of the research publication describing the cloning and sequencing of *dniR* protein from *E. coli*, have not noted the conserved repeat region within their sequence.

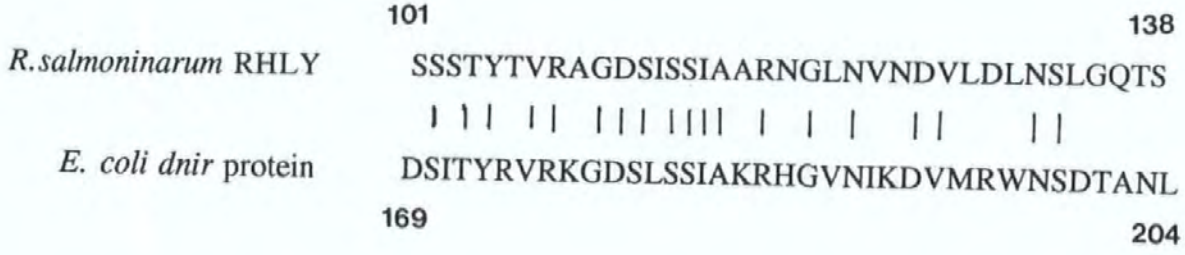


Figure 9.10 Regions of amino acid sequence homology between *R.salmoninarum* RHLY protein and *E. coli dniR* protein. Sequence homology was detected using the AALIGN program on the DNASTAR sequence analysis system. The respective amino acid residue numbers are shown, and ( ) indicates an identical amino acid.

Figure 9.11 Homology between repeated regions in the amino acid sequences of the RHLY protein of *R.salmoninarum* and the *dniR* protein of *E. coli* . Part A) shows the amino acid sequence of the two proteins, the repeated regions are boxed. The 5 repeated regions alone are shown in part B), and the alignment of the five regions is shown in part C) where only homologous residues are shown.

A)
R.salmoninarum RHLY (this study)

MTSPPQPNSKAAKQAKQLAVVSTAALPVIMLSSLALAQPANAPPPALAIAGTLNA
AVKNAVAKVLANRPAGSFIPAGNVAASLPNLKTATSPSAIKPAAPSSSTYTVRAG
DSISSIAARNGLNVNDVLDLNSLGQTSLIFPGQQIKANRRRSAAAAAAGPGVRRR
AALPPTRFAPAITVSAIAAKNGLKLNDVLRLNGLGMTSIIDRDRYFSLVAIRTSRA
CTSSQHASASTPSAPAPSGGYKIKSGDTLSSIAARNNVSLNDLLQANGLSMSSVIY
AGKTHSIPCAVTPGDVNVTPAAPASGPGTFGNYTYPEAVVSQANTNRAALAAAP
LPSQAEMKQLIAATAAQMGVDPSLALAFAFQESGFQAQVVSPANAIGAMQVIPQ
SGQWASEMVGPDL NLLNPQDNVVAALPSSAR

E. coli dniR protein (Kajie *et al.*, 1991)

MPKMLALSDILKNSKRYGVRLPTTDESRALARVHLSSPVEMAKVADMAGISVSK
LKTFNAGVKGSTLGASGPQYVMVPKKHADQLRESLASGEIAAVQSTLVADNTPL
NSRVYTVRSGDTLSSIASRLGVSTKDLQQWNKLRGSKLKPGQSLTIGAGSSAQRL
ANNSDSITYRVRKGDSLSSIAKRHGVNIKDVMRWNSDTANLQPGDKLTLFVKNN
NMPDS

B)
RHLY repeat regions.

1. SSTYTVRAGDSISSIAARNGLNVNDVLDLNSL
2. LPTTRFAPAITVSAIAAKNGLKLNDVLRLNGL
3. SGGYKIKSGDTLSSIAARNNVSLNDLLQANGL

***dniR* repeat regions.**

4. SRVYTVRSGDTLSSIASRLGVSTKDLQQWNKL
5. SITYRVRKGDSLSSIAKRHGVNIKDVMRWNSD

C)
Alignment of sequences.

1. STYTVR GDS SSIAARNGLN NDVL LNSL
2. R T S IAA NGL LNDVLRLNGL
3. S Y SGDTLSSIAARN VSLNDLLQ NGL
4. S YTVRSGDTLSSIA R GVS KDL QWN L
5. STYRVR GDSLSSIA R GVN KDV RWNS

At this point, the analysis of the RHLY protein and the experimental work of this research project was ended.

9.7 DISCUSSION.

The first portion of this chapter describes the determination of the complete nucleotide sequence of the *R.salmoninarum* DNA fragment contained within the plasmid pRHLY11, a fragment containing a coding sequence for the haeomolytic RHLY protein. The enzymic chain-termination method of DNA sequencing was utilized, using the commercially available Sequenase 2.0 kit to determine the sequence of three subclones, made from of the 1.6 kbp *Sal I*-generated *R.salmoninarum* DNA insert in pRHLY11. Once familiarised with the different portions of the sequencing protocol, the method was found to be relatively straightforward and yielded high quality sequence information. A few problems however were encountered during the sequencing of the 450 bp *Ava I*-generated fragment from the centre of the pRHLY11 insert. This region, as can be seen in the nucleotide sequence presented in Fig. 9.3 has a high G + C ratio compared to the other portions of the sequence as well as a long run of guanine and cytosine residues between nucleotide 600 and nucleotide 690. The composition of bases in this area provided two problems during sequencing. Firstly, where there are long stretches of guanine and cytosine residues, there will tend to be stable secondary structure within the DNA molecule, as the two bases will bond together by complementary base pairing giving ambiguous sequence information. If this secondary structure is not removed by complete denaturation of the DNA, the DNA polymerase enzyme will have difficulty in moving along the template during DNA synthesis. This inefficiency is reflected in the final sequencing gel in the phenomena known as compressions, closely packed bands in the lanes containing G and/or C terminated polynucleotides. However it was found, that by ensuring that the sequencing template was completely denatured (by increasing the time and temperature of denaturation) this problem could be overcome. The same region of DNA template was

also difficult to sequence, as it was the overlap point for sequence information. Sequencing was performed using both forward and reverse primers, because the templates were too large to sequence efficiently in one direction. The overlap point between information gained using these different primers, occurred in the high G + C region, and was consequently difficult to resolve. Although it took a long time, this problem was solved however by running a number of duplicate gels to confirm the base sequence in this region.

Once all the sequence information had been obtained, the complete nucleotide sequence of the *R.salmoninarum* DNA was compiled. At this point the Recombinant Toolkit computer package was ultilized to analyse the sequence for aspects of its structure and function. An extensive restriction endonuclease cleavage map was derived (Fig. 9.4) and confirmed the location of cleavage sites for *Ava I, Hpa I,* and *Pvu I* previously established in chapter 7 of this thesis (Fig. 7.14, p. 162).

The next task was to search for an open reading frame on the RHLY11 insert, which when transcribed and translated, would produce the RHLY protein. As previously mentioned, without conclusive evidence such as the information gained from deletion mutation experiments, the detection of the ORF of interest amongst a nucleotide sequence can be difficult. More than often it is left to the investigator to glean a number of clues from within the DNA sequence and from previous biochemical characterization of the encoded protein. This was certainly the case with the detection of the ORF for the *R.salmoninarum* RHLY protein.

Prior to nucleotide sequence analysis, it was known that the RHLY protein encoded by the cloned DNA is approximately 48-50 kDa in size, and that the expression of the coding sequence (the *rsh* gene), occurs under the influence of endogenous transcriptional and translational control elements. By noting these findings, and by using the Recombinant Toolkit software, the detection of a putative ORF for the RHLY protein was relatively

straightforward. Fortunately, the nucleotide sequence was seen to contain only one reading frame of suitable size for the RHLY protein, and the presence of gene expression machinery both proximal and distal to the putative ORF would seem to confirm its presence. The SD sequence, -10 and -35 regions and transcriptional start nucleotide (+1), were all found in the appropriate positions relative to the putative RHLY ORF, and were found to be analogous to the same sequences found in other prokaryotes (Hawley and Mc Lure, 1983), as well as a putative termination loop sequence (Singer and Berg, 1991). Interestingly, the putative SD region (GAGGA), -10 sequence (TTAACT) and -35 sequence (TTCACA) found proximal to the putative RHLY reading frame are very similar to equivalent sequences in *E. coli* genes (Hawley and McLure, 1983; Singer and Berg, 1991). As the expression of cloned genes in *E. coli* is heavily influenced by the structure and conformation of the transcriptional/translational control regions present on the foreign DNA sequence (Old and Primrose, 1989), this may account for the stable production of the RHLY protein in the *E. coli* host.

Once detected, the open reading frame in the *rsh* gene was used to derive an amino acid sequence for the RHLY protein (Fig. 9.5). The RHLY protein was seen to contain 416 amino acid residues and have a predicted molecular weight of approximately 42 kDa. The calculated size for the RHLY protein appears to be somewhat smaller than the size estimated for RHLY from minicell analysis of pRHLY11, however this may be explained by factors such as the presence in the RHLY polypeptide of a high proportion of certain amino acids such as proline, which can affect significantly, the mobility of proteins in polyacrylamide gels (Hames and Rickwood, 1986). Computer analysis of the ORF for RHLY protein also generated a table of codon usage. Although this table does not yield information valuable to the present study of RHLY, it is included for reference purposes, and may aid future studies on RHLY gene origins or in future molecular studies of *R.salmoninarum* components.

Once an amino acid sequence had been derived for the RHLY protein, more sophisticated computer analysis was performed using the DNASTAR suite of computer programs. The first analysis performed on the RHLY amino acid sequence was a prediction of the secondary structure for the complete RHLY protein (Fig. 9.7). Computer aided secondary structure prediction, must be treated with caution for a number of reasons, but most importantly, that the algorithms employed (Chou and Fasman, 1974; Garnier *et al.*, 1978) are based on data obtained from a limited number of three dimensional protein models, and data based on structure models for globular proteins only (Doolittle, 1986). Computer-aided structure predictions may however, confirm assigned structure/function aspects of a protein, aid the protein characterization process and also give impetus to further biochemical studies. It is for these reference purposes that the structure prediction data for the cloned RHLY protein is included in this thesis. Also included in Fig. 9.7 is DNASTAR-generated data for hydropathy, surface interaction (amphipathic regions) and antigenic site analysis, this data was not used for the characterization of RHLY protein, but is also displayed for future reference.

The main aim of the analysis by DNASTAR performed on the RHLY amino acid sequence, was to compare the sequence with the thousands of sequences lodged in the PIR database, to search for homologous sequences in an attempt to characterize the product of the cloned *rsh* gene in more detail. The PROSCAN search of the PIR database, produced 200 matched amino acid sequences, but on further analysis, all but one of these were found to be poor matches based on sequence homology over the whole amino acid sequence and not on homology between certain regions of the protein.

One sequence however, the *dniR* protein of *E. coli*, was found to show homology with RHLY over a stretch of approximately 30 amino acid residues (Fig. 9.10), and therefore warranted further investigation. Further comparisons of the two sequences revealed that the homologous regions were conserved and repeated in both molecules, twice in RHLY

and once in *dniR* (Fig. 9.11). As it has been found, that repeated amino acid motifs, especially conserved motifs, often have important structure/function implications for a protein (Doolittle, 1986), more information was sought on the function of *E. coli dniR* protein as a route to possible clues for the characterization of the cloned RHLY molecule.

The *dniR* protein (Kajie *et al.*, 1991), containing 222 amino acids and of a size in the region of 24 kDa, is the product of the *dniR* gene, and has a positive regulatory action on induced synthesis of the dissimilatory hexaheme nitrite reductase (cytochrome c_{552}) of *E. coli*. This enzyme has both nitrite and hydroxylamine reductase activities and is part of the metabolic response to anaerobiosis, being inactivated by molecular oxygen and stimulated anaerobically by the presence of nitrate or nitrite in the medium.

The publication by Kajie and his colleagues, displays the full nucleotide sequence of the *dniR* gene, and a derived amino acid sequence. Examination of this published sequence revealed the repeating amino acid motifs found to be homologous to repeats in the *R.salmoninarum* RHLY protein, but these repeats and their possible function are not mentioned in the text of the publication. In an attempt to characterize the repeating motifs, contact was established by letter with Dr. Kajie displaying the data shown in Fig. 9.11 relating to the position and composition of the homologous motifs in *R.salmoninarum* RHLY and *E. coli dniR*, and asking if there was any indication of their function. In a prompt reply, Dr Kajie outlined the results of some amino acid sequence homology searches he had performed using the repeating motifs in the RHLY and *dniR* proteins. The first search performed was a search of a protein database for protein motifs having homology to the five amino acid sequences analogous in RHLY and the *dniR protein*. No homologous protein motifs were found. Dr. Kajie had also previously searched for sequence homology between *dniR* and a number of transcriptional regulators, he found that the *dniR* protein contained two homologous regions of 22 amino acids with the characteristic structure of *E. coli* DNA binding proteins (helix-turn-helix structure) at

positions 39-60 and 119-140. This latter sequence is included in one of the repeating amino acid sequences (No. 4 in Fig. 9.11) of *dniR* homologous to the RHLY motifs.

It is therefore possible, that the RHLY protein has a regulatory function as well as a haemolytic property, whether the expression of the RHLY gene is under the control of environmental conditions (aerobic/anaerobic) as is the case with *dniR* in *E. coli*, is debatable, but it does warrant further investigation. The biosynthesis of the haemolysin found in the swine pathogen *Actinobacillus pleuropneumoniae*, is regulated by the product of the *HlyX* gene, which in experimental studies has been shown to have functional homology to the *E. coli* global anaerobic regulator protein FNR (MacInnes *et al.*, 1990). The *dniR* gene of *E. coli* contains an FNR-binding site proximal to the open reading frame, and is thought to be regulated in the same manner. The possibility exists, that although no FNR-binding site can be detected proximal to the *R.salmoninarum* RHLY gene, the production of the encoded haemolytic molecule may be regulated in a similar manner.

It is clear from the results of the investigations undertaken so far on the RHLY protein of *R.salmoninarum*, that its true role has yet to be established. However, the clues provided by the experimental work performed on RHLY in this chapter and in the other chapters of this thesis, will give both impetus and direction to further studies.

CHAPTER 10. GENERAL DISCUSSION.

The experimental work of this research project is discussed throughout this thesis at the end of each phase of practical investigation. The purpose of this, the final chapter, is to bring together all the information in a general discussion.

The initial aims of this research project, as stated in the introduction of this thesis, were to assess the feasibility of the application of gene cloning techniques to future studies of *R.salmoninarum*, and by using these techniques, attempt the isolation and characterisation of putative determinants of pathogenicity from the bacterium. In considering the results obtained during this research, it can be said that the gene cloning approach to studies of *R.salmoninarum* has been validated, as a previously uncharacterised molecule has been 'dissected' from the bacterium, and a reliable source of this molecule is now available for further study. However, despite this fact, no solid information regarding the pathogenic processes of *R.salmoninarum* has been gained.

In stating this, the author is aware that it would be somewhat naive to make glib statements about relative success or failure. Science is better served by the critical evaluation of the large body of information gathered as a result of any dedicated research endeavour, whether this information is gathered from specific experimentation, from discussion with other research workers or gleaned from literature published on the general subject area.

The first part of this research project was concerned with the culture and initial characterisation of a number of *R.salmoninarum* isolates, prior to their use in gene cloning experiments. Considerable difficulty at this stage was experienced in maintaining contamination-free cultures of the bacterium, the overgrowth of other microorganisms being a common occurrence and preventing an unadulterated source *R.salmoninarum* macromolecules from being obtained. The long incubation periods required for the culture of the organism *in vitro*, means that particular attention has to be paid to aseptic practices.

These problems appear to hamper the majority of studies performed on *R.salmoninarum*, and are described frequently in the published literature concerning the bacterium (Fryer and Sanders, 1981; Austin and Rayment, 1985; Austin and Austin, 1987; Pascho and Mulcahy, 1987). A review of these publications also reveals a perceived deficiency in the culture media available, as many studies have been reported for the preparation and evaluation of new media formulations and comparative analysis of those media formulations that already exist (Evelyn, 1977; Embley *et al.*, 1982; Austin *et al.*, 1983; Daly and Stevenson, 1985). In commenting on the culture of *R.salmoninarum*, it is obvious that culture methods to date are somewhat inadequate. The obligate intracellular parasitic nature of the pathogen, may mean that its *in vitro* growth may be restricted, but further studies on possible culture methods are vital to further progress in the characterisation of the bacterium. Two approaches to the culture of *R.salmoninarum* surely need to be sought if true benefits are to accrue; a truly defined growth medium for both biochemical and genetic studies of the organism, and, the adoption of a culture system more akin to the conditions experienced in host tissues for the analysis of determinants of BKD pathogenesis. A defined medium could be developed utilising the semi-defined formulation of Embley *et al.* (1982). Removal, addition and substitution of various components may lead to the elucidation of the metabolic processes of the bacterium, especially with regard to its carbon and nitrogen sources. The use of fish cell lines and tissue culture techniques may be of benefit, as well as the possible investigation of growth media based on isolated fish materials such as coelomic fluid.

The initial characterization of the *R.salmoninarum* isolates, by SDS-PAGE profiles and immunoblotting with antiserum raised against both whole cells and ECP of the bacterium revealed a similarity between all of the isolates analysed. This uniformity in both biochemical and antigenic properties of the organism has been widely reported, and has led to the conclusion that, taxonomically, the bacterium comprises a homogeneous

genospecies (Austin and Rodgers, 1980; Sanders and Fryer, 1980; Getchell *et al.*, 1985; Goodfellow *et al.*, 1985). The main purpose of the initial characterization of *R.salmoninarum* isolates performed in this research project, was the detection of components with possible implications for BKD pathogenesis, so that these molecules could then form a focus for gene cloning experiments. Therefore, the different isolates were studied for the presence of recognisable cell-associated antigens as well as possible determinants of both haemolytic and proteolytic activity produced by the bacterium.

A number of possible target antigens were detected by immunoblotting experiments, and of particular importance, a 57 kDa protein, corresponding to the major surface antigen of the bacterium was detected. This molecule, referred to variously as p57, MSA and haemagglutinin, is undoubtedly the best characterized component of *R.salmoninarum*, it is thought to play an important role in the pathogenesis of BKD, is highly immunoreactive (Turaga *et al.*, 1987), produced in relatively large amounts during *R.salmoninarum* infections (Kaattari *et al.*, 1987) and has been implicated in the generation of the systemic granulomatous lesions found in BKD (Villena *et al.*, 1989). The molecule has been shown to breakdown to highly immunogenic fragments (Wiens and Kaattari, 1991) and possesses has hydrophobic and leucocyte agglutinating properties (Bruno, 1988b).

Immunoblots performed during this project, confirmed both the abundance of p57, and its ability to break down to many fragments. Although the characterization of this molecule may go a long way in increasing our understanding of the initiation, progression and outcome of *R.salmoninarum* infection, its abundance, ubiquity and strong immunostimulatory properties may prove problematical in future characterization of other antigenic components of the pathogen, as its presence in all crude protein preparations, could quite easily mask other important and possibly protective antigens. Future antigenic analyses of *R.salmoninarum* therefore, would benefit from the availability of mutant strains of the bacterium, which are unable to produce p57 or that synthesize reduced

amounts of the protein.

All attempts to detect both haemolytic and proteolytic properties in preparations of the five *R.salmoninarum* isolates failed, and these negative results can be compared with those of similar studies. The presence of these properties has however been intimated by other workers, who have through both *in vitro* and *in vivo* experiments, concluded that these activities are produced by the bacterium (Austin and Rodgers, 1980; Bruno, 1985; Goodfellow *et al.*, 1985). Other workers have used pathological data to support their hypotheses that *R.salmoninarum* has both toxic/haemolytic and strong proteolytic activities (Young and Chapman, 1978; Turaga *et al.*, 1987). Future attempts to isolate and characterise these properties should be made, and may not only be aided by any developments towards a more appropriate culture system for *R.salmoninarum*, but by the application of alternative assay methods. These methods could include the assessment of cytotoxicity in cell culture, and the employment of a much wider range of substrate materials, both natural and synthetic, labelled and unlabelled. A better understanding of the growth cycle of *R.salmoninarum* may also aid the isolation of these putative pathogenicity determinants, as their production may be facilitated by factors linked to different phases of growth.

Apart from the *R.salmoninarum* components that formed the focus for the initial characterization of the bacterium made during this project, there are other properties and molecules that warrant further investigation. Catalase, esterase and lipase activities (Austin and Rodgers, 1980; Goodfellow *et al.*, 1985), would be suitable targets along with cell wall components such as glycolipids (Embley *et al.*, 1983). Other molecules may also be detected in *R.salmoninarum*, by assaying for molecules found in other intracellular bacterial pathogens such as *Listeria monocytogenes, Legionella pneumophila, Chlamydia spp.* and *Rickettsia spp.*. Molecules similar in *R.salmoninarum* may be detected and isolated using methods such as immunodetection of antigenically related components,

nucleic acid probes for related genes, or by PCR cloning with degenerate oligonucleotide primers.

The next stage of the research project, involved the application of recombinant DNA techniques to *R.salmoninarum*, and centred on the cloning of fragments of the organism's genome in *E. coli* host/vector systems to facilitate the construction of representative gene libraries. It was hoped that, by screening these gene libraries for antigens or specific activities of *R.salmoninarum*, that gene sequences encoding important molecules of the bacterium would be isolated. In the absence of any published work on the application of gene cloning to *R.salmoninarum*, the choice of *E. coli* host/vector systems for this purpose was based on the expertise present in the laboratory, and on the fact that the vast majority of similar studies made on bacterial pathogens have been made utilising cloning experiments in these systems. Therefore, a large body of information concerning the theory and practice of *E. coli*-based gene cloning was available, and comprehensive laboratory protocols exist for the techniques involved (Sambrook *et al.*, 1989). The choice of cloning vectors and *E. coli* host strains for use in this project was made using several criteria, and a full account of these can be found in chapters 4 and 5 of this thesis. Although the results presented in this thesis, and the work of Grayson (1993) and Chien *et al.* (1992) would appear to support this choice, each reporting the successful cloning of *R.salmoninarum* genes in *E. coli* host/vector systems, the merits in alternative cloning systems should also considered. Alternative cloning systems, utilising species of the Gram-positive genera *Bacillus*, *Staphylococcus*, *Streptomyces*, *Streptococcus* and *Corynebacterium* have also been developed (Old and Primrose, 1989). In conjunction with these systems a number of 'shuttle-vectors', capable of replication and gene expression in different host bacteria have also been constructed (Pouwels *et al.*, 1985). Studies on *R.salmoninarum* using these alternative cloning systems may also be beneficial because of their similar Gram-positive nature. Another recombinant DNA approach that may also

be considered is the possibility of constructing cloning vectors for *R.salmoninarum*, or attempting to use other available Gram-positive replicons for self cloning experiments. Transposon mutagenesis and allelic replacement experiments are also powerful tools for studying bacterial pathogens (Foster, 1991; Finlay, 1992), and the existence of a number of sequenced genes from *R.salmoninarum*, means that studies can now be made into the feasibility of using these techniques with the bacterium.

Once a number of *R.salmoninarum* gene libraries had been constructed in *E. coli* host/vector systems, these libraries were screened for recombinant viral or bacterial clones producing antigens or exhibiting biochemical properties originating from the expression of *Renibacterium* genes. A number of screening procedures were utilised for this purpose, however, only one stable recombinant (that bearing the *rsh* gene) was isolated for further analysis. The failure to detect clones producing *R.salmoninarum* antigens, or proteolytic components, could be attributed to a number of events, reflecting incompatibilty between either cloned DNA and the machinery of gene expression in *E. coli,* or between a product encoded by *R.salmoninarum* DNA and the metabolic activities of the *E. coli* cell. Failure to detect a clone of interest may also reflect defficiencies in the gene library screening procedure.

Incompatibility at the level of gene expression, can be the result of a number of attributes of the cloned gene, these include promoter strength (Dueschle *et al.*, 1986), structure of the ribosome binding site (de Boer *et al.*, 1983a) and choice of codons for amino acid synthesis (Kurland, 1987). Gene dosage effects which may vary with the copy number of the cloning vector employed, also affects whether a product produced by the cloned gene is detectable.

These possible events, may explain the failure in this study, to detect more than one *R.salmoninarum* component cloned in *E. coli,* however, this must be considered carefully in the light of the exploitation of gene fusion vectors and vectors containing strong *E. coli*

promoters in the construction for *R.salmoninarum* gene libraries. These vectors were also chosen with both high and low copy numbers to in an attempt to take into account gene dosage effects. Careful study of the nucleotide sequences of the three reported cloned *R.salmoninarum* genes, RHLY, (this project), MPR (Grayson, 1993) and MSA (Chien *et al.*, 1992)) would also indicate that incompatibility between the mechanisms of gene expression in *E. coli* and *R.salmoninarum* is unlikely, as all these genes have *E. coli*-compatible promoters and ribosome binding sites.

Failure to detect cloned components may be the result of differences in the biochemical processes of *R.salmoninarum* and *E. coli*. The host bacterium may not posess certain factors needed for any post-translational modification of *R.salmoninarum* proteins, or cloned products may be inactivated or degraded by enzymes present in *E. coli*. Conversely, cloned *R.salmoninarum* components may be lethal to *E. coli*, or seriously affect its metabolic processes, preventing the propagation of a recombinant clone and thus preventing its detection and isolation.

The inability to recover recombinant clones from *R.salmoninarum* gene libraries may also reflect defficiencies in the screening protocols adopted. Erythrocytes used as substrates for the detection of haemolytic clones were washed prior to use, to slow any deterioration of the cells *in vitro* and in an attempt to remove other blood constituents that could have interfered with the activity of cloned haemolytic agents. It may also be the case that components in the blood may have enhanced the activity of such agents, and therefore future protocols for screening *R.salmoninarum* gene libraries may be more successful with whole blood, especially whole salmonid blood, is included. Defficiencies in the screening of gene libraries for proteolytic activity may also be overcome by the use of different substrates, not only natural substrates such as elastin, fibrin and haemoglobin but a wider variety of chromogenic or fluorogenic synthetic substrates. Gene cloning studies on *R.salmoninarum* could also benefit from more selective screening methods. Protein

antigens, or molecules exhibiting an enzyme activity of interest could be purified, and a specific monoclonal antibody raised against them. Alternately, the N-terminal sequence of a purified protein may be determined, and by using the amino acid sequence, a specific oligonucleotide may be synthesised for gene library screening by DNA hybridization.

As a result of gene library screening, a stable haemolytic cosmid-containing *E. coli* clone was obtained for further analysis. The first stage of this analysis, was represented by attempts to subclone the haemolysis-conferring DNA fragment contained in the recombinant cosmid pRHLY1. The recombinant cosmid was found to contain a DNA insert of approximately 32 kilobase pairs. The haemolysis-conferring sequence of DNA was then subcloned on a 7.5 kbp fragment of DNA generated by *HindIII* cleavage. This fragment was ligated to the plasmid vector pBR328, forming the recombinant plasmid pRHLY2. One further round of subcloning resulted in the construction of pRHLY20, a recombinant plasmid consisting of a 1.65 kbp *Sal I*-generated DNA fragment contained within the low-copy plasmid pGD103. The purpose of the subcloning procedure was to remove DNA that was not essential to the production of the encoded haemolytic molecule, and may prevent its successful characterization. However, the recombinant cosmid pRHLY1 and the smaller plasmid pRHLY2, may be invaluable in further studies of the RHLY gene. Studies in the genetic control of bacterial pathogenicity and virulence, have revealed that important genes are often clustered together on 'virulence operons' and these are found in a number of important bacterial pathogens (Miller *et al.*, 1989; Mengaud *et al.*, 1991). Control regions for genes may also be found some considerable distance from the coding sequence of nucleotides (Dorman and Ni'Bhriain, 1992; Mekalanos, 1992), so by using the RHLY gene as a reference point, and sequencing the DNA inserts in pRHLY1 and pRHLY2, novel gene sequences and possible control regions for the expression of *R.salmoninarum* genes may be found.

The initial structural analysis of the haemolysis-conferring DNA fragment, also included

the construction of a restriction endonuclease cleavage map. This map was constructed utilising the recombinant plasmid pRHLY11 and its purpose was to determine restriction enzyme cleavage sites for a number of enzymes which would aid the further analysis of the cloned DNA. The map once constructed, proved invaluable for the formation of plasmid sequencing templates, and may in future be useful in the characterization of DNA sequences thought to be similar to that containing the *rsh* gene.

Southern blot hybridization using the insert in pRHLY11 as a DNA probe, revealed that the cloned 1.65 kbp *Sal I*-generated fragment of DNA was homologous to a sequence found in the genome of *R.salmoninarum*, and hence established the fidelity of the cloning procedure. It was also established that the sequence was present in all of the *R.salmoninarum* isolates used in this research project. The cloned *R.salmoninarum* DNA fragment, was not seen to hybridise to the negative control genomic DNAs extracted from *Aeromonas salmonicida* strain CM30, *Staphylococcus aureus* Oxford strain or *E. coli K12* strain DH1. The possibility exists that the cloned *R.salmoninarum* DNA fragment may comprise a specific hybridization probe for the bacterium (Macario and de Macario, 1990), and that this probe may be useful for the sensitive and specific detection of *R.salmoninarum* and the detection of homologous sequences in other bacteria. It is therefore important that further studies should be made, using the cloned DNA in hybridization experiments with DNAs from a wide range of bacterial species, so as to assess its specificity and to isolate related sequences from other organisms.

Once it had been confirmed that the cloned haemolysis-conferring fragment of DNA was representative of a sequence contained in the genome of *R.salmoninarum*, the characterization of the molecular determinant of haemolytic activity could begin.

Attempts at the biochemical characterization of the *R.salmoninarum* haemolysin (RHLY), were made using an experimental strategy designed firstly to detect and locate the molecule in haemolytic *E. coli* clones followed by its isolation and purification away from

E. coli components, and ultimately to analyse the purified molecule with respect to its structure, function and mode of action against erythrocytes. Another important analysis attempted at this stage of the research project, was aimed at confirming that the cloned haemolytic molecule was a true representative of a component present in preparations of *R.salmoninarum*. If this could be acheived, it would go a long way to validating the use of recombinant DNA techniques as applied to this organism, and at the same time, establish the fidelity of cloning procedures performed during this research project

SDS-PAGE and immunoblot analysis of protein preparations from a haemolytic *E. coli* clone and a comparative control bacterium, were not conclusive. Supplementary protein bands were not visible in gels stained with coomassie blue, and although immunoblotting with anti-*R.salmoninarum* sera indicated the presence of 3 extra protein bands in preparations of the *E. coli* clone containing pRHLY11, the apparent high titre of cross reacting antibodies prevented the conclusive detection of RHLY. It is difficult to say whether or not, with further experimentation, that electrophoresis-based separation techniques could have conclusively established the presence of RHLY. However, although it appeared that the molecular determinant of haemolytic activity was not produced in large amounts in the *E. coli* DH1/pRHLY11 clone, it is possible that the production of the molecule is linked in some way to the growth cycle of the *E. coli* host, and that its detection using SDS-PAGE could have been facilitated by the utilization of older (or younger) cultures of the *E. coli* clone. Detection of RHLY by SDS-PAGE analysis, may also have been possible, if the RHLY-encoding DNA fragment had been cloned into a vector with a higher copy number in *E. coli,* the increased production of the molecule brough about by the increased gene dosage effect, may have made a RHLY protein visible by this method.

The conclusive detection of RHLY utilizing anti-*R.salmoninarum* sera, may also have been possible, especially if cross reactive antibodies could in some way be removed.

Possible ways in which this could be acheived, are by either preabsorbing antibodies to *E. coli* components using a lysate of the bacterium (Harlow and Lane, 1989), or by the removal of these cross reacting antibodies by affinity chromatography of the rabbit antiserum through a matrix containing immobilised *E. coli* antigens (Dean *et al.*,1985).

The detection of a haemolytic molecule in the *E. coli* clone containing pRHLY11 was also attempted using an erythrocyte/agarose overlay of PAGE separated proteins in a zymogram experiment (Titball, 1983). However, no haemolytic activity was observed on any of the overlaid gels. Although the zymogram method is theoretically an effective means of detecting a protein by virtue of its biochemical properties, as the protein is effectively concentrated at on location in the gel by electrophoretic separation, the possible interference in the activity of the molecule by the conditions present during electrophoresis detracts from its application.

In the absence of a selective detection method for RHLY, the presence of protein encoded by the cloned haemolysis-conferring *R.salmoninarum* DNA fragment was studied using *in vivo* transcription and translation of the plasmid pRHLY20 in *E. coli* minicells. An autoradiograph of radiolabelled plasmid encoded products subjected to SDS-PAGE, revealed the presence of at least three supplementary protein bands in minicells containing the pRHLY20 plasmid when compared with minicells containing pGD103 vector DNA only. These three protein bands were found to be of similar size to those detected in preparations of the haemolytic *E. coli* clone DH1/pRHLY11 probed with anti-*R.salmoninarum* serum and were tentatively identified as representatives of the RHLY protein. Because the maximum theoretical size of the protein encoded by the cloned *R.salmoninarum* DNA fragment was considerably smaller than the sum total of sizes calculated for the protein bands detected in both immunoblot and minicell analysis, it was suspected that the complete RHLY molecule was represented by the protein detected in these analyses of a size 48-50 kDa. The other protein bands were thought to represent

truncated RHLY protein produced by incomplete synthesis of the molecule or degradation by proteolysis in the *E. coli* host.

Once the putative RHLY molecule had been detected, a sensitive and specific assay was required for use in any subsequent purification protocol. In the absence of a specific antiserum to the protein, or the direct biochemical detection of the molecule in electrophoresis gels, it was decided to investigate the use of a microtitre plate haemolytic assay for the detection of RHLY in cell-free preparations of the haemolytic *E. coli* clone. However, no haemolytic activity could be detected in either whole cell or concentrated ECP fractions, despite varying both the initial protein concentration, and the temperature of assay plate incubation. Haemolysis assays are one of the most widely used method for the detection and characterization of toxins and membrane-active agents of both bacterial and non-bacterial origins (Bernheimer, 1988), are simple to perform without complex reagents and if set up in microtitre trays, can be used with large numbers of test samples. Damage to the erythrocyte membrane results in the leakage of haemoglobin which in qualitative experiments can be detected by eye, and can be quantified by colorimetry or other spectrophotometric methods. The use of a microtitre plate haemolytic assay for the detection of RHLY, would provide a simple and efficient means of tracing the molecule during purification experiments, and could then aid the characterization of the molecule with respect to its biochemical nature. Further experimentation on the use of this method for the assay of RHLY is implicated. An excellent review on the haemolytic assay of bacterial toxins has been published by Bernheimer (1988). In this work, the various optimal conditions for the assay of cytolysins are presented. It is noted that in the haemolysis assay of a number of toxins, a protective protein is included in the assay mixture. These protein molecules, usually either gelatin or bovine serum albumin are used to prevent denaturation of the toxin in the presence of other bacterial components. The presence of certain metallic ions such as those of magnesium and calcium is also

considered, these ions are required for the maximal activity of many cytolysins such as the phospholipases (Reynolds *et al.*, 1991). Apparently the use of phosphate buffers in haemolytic assays often affects the activity of the toxin under study, as important divalent cations can be chelated by the phosphate compounds. This is especially relevant whilst considering the failure of the RHLY haemolytic assay, as PBSA was the diluent used in these experiments. In the case of the toxins that belong to the group known as the thiol-activated cytolysins, antigenically related membrane-damaging toxins of a variety of Gram-positive bacteria (Alouf and Geoffroy, 1991), the addition of reducing agents such as cysteine and dithiothreitol (DTT) is required for activation of the toxin molecule.

Further attempts at exploiting haemolytic assay in the study of RHLY, may be better served by taking these facts into account. Without a suitably sensitive, selective and specific assay, the isolation and purification of the RHLY protein away from the large number of *E. coli* proteins was thought too difficult to attempt using routine purification methods such as gel filtration and ion-exchange chromatography (Harris and Angal, 1990). This, and the time constraints placed on the research prevented the performance of a comprehensive program of experimental work to characterize the mode of haemolytic action mediated by RHLY and to determine the biochemical nature of the molecule. Whilst the RHLY protein was in the presence of possibly interfering *E. coli* host cell components, an objective study of the structure/function aspects of RHLY could not be performed. However a number of experiments were performed in an attempt to gain an insight into the possible nature of the haemolytic activity mediated by the RHLY protein. These experiments utilised erythrocyte/agarose overlays of the haemolytic *E. coli* clone DH1/pRHLY11 and the DH1/pBR328 comparative control. Of most value to further studies of the RHLY molecule, was the finding that the RHLY-containing *E. coli* was able to lyse erythrocytes from a number of animal species. This would seem to imply that haemolysis mediated by the RHLY protein, is a reflection of cytolytic damage to

eukaryotic membranes in general and not specific to the tissues of salmonid fish.

The site of action for bacterial membrane-active cytolysins, is commonly that of one of structural molecules of the eukaryotic membrane (Stephen and Pietrowski, 1986), and usually, means either the protein or lipid components. Overlay experiments on the haemolytic *E. coli* clone, using both egg yolk for the detection of lipolytic/phospholipolytic activities and casein for the detection of possible proteolytic activity, but none of these properties were found to be present. Once again, these experiments can only be seen as comprising a cursory study in to the mode of action of RHLY. A more comprehensive analysis would desirably utilize purified RHLY molecules and could consist of a wider range of substrates for proteolytic and lipolytic activity. Of particular relevance, is the use of membrane phospholipids in degradation experiments. Phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and sphingomyelin can be exposed to the test substance, and the degradation of the various substrates detected by thin layer chromatography.

Methods for the characterisation of the mode of RHLY action, could involve the use of erythrocyte ghosts or synthetic membranes of constructed multilamellar vesicles or liposomes, which would be examined by electron microscopy after exposure to the putative cytolysin (Tranum-Jensen, 1988). Such experiments could reveal whether RHLY-mediated haemolysis is a result of generalised membrane damage, or induced pore formation.

The haemolytic activity mediated by RHLY in the *E. coli,* may possibly not reflect the action of one molecule alone. Cell lysis may be the result of a cooperative process, which involves two or more components of bacterial origin acting on a target membrane either at the same time or consecutively. Cooperative lysis is predominantly a function of interactions within membrane lipid components (Fehrenbach and Jurgens, 1991), and involves a first step in which outer membrane phospholipids are hydrolysed by a

phospholipase and a consecutive lytic step involving enzymes such as cholesterol oxidase or a second phospholipase. Second step components may also consist of non-enzymic factors, the most studied being the CAMP-factor of group β-streptococci (Christie *et al.*, 1944; Bernheimer *et al.*, 1989). Other non-enzymic second stage lytic factors are found in *Bacillus spp.* Corynebacteriae, members of the Enterobacteriaceae, *Staphylococcus aureus* (Fraser, 1964) and *Actinobacillus pleuropneumoniae* (Frey *et al.*, 1989). Future studies of the haemolytic activity mediated by the cloned RHLY should be made to determine wether cell lysis results from the action of RHLY alone, or from the concerted action of RHLY and a component of *E. coli.*

More information about the RHLY protein, and the establishment of a similar molecule in preparations of *R.salmoninarum,* has been facilitated by the work of Grayson (1993). During this project, experiments were undertaken to construct a fusion of the gene coding for RHLY (*rsh*) with the β-galactosidase gene (*lac*) present on the plasmid expression vector (pEX). As a result of this gene fusion, a hybrid protein, representing portions of both the β-galactosidase protein and the RHLY protein was produced. As the presence of β-galactosidase could be detected in a simple assay, the hybrid protein could be readily purified. Subsequently, antibodies to the purified hybrid protein were raised in a rat. These antibodies were used in immunoblots of both whole cell and ECP preparations of a number of *R.salmoninarum* isolates. It was found that anti-β-galactosidase/RHLY antibodies bound to a number of protein bands in the whole cell preparations of *R.salmoninarum*, but not to proteins in the ECP fraction. Two distinct bands were visible at approximately 80 kDa whilst two other bands were detected at sizes 49 kDa and 28 kDa.

Sometime after the practical phase of this research project, an immunoblot was performed using the antiserum raised to the β-galactosidase/RHLY fusion to probe a whole cell preparation of the *R.salmoninarum* isolate MT444. This blot was performed to both

establish the fidelity of the RHLY cloning procedure, and in some way to validate the whole research project with respect to the application of cloning to studies of *R.salmoninarum*. The developed immunoblot revealed antibody bound-proteins in the *R.salmoninarum* preparation, of sizes equivalent to those reported above in immunoblots of RHLY-containing *E. coli* probed with anti-*R.salmoninarum* whole cell serum, and in the immunoblots performed by Grayson (1993). These results support those obtained in minicell analysis of a RHLY encoding plasmid, results that indicate that the RHLY protein is of a size in the region of 48-50 kDa. These results also confirm that the cloned RHLY molecule is similar to a native protein component of *R.salmoninarum*.

In the absence of any conclusive evidence for the structure, function or mode of action of the RHLY molecule, it was decided to take another possible route to its characterisation. By determining the nucleotide sequence of the cloned DNA fragment encoding the RHLY protein, and analysing the results obtained, it was hoped that the coding sequence for RHLY could be studied, and clues found to the structure/function aspects of the molecule itself.

The 1.65kbp cloned *R.salmoninarum* DNA insert of pRHLY11, coding for the production of the RHLY protein, was subcloned in three portions to form sequencing templates in the vector pUC18. These inserts were then sequenced utilizing the dideoxy chain-terminating method of Sanger *et al.*, (1977) with both forward and reverse pUC/M13 sequencing primers. A complete nucleotide sequence for the entire DNA insert of pRHLY11 was compiled by comparing and joining at regions of overlap the base sequences obtained from the three sequencing subclones. Sequencing using double-stranded plasmid templates (Chen and Seeburg, 1985) has been found by some workers (Dr. S.E. Hunter and Prof. G. Dougan, pers. comm.) less reliable for generating DNA sequence information that the widely used single stranded templates generated by the M13 phage-derived vectors. The problems encountered by these workers was seemingly related to the purity of the plasmid

preparation and the incomplete denaturation of the template DNA. The plasmid templates used in this research project however were purified by caesium chloride gradient ultracentrifugation and care was taken in any denaturing steps in the protocol. Despite a few problems encountered in obtaining unambiguous sequence information from G and C rich regions of the DNA being sequenced (a problem incidently, also experienced with M13 vectors (Sambrook *et al.*, 1989)), double-stranded templates were found to perform well in sequencing reactions.

The complete sequence was found to be 1,674 nucleotides in length and a computer-constructed restriction endonuclease cleavage map, verified the positions of recognition sequences for the enzymes *Ava I, Hpa I* and *Pvu I* previously established. No recognition site however, was detected for *Kpn I* an enzyme for which a cleavage site was ascribed in the preliminary restriction map. The recognition sequence for *Kpn I* is GGTACC, and no such combination of nucleotides can be detected in the sequence information obtained for the cloned *R.salmoninarum* DNA fragment. Possible reasons for the absence of this sequence are a deletion event in the original cloned DNA, or that *Kpn I* cleavage occurred due to the relaxation of recognition sequence specificity (star activity) sometimes found with this enzyme (Kessler and Holtke, 1986).

The next computer-aided analysis performed on the compiled nucleotide sequence, was a search for an open reading frame of nucleotide bases, that could signify a region of the cloned *R.salmoninarum* DNA that was large enough to code for the RHLY protein. Only one ORF of suitable size was detected in the nucleotide sequence, a 1248 bp region, commencing at a putative initiation sequence (ATG) at nucleotide 154, to a putative termination sequence (TAG) at nucleotide 1402. Whether or not this represents the true ORF for the RHLY protein, has not been conclusively proved, however two pieces of evidence exist to support this finding. Firstly, and detected at the time of sequence analysis, putative regions of transcriptional and translational control were found

surrounding this ORF in the appropriate positions, and secondly, a β-galactosidase/RHLY fusion protein (Grayson, 1993) was constructed at a later date using this sequence information. Future studies may be warranted to confirm the position of the RHLY ORF, and such studies should involve the deletion of DNA from positions supposedly distal and proximal to the putative ORF and the subsequent effect on the products encoded in coupled transcription/translation analysis such as minicell experiments. The removal of DNA from the ORF, distal to the initiation sequence, should result in the truncation of the RHLY protein, whilst deletion events that interfere with the transcription and translation control sequences should prevent the expression of the putative *rsh* gene and result in lack of RHLY synthesis. Further experimentation would also confirm the location and operation of the putative *rsh* promoter region. Removal of DNA downstream from the ATG initiation codon, and its replacement with, in frame, a coding sequence for a protein whose production can be readily assayed (de Boer *et al.*, 1983b), would enable the operation of the putative *rsh* promoter region to be detected.

By utilizing the putative ORF in the *rsh* gene, an amino acid sequence for RHLY was derived. Once again, the accuracy of this sequence, derived from the nucleotide sequence of the *rsh* gene may be ascertained by future experimentation. Enzyme cleavage of purified RHLY protein could be used to generate peptide molecules, for which N-terminal amino acid sequences could be determined (Creighton, 1990). The sequences of these peptides should correspond to portions of the computer-derived amino acid sequence for RHLY. Once the presence of the *rsh* gene had been established, and a putative amino acid sequence for the RHLY protein determined. Attempts were made to further characterize structure/function aspects of the cloned RHLY molecule by computer-aided analyses of the amino acid sequence and its comparison with those lodged in the PIR database.

A helpful guide to the analysis of nucleotide and sequence data has been published by Doolittle (1986), not only does this work indicate the value of sequence analysis, it also

points out the relevance of the large amount of data produced.

The data generated on the codon usage, the prediction of secondary structure, hydropathy and possible antigenic sites of the RHLY protein, although displayed in this thesis, is included solely for reference purposes. It serves to indicate the versatility of computer-aided sequence analysis and to aid the future 'fine tuning' of the biochemical characterization performed on the RHLY protein. Without comparative data for similar protein molecules, or homologous amino acid sequences, this comprehensive analysis of the cloned molecule is not possible. The nuances in the variation of α-helix, β-sheeting, turns and coils, are in the realm of the biophysicist, and comprise a lengthy research programme in their own right. Hopefully, in the future, when the structure, function and mode of action for RHLY has been elucidated, this secondary structure data will be valuable.

A search of the PIR protein sequence database, was undertaken to look for amino acid sequences of proteins with known structure and/or function, that were homologous to RHLY. In this way, it was hoped that clues to the structure and biochemical properties of the cloned protein could be gained.

A search of the PIR database, revealed 200 sequences similar to RHLY, but when these sequences were aligned, it was found that the similarity was largely based on homology between single amino acids spaced along the length of the two sequences, and not the result of homology between extended sequences, a possible indication of structural and/or functional homology.

The reaction to this finding can be one of frustration or great excitement, as the inability to detect significant homology can be seen, as a pessimist, the reflection of an incorrectly derived amino acid sequence, or as an optimist, indication of a novel protein awaiting further study. In the case of this research project, feelings were mixed.

One sequence detected in the database however, showed homology with RHLY in a

region consisting of over 30 amino acids, this protein bieng the product of a regulatory gene (*dniR*) for the dissimilatory nitrite reductase of *E. coli K12*. Further alignment of RHLY and the *dniR* protein revealed that the homologous regions were conserved and repeated in both molecules, twice in RHLY and once in *dniR*. As conserved amino repeated amino acid motifs often have important structure/function implications for a protein, information gained on the *E. coli dniR* protein may have aided the further characterization of the cloned RHLY molecule. Correspondence with Dr. Sinichi Kajie, who was one of the workers involved in the cloning and sequencing of *dniR*, revealed that one of the repeated motifs in the *E. coli* protein, contains a region similar in structure to *E. coli* DNA binding proteins. The presence of a putative DNA binding region in RHLY is debatable, but requires further investigation. Whether the *rsh* gene of *R.salmoninarum* is under the same environmental control as *dniR* in *E. coli* is unknown, however the synthesis of a haemolysin found in *Actinobacillus pleuropneumoniae,* is regulated by the product of the *HlyX* gene which in turn is functionally homologous to the global anaerobic regulator protein FNR (MacInnes *et al.*, 1990). An FNR binding site can be found proximal to the ORF in the *dniR* protein and is thought to be regulated in the same manner. Although no FNR-binding site was detectable proximal to the *rsh* gene of *R.salmoninarum*, further sequencing upstream of the RHLY-encoding gene may reveal such a site.

It should be noted that the results of such exercises should be approached with caution, as the dangers of extrapolating from sequence homology information, are great (Doolittle, 1986). Although information on the combination and relative amounts of specific amino acid residues, may give clues to the characteristics of a protein, there is no substitution for actual biochemical and molecular biological experimentation. Information on the structure/function aspects of the *dniR* protein of *E. coli* and other functionally homologous molecules, may give impetus for further studies on the cloned RHLY protein and the

molecule as found in *R.salmoninarum*, but no concrete evidence is yet available to ascribe any indisputable property to the molecule.

As to the role of the RHLY protein, if an identical molecule exists in *R.salmoninarum*, one can only speculate. RHLY may only play a role in the metabolism of the bacterium, being either an enzyme with catabolic or anabolic significance, or even merely a structural component. The indicated cell associated nature of the molecule sheds little light on its function. However, if the protein is surface-exposed, this may reflect a role in the pathogenesis of *R.salmoninarum* infections and may even be vital for the successful colonisation of the host. The apparent membrane active properties could play a significant part in the establishment and progression of BKD and may even have significance in the invasion of host cells. The electron microscopic studies of Gutenberger (1991) reveal that once inside the phagolysosome of the salmonid macrophage, *R.salmoninarum* cells appear to disrupt the surrounding membrane on contact, it would be interesting to discover whether the cell-associated determinant of membrane lysis is RHLY itself.

The immunological significance of RHLY is also unknown, its cytolytic activities may play a part in the immunosuppression detected in *R.salmoninarum* infections (Turaga *et al.*, 1987) by disabling or even destroying cells of the immune repertoire.

Future characterization of the RHLY protein may answer some of these questions, and the possible construction of RHLY-negative mutants of *R.salmoninarum* could determine its role in pathogenicity and virulence.

It is clear from the information gathered during this research project, that the techniques of the recombinant DNA technology, offer new direction to the study of *R.salmoninarum*, an important pathogen of salmonid fish which has for decades resisted the many attempts made to solve the many mysteries of its biology. If the bacteria is truly an obligate intracellular parasite, that has through evolution, formed ways of surviving in the tissues of its host, it surely must possess mechanisms and molecules equivalent to those in similar

animal pathogens. Just as gene-cloning and the allied techniques of molecular biology have brought greater understanding of other 'difficult' bacteria, the time must be upon us to follow a similar path with *R.salmoninarum.*

Since the commencement of this research project, the author has noted the other successful gene cloning based studies of *R.salmoninarum.* In addition to the RHLY protein, the major soluble antigen (MSA, p57 or Haemagglutinin), to date the only *R.salmoninarum* molecule with proven roles in BKD establishment and progression, has also been cloned (Chien *et al.*, 1992). A co-worker in the authors laboratory has also succeeded in cloning a 65 kDa haemolytic protein (unrelated to RHLY), with the structural properties of bacterial zinc-metalloproteases (Grayson, 1993).

There is no doubt, that more *R.salmoninarum* molecules will be isolated in this manner, and studied not only specifically for their roles in BKD, but as a vital route to the better understanding of this enigmatic bacterium.

In concluding this thesis, the author accepts that such sweeping praise for molecular biology, must smack of reductionism. However, let us reflect on the fact that often, the most beautiful paintings have the most beautiful brushwork.

APPENDIX I

Formulation and preparation of reagents, solutions and growth media.

BACTERIOLOGICAL MEDIA.

LURIA BERTANI (LB) MEDIUM.

per litre of ddH_2O:

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

LB agar as above, with the addition of 1.5% w/v Agar No.2
Sterilized by autoclaving 15 min. at 15 lb/in^2.

ANTIBIOTIC STOCK SOLUTIONS.

Ampicillin (sodium salt)	50 mg/ml in ddH_2O
Chloramphenicol	25 mg/ml in ethanol
Kanamycin	25 mg/ml in ddH_2O
Tetracycline.	10 mg/ml in 50% v/v ddH_2O/ethanol

Sterilized by filtration (0.22 μm).

MHC MEDIUM.

per litre of ddH_2O:
Mueller-Hinton medium 21 g.
Cysteine hydrochloride 1 g.
pH adjusted to 6.8 with NaOH as required.

MHC agar as above, with the addition of 15 g/l Agar No.2
Sterilized by autoclaving 15 min. at 15 lb/in^2.

SELECTIVE KIDNEY DISEASE MEDIUM (SKDM).

per litre of ddH_2O:

Tryptone	10 g
Yeast extract	0.5 g
Cycloheximide	0.05 g
Agar No.2	10 g

The components were dissolved, the pH adjusted to 6.8 with NaOH and the medium sterilized by autoclaving for 15 min. at 15 lb/in^2. The medium was allowed to cool, before the addition of the following filter sterilized (0.22μm) components:

Foetal calf serum	100 ml
Cysteine hydrochloride (10% w/v)	1 ml
D-cycloserine (0.125% w/v)	1 ml
Polymyxin B sulphate (0.25% w/v)	1 ml
Oxolinic acid (0.025% w/v)	1 ml

KIDNEY DISEASE MEDIUM VERSION 2 (KDM2).

Tryptone	10 g
Yeast extract	0.5 g
Cysteine hydrochloride	1 g
Agar No.2	15 g

The components were dissolved, the pH adjusted to 6.8 with NaOH and the medium

sterilized by autoclaving for 15 min. at 15 lb/in^2. The medium was allowed to cool, before the addition of 25 ml of filter sterilized (0.22μm) foetal calf serum.

BACTERIOPHAGE METHODS.

SM BUFFER.

Per litre ddH_2O:

NaCl	5.8 g
$MgSO_4.7H_2O$	2 g
1M. TRIS pH (7.5)	50 ml
2% Gelatin solution	5 ml

Made up in 100 ml batches, sterilized by autoclaving for 15 min. at 15 lb/in^2.

SOFT AGAR.

Per litre ddH_2O.

Tryptone	10g
NaCl	5g
Agar No.2	6g
1M $MgSO_4$	10ml

Made up in 100ml batches, sterilized by autoclaving for 15 min. at 15 lb/in^2

PROTEIN ANALYSIS METHODS.

CONCENTRATION OF BACTERIAL PROTEIN SOLUTIONS.

PBSA

1 tablet PBSA (Oxoid,Basingstoke) dissolved in 100 ml ddH_2O.
Sterilized by autoclaving for 15 min. at 15 lb\in.^{2}

SDS/PAGE

SDS/PAGE GEL MIXES.

Per gel (LKB 2001 system):

Stacking gel (4%)

Acrylamide/Bis-acrylamide	1.3 ml
stacking gel buffer	2.5 ml
10% SDS	100 μl
ddH_2O	6.1 ml
10% APS	50 μl

Separating gel (5%)

Acrylamide/Bis-acrylamide	1.75 ml
Separating gel buffer	1.25 ml
10% SDS	100 μl
ddH_2O	6.85 ml
10% APS	50 μl

Separating gel (10%)

Acrylamide/Bis-acrylamide	3.5 ml
Separating gel buffer	1.25 ml
10% SDS	100 μl
ddH_2O	5.1 ml
10% APS	50 μl

SDS/PAGE SOLUTIONS.

SEPARATING GEL BUFFER.

TRIS	36.3 g
N,N,N',N',- Tetramethylethylenediamine	230 μl

Dissolved in approx. 90 ml ddH_2O, pH adjusted to 8.9 and diluted to 100 ml with ddH_2O.
Stored at 4°C for no more than 3 weeks.

STACKING GEL BUFFER.

TRIS	5.98 g
N,N,N',N',- Tetramethylethylenediamine	460 μl

Dissolved in approx. 90 ml ddH_2O, pH adjusted to 6.7 and diluted to 100 ml with ddH_2O.
Stored at 4°C for no more than 3 weeks.

ACRYLAMIDE/BIS-ACRYLAMIDE SOLUTION.

Acrylamide	28.0 g
N,N'-Methylenebisacrylamide	0.74 g

Dissolved and diluted to 100ml with ddH_2O and filtered using Whatman No.1 paper filter.
Stored in a dark bottle at 4°C for no more than 8 weeks.

AMMONIUM PERSULPHATE (APS).
10% w/v solution in ddH_2O, made immediately prior to use in 1ml volumes.

SODIUM DODECYL SULPHATE (SDS) SOLUTION
10% w/v solution in ddH_2O

Made in 100 ml volumes, filtered using Whatman No.1 paper filter and stored at ambient temperature for no more than 12 weeks.

ELECTRODE BUFFER.
Per litre of ddH_2O:

TRIS	3.025 g
Glycine	14.4 g
SDS	1 g

pH adjusted to 8.3 using NaOH.

SAMPLE BUFFER.

TRIS	1.51 g
glycerol	20 ml

Dissolve with 35 ml ddH_2O, pH adjusted to 6.75, followed by the addition of:

SDS	4.0 g
Bromophenol blue	0.002 g

dilute to 90 ml with ddH_2O Store at ambient temperature for no more than 12 weeks. Sample buffer for electrophoresis under reducing conditions was made prior to use by adding 2-β-Mercaptoethanol to a final concentration of 10% (v/v).

STAINING OF POLYACRYLAMIDE GELS.

COOMASSIE BLUE STAINING SOLUTION.

Coomassie brilliant blue-R	1.25g
in a 500ml volume of:	
Methanol	400 ml
Glacial acetic acid	70 ml
Distilled water	530 ml

Filtered using a Whatman No.1 paper filter.

DESTAINING/FIXING SOLUTION.

Methanol	400 ml
Glacial acetic acid	70 ml
Distilled water	530 ml

IMMUNOBLOTTING (WESTERN BLOTTING).

TRANSFER BUFFER.

per 2 litre volume:	
TRIS	6.1 g
Glycine	28.8 g
Methanol	400 ml
ddH_2O	1600 ml

Adjust pH to 8.3, store at 4°C.

This is the buffer of choice for the Bio-Rad Transblot system but at 0.5 x concentration it will also mediate protein transfer using the Milliblot semi-dry system.

IMMUNOSTAINING.

DAB/NICKEL DEVELOPMENT SOLUTION.

PBSA	100 ml
DAB	50 mg
$NiCl_2$	300 mg

Filtered using Whatman No.1 paper filter.
The solution was made fresh immediately prior to blot development and the chromogenic reaction was initiated by the addition of 100 μl of hydrogen peroxide solution.

DNA ANALYSIS METHODS.

MARMUR GENOMIC DNA ISOLATION.

All solutions made in ddH_2O.

SUCROSE/TRIS SOLUTION.
Sucrose 25% w/v in 0.05 M TRIS-HCl pH 8.0

LYSOZYME SOLUTION.
Lysozyme 10 mg/ml in 0.25 M TRIS-HCl pH 8.0

EDTA SOLUTION.
EDTA (sodium salt) 0.25 M solution pH 8.0

TRITON LYSIS SOLUTION.

Triton X100	2% w/v
TRIS/HCl	0.05 M
EDTA (sodium salt)	0.063 M

pH adjusted to 8.0

ACETATE-EDTA SOLUTION.

Sodium actetate	3 M
EDTA (sodium salt)	1 mM

pH adjusted to 7.0

SALINE EDTA SOLUTION.

NaCl	0.15 M
EDTA (sodium salt)	0.1 M

pH adjusted to 8.0

PERCHLORATE SOLUTION.
Sodium perchlorate 5 M

ETHANOL.
Solutions of 70%,80%,90% and 95% v/v ethanol in analytical grade H_20.

STANDARD SALINE-CITRATE SOLUTION.

NaCl	0.15 M
Trisodium citrate	0.015 M

DILUTE SALINE-CITRATE SOLUTION.

NaCl	0.015 M
Trisodium citrate	0.0015 M

CONCENTRATED SALINE-CITRATE SOLUTION.

NaCl	1.5 M
Trisodium citrate	0.15 M

RIBONUCLEASE (RNase).
A 2% w/v solution in 0.15 M NaCl

pH adjusted to 8.0. The solution was incubated at 95°C for 10 min. to inactivate any

contaminating deoxyribonuclease.

ISOLATION OF PLASMID DNA FROM *E. coli.*

MINIPREP ISOLATION.

All solutions made in ddH_2O.

TE BUFFER.

TRIS	10 mM
EDTA (sodium salt)	1 mM

Made up in 100 ml volumes, sterilized by autoclaving for 15 min. at 15 lb/in.^{2}

B & D SOLUTION I.

Glucose	50 mM
TRIS/HCl	25 mM
EDTA (sodium salt)	10 mM

pH adjusted to 8.0, made up in 100 ml volumes and sterilized by autoclaving for 15 min. at 15 lb/in.^{2}

Prior to use add 5 mg/ml lysozyme to sterilized solution.

B & D SOLUTION II.

ddH_2O	4.3 ml
NaOH (5 M)	0.2 ml
Sodium dodecyl sulphate (10% solution)	0.5 ml

B & D SOLUTION III.

Sodium acetate	3 M

pH adjusted to 4.8 with glacial acetic acid, stored at 4°C

RNase SOLUTION.

Ribonuclease A (pancreatic RNase) 1 mM in a 50 mM TRIS buffer (pH 8.0)
Before use, this solution is incubated at 95°C for 15 min. to remove any contaminating deoxyribonuclease.

AGAROSE GEL ELECTROPHORESIS OF DNA

TBE BUFFER (x10).

TRIS	0.9 M
Boric acid	0.9 M
EDTA (sodium salt)	0.025 M

pH adjusted to 8.5, sterilized by autoclaving for 15 min at 15 lbs/in^2. This x10 stock is diluted as required.

FICOLL LOADING BUFFER.

per 10ml ddH_2O:

Bromophenol blue	0.025g
Ficoll 400	1.5g

ETHIDIUM BROMIDE SOLUTION.

Ethidium bromide 10 mg/ml in ddH_2O
Stored at 4°C in dark bottle.

DNA MANIPULATION METHODS.

TRANSFORMATION OF *E.coli* K12 STRAINS.

SOB MEDIUM.
per litre of Analar H_2O:

NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM

Combine Tryptone, Yeast extract, NaCl and KCl in water and sterilize by autoclaving for 15min. at 15lb/in^2.
$Mg^{2+}$are added prior to use, from a filter sterilized (0.22μm) stock of 1M. $MgCl_2$ plus 1M. $MgSO_4$ in ddH_2O.

SOB agar as above, with the addition of 1.5% w/v Agar No.2

SOC MEDIUM is identical to SOB medium, with the addition of glucose to a final concentration of 20 mM.

RF1 MEDIUM.
per litre ddH_2O:

RbCl	12 g
$MnCl_2.4H_2O$	9.9 g
Pottassium acetate (1M, pH 7.5)	30 ml
$CaCl_2.2H_2O$	1.5 g
Glycerol	150 g

pH adjusted to 5.8 with 0.2M acetic acid and filter sterilized (0.22 μm)

RF2 MEDIUM.
per litre of ddH_2O:

MOPS (0.5M, pH 6.8)	20 ml
RbCl	1.2 g
$CaCl_2.2H_2O$	11 g
Glycerol	150 g

BLUE/WHITE SCREENING.

MINIMAL MEDIUM (M9).
per litre of ddH_2O:

Na_2HPO_4	6 g
KH_2PO_4	3 g
NH_4Cl	1 g
Agar No.2	15 g

Sterilize by autoclaving at 15 lb/in^2, allow to cool to 50°C then add the following

filter sterilized (0.22 μm) solutions:

$MgSO_4$ (1M)	2 ml
$CaCl_2$ (1M)	0.1 ml
Glucose (20% w/v)	10 ml
Thiamine HCl (1M)	1 ml

X-GAL SOLUTION.

A 2% w/v solution of 5-bromo-4-chloro-3-indolyl-β-$_D$-galactopyranoside in dimethylformamide.

IPTG SOLUTION.

A 24 mg/ml solution of isopropyl-β-$_D$-thiogalactopyranoside in ddH_2O

DNA SEQUENCING.

SEQUENCING GEL MIX (4%)

Urea	21 g
40% w/v acrylamide [19:1] bisacrylamide	5 ml
TBE buffer x10	5 ml
ddH_2O	22.5 ml

SEQUENCING GEL MIX (6%)

Urea	21 g
40% w/v acrylamide [19:1] bisacrylamide	7.5 ml
TBE buffer x10	5 ml
ddH_2O	20.25 ml

dissolve the urea, make up to 46 ml with ddH_2O and filter (0.45μm membrane). De-gas for 15 min followed by the addition with gentle mixing of:

10% APS	400μl
TEMED	40μl

APPENDIX II

The genetic code, and amino acid data.

First Position (5′)	Second Position								Third Position (3′)
	U		C		A		G		
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys	C
	UUA	Leu	UCA	Ser	UAA	Ter[1]	UGA	Ter[3]	A
	UUG	Leu	UCG	Ser	UAG	Ter[2]	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC	Leu	CCC	Pro	CAC	His	CGC	Arg	C
	CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	A
	CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg	G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser	C
	AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	A
	AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg	G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly	C
	GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly	A
	GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly	G

GENETIC CODE

[1] Chain terminating Ochre
[2] Chain terminating Amber
[3] Chain terminating Umber

Amino acid	3 Let Abbrev.	1 Let Abbrev.	Mol Weight	Codon	Functional	Hydrophobic	Charge	Chemical
Alanine	Ala	A	89	GCA GCC GCG GCU	HNP	Pho	0	Aliphatic
Arginine	Arg	R	174	AGA AGG CGA CGC CGG CGU	+	Phi	+	Basic
Asparagine	Asn	N	132	AAC AAU	POU	Phi	0	Amide
Aspartic acid	Asp	D	133	GAC GAU	-	Phi	-	Acidic
Cysteine	Cys	C	121	UGC UGU	POU	Phi	0	Sulfur
Glutamic acid	Glu	E	147	GAA GAG	-	Phi	-	Acidic
Glutamine	Gln	Q	146	CAA CAG	POU	Phi	0	Amide
Glycine	Gly	G	75	GGA GGC GGG GGU	POU	Phi	0	Aliphatic
Histidine	His	H	155	CAC CAU	+	Phi	+	Basic
Isoleucine	Ile	I	131	AUA AUC AUU	HNP	Pho	0	Aliphatic
Leucine	Leu	L	131	CUA CUC CUG CUU UUA UUG	HNP	Pho	0	Aliphatic
Lysine	Lys	K	146	AAA AAG	+	Phi	+	Basic
Methionine	Met	M	149	AUG	HNP	Pho	0	Sulfur
Phenylalanine	Phe	F	165	UUC UUU	HNP	Pho	0	Aromatic
Proline	Pro	P	115	CCU CCC CCA CCG	HNP	Pho	0	Imino
Serine	Ser	S	105	AGC AGU UCA UCC UCG UCU	POU	Phi	0	Hydroxyl
Termination	End	*	-	UAA UAG UGA				
Threonine	Thr	T	119	ACA ACC ACG ACU	POU	Phi	0	Hydroxyl
Tryptophan	Trp	W	204	UGG	HPH	Pho	0	Aromatic
Tyrosine	Tyr	Y	181	UAC UAU	POU	Phi	0	Aromatic
Valine	Val	V	117	GUA GUC GUG GUU	HNP	Pho	0	Aliphatic

HNP = Hydrophobic nonpolar
POU = Polar uncharged
Pho = Hydrophobic
Phi = Hydrophillic

AMINO ACID DATA

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