MOLECULAR ANALYSIS of the SALMON PATHOGEN *Piscirickettsia salmonis*

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ABBREVIATIONS

APS	Ammonium persulphate
BSA	Bovine serum albumin
$CHCl_3$	Chloroform
CSPD®	Disodium 3-(4-methoxyspiro{1,2,dioxetane-3,2,-(5'-chloro)tricyclo[3.3.1.1]decan}-4-yl [phenyl phosphate
dH_2O	Distilled water
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
H_2O_2	Hydrogen peroxide
IPTG	Isopropyl-β-D-galactopyranoside
LPS	Lipopolysaccharide
MEM	Minimal Essential Media
$MgCl_2$	Magnesium chloride
MIP	Macrophage infectivity potentiator
NaOH	Sodium hydroxide
OPD	O-Phenylenediamine
PBS	Phosphate buffered saline
PFU	Plaque forming units
RLO	Rickettsial-like organism
SDS	Sodium dodecyl sulphate
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	N, N, N, N- tetra-methylenediamine

SUMMARY

Piscirickettsia salmonis is the causative agent of Piscirickettsiosis, a disease causing significant losses in salmon aquaculture in Chile and which has also been isolated in salmonids in several other countries. This investigation was designed to prepare a recombinant library of *P. salmonis* DNA in a phage lambda vector. *P. salmonis* LF-89 was grown in CHSE-214 and RTG-2 tissue culture cells and morphological changes monitored by light microscopy and staining. Cytoplasmic vacuoles were visible in infected cells by 5 days post-infection and these expanded in size until cells burst, releasing bacteria into the culture fluid. Within 14-17 days the complete cell monolayer showed cytopathic effects and cells detached from the culture surface. Bacteria were collected from large volumes of culture fluid by centrifugation, resuspension and brief homogenisation to disperse the material, low speed centrifugation to remove large cellular debris, followed by purification of bacteria from cell debris by centrifugation on a Percoll density gradient. DNA was extracted from the bacteria, partially digested with *EcoRI* and ligated into λExCell. The library appeared to contain approximately 1.79 x 10^5 recombinants/μg DNA, and the recombinant library was screened with rabbit antiserum to purified *P. salmonis* and antisera raised in fish. No reactive clones were detected in screening with antisera, or with oligonucleotide probes against both *P. salmonis* 16S rRNA gene and *Legionella pneumophila* Mip gene.

A representative selection of recombinant phage was analysed. None of these recombinant phage inserts could be released with *EcoRI*, the restriction enzyme used in preparation of the library. Release of inserts with restriction enzymes flanking the *EcoRI* site was successful and several inserts, ranging in size from 0.4 to 3.5kb were detected. Sequence analysis of two inserts showed that the expected *EcoRI* site in pExCell recombinants was corrupted to the sequence CCATTC or GCGTTC. Limited sequence analysis of inserts revealed very high identity with *E. coli* gene sequences in the databases, and little similarity with rickettsial sequences.

During this project an outbreak of a suspected rickettsial infection was identified in Scotland and the Ardintoul isolate was characterised. The organism replicated readily

in CHSE-214 and RTG-2 cells and was also cultivated in XTC cells (a frog-derived cell line).The organism was viewed by light microscopy, electron microscopy and fluorescence microscopy. The 16S rRNA sequence of the organism was compared with those of previously characterised piscirickettsia and found to differ by at least 8 bases from other isolates. Closest similarity was to isolates SLGO-94 (British Columbia) and LF-89 (Chile, type strain). Bacteria were purified from cells and a recombinant library prepared in λFixII. Inserts of 4 to 9kb were detected in the library and preliminary screening was carried out with oligonucleotide probes, although no true positive clones were identified. Antiserum to purified Ardintoul rickettsiae was raised in a sheep. Analysis by ELISA showed the serum to be of high titre, and in immunoblotting it recognised at least 15 *P. salmonis* antigens, and had very little reactivity with cellular material. The dominant antigen recognised was a polypeptide of c. 70kDa, with other antigens of 80, 55, 50 and 20kDa also inducing a significant response. The antiserum will be useful in further analysis of the λFixII library for identification of surface-exposed antigens which could form the basis of future vaccine development.

INTRODUCTION

Aquaculture is a growing industry, with production amounting to an estimated 15 million metric tons in 1990 (FAO 1992) accounting for roughly 15% of the total world production of fish. As part of this growth, salmon aquaculture has developed into a major industry in Norway, Scotland, Chile and several other countries making a significant contribution to the fisheries industry. In Scotland the production of Atlantic salmon was over 87000 tonnes in 1997, and in Norway was over 300000 tonnes (G. Rae, personal communication). One of the limiting factors in the development of aquaculture has been infectious disease, and the rapid expansion of fish culture has been accompanied by increased mortalities due to disease. The effects on fish can cover an entire spectrum from epizootics with mass mortalities to minor chronic losses. Disease control in aquaculture depends on correct diagnosis and understanding of the life cycle of the causative agent, preventative measures, such as maintenance of water quality, provision of an adequate diet, prophylactic immunisation and reduction of environmental stress, as well as treatment, usually in the form of chemotherapy, as a last resort. Stress is an important factor in the outcome of an infection within a fish population; it may result from overcrowding, inadequate diets, oxygen deficiency, build-up of metabolic by-products, toxic chemicals from external sources, temperature variations, and other less obvious features of what is at best an abnormal environment for aquatic animals. A more positive approach to disease control is that of prophylactic immunisation. With newer methods of vaccine application, treatment of entire cultured fish populations has become feasible (Sindermann, 1989)).

1.1 Microbial diseases in salmon aquaculture

There is extensive literature on diseases that are important in sea water culture of salmonids, the most significant of which are summarised in Table 1. Although bacteria are important pathogens of fish, viruses and parasites also cause serious problems for the industry.

Table 1 Important Diseases of Farmed Salmon

	ORGANISM	DISEASE
BACTERIAL	*Vibrio anguillarum*	VIBRIOSIS
	Vibrio ordalii	
	Renibacterium salmoninarum	BACTERIAL KIDNEY DISEASE
	Aeromonas salmonicida	FURUNCULOSIS
SEA LICE	*Lepeophtheirus salmonis*	
VIRAL	Infectious Pancreatic Necrosis Virus	

A small selection of diseases affecting farmed salmon is shown in Table 1; (information adapted from Leong, (1993 & 1995) who reviewed fish vaccine advances)

Sea pen culture of Atlantic salmon began in Scotland and Norway over 30 years ago and culture of several species, particularly of Coho salmon, *Oncorhynchus kisutch*, began on the West coast of America in the early 1970s. Mortalities due to vibriosis, caused by *Vibrio anguillarum,* were soon noted (Sindermann, 1989). Outbreaks of the disease were initially controlled by the use of antibiotics (Fryer *et al.*, 1978), but effective vaccines have since been developed against this organism .

Another disease which has caused severe problems for salmon aquaculture is furunculosis, caused by *Aeromonas salmonicida.* This disease was first recognised over 100 years ago (Emmerich and Weibel, 1894, cited by Bernoth, 1997) and caused significant losses in wild salmon in Scotland in the 1930s (Mackie *et al.,* 1985). Although the incidence of disease declined in wild fish, furunculosis became an acute problem for salmon culture; it was estimated that in the Highland region of Scotland over 50% of smolts put to sea in 1991 were lost due to furunculosis (Munro and Gauld, 1996). Again, the introduction of effective vaccines, coupled with improved husbandry and management, have reduced the impact of this disease, such that smolt losses in the Highland region fell to an estimated 4% by 1993 (Munro and Gauld, 1996).

1.2 Emerging pathogens in salmon aquaculture

Whereas the bacteria causing the major diseases which affected the early development of salmon aquaculture (vibriosis, furunculosis and bacterial kidney disease) have been recognised as pathogens of wild fish for a century, a number of new diseases have emerged more recently (Fryer and Bartholemew, 1996, Fryer and Mauel, 1997). The most important are Hitra disease, caused by *Vibrio salmonicida* (Egidius *et al.*, 1986), infectious salmon anaemia, caused by an orthomyxo-like virus (Thorud and Djupvik 1988; Falk *et al.*, 1997), and piscirickettsiosis, caused by *Piscirickettisa salmonis* (Fryer *et al.*, 1992).

1.3 Piscirickettsiosis- early reports

Coho salmon were introduced for commercial aquaculture in Southern Chile approximately 15 years ago, and other salmonid species introduced include Chinook salmon, Atlantic salmon and Rainbow trout. In the winter of 1989, high levels of mortalities occurred among farmed Coho salmon, *Oncorhynchus kisutch* (Walbaum) in Southern Chile (Branson and Diaz-Munoz, 1991). At this stage other year classes and other species were not affected, and the disease was reported mainly in the Gulf of Ancud, an area between Chiloe Island and the mainland south of Puerto Montt; other farms were involved, but to a much lesser extent. Losses as high as 90% were reported, with an average loss of approximately 60% occurring on many farms. There appeared to be no common factor linking the diseased fish, as fish from various hatchery stocks, water supplies during the freshwater phase of production, and different types of food, were all affected.

The epizootic of 1989 was estimated to have killed approximately 1.5 million Chilean salmon, ranging from around 200g to 2 kg (market size), resulting in significant economic losses. The disease was only observed in Coho salmon reared in seawater net pens around Puerto Montt and Chiloe Island. In the autumn of that year, in the area described above, there was a period of fluctuating sea temperature which coincided with a non-toxic algal bloom in March. When the algal bloom had disappeared and the temperature had stabilised the losses continued to escalate until May/June, when a steady

decline in the number of mortalities resulted until a normal level was reached in the following July/August. This disease had been recognised as causing high mortalities in Coho salmon since 1981 at these farms (Bravo, 1994), and farms with infected fish reported monthly mortalities, ranging from 1-20%, and up to 40% during epizootics. Epizootics occurred from April to August (autumn to mid-winter in Chile), 10-12 weeks following saltwater introduction, and lasted up to 10 weeks before subsiding. Repeat outbreaks often occurred from October to December. The number of farms identified with infected Coho salmon increased from four in April 1989 to 32 in April 1990 (Fryer *et al.*, 1990).

The initial investigation of the disease in 1989 indicated a systemic infection with a non-culturable, bacteria-like organism, sometimes replicating, that could be observed within intracytoplasmic vacuoles of host cells. This suggested a chlamydial or rickettsial organism. The organism was confirmed as the aetiological agent of the disease by fulfilling Koch's postulates (Cvitanich *et al.*, 1991), and the authors suggested the disease be called Salmonid Rickettsial Septicaemia (SRS).

The condition has since been reported in other salmon species, including Sakura, Atlantic and pink salmon and also rainbow trout. Affected fish with similar characteristics have been reported in Canada (Brocklebank *et al.*, 1992), Ireland (Rodger and Drinan, 1993) and Norway (Olsen *et al* ., 1993 & 1997), with the outbreaks of disease in Norway occurring during the late 1980s. This condition, therefore, poses a significant threat to the entire salmon industry.

1.4 Clinical signs and gross pathology in piscirickettsiosis

The pathology of this disease has been reviewed by Fryer *et al.*, (1992). The first evidence of the disease among a fish population appears to be small white spots on the gills, as first reported in March 1989 on affected fish in Chile. Most of the affected salmon displayed skin lesions ranging from small areas of raised scales, which were often haemorrhagic, 0.5cm diameter or less, through to white raised plaques and shallow haemorrhagic ulcers, approximately 2cm in diameter. Some affected fish showed no evidence of skin lesions. Affected fish were dark and lethargic, being easy to spot hanging

at the net sides. Many had some degree of ascites, varying degrees of peritonitis, general pallor (suggesting anaemia) and slightly enlarged spleens. In all cases the fish had swollen grey kidneys and livers, often with pale focal lesions. All fish had good body fat reserves, but there was no food present in the gut of any fish.

Blood smears prepared from the affected fish showed a large number of macrophages, most of which contained either degenerate cellular debris or organisms ranging from few to many within cytoplasmic vacuoles (Fryer *et al.*, 1992). Due to the systemic nature of the disease, infected cells were commonly found in many tissues. The severity of the gastrointestinal, haematopoietic and hepatic lesions probably accounts for the high mortality associated with this disease. The pathological lesions described are similar to those described for rickettsial diseases of other animals. The anaemia associated with this disease appears to be haemolytic. Rickettsiae causing the disease typhus in humans are capable of haemolysing erythrocytes of several animal species. The haemolytic activity is correlated with infectivity of rickettsia and with their metabolic activities.

1.5 Identification of *Piscirickettsia salmonis*

To identify the organism causing SRS, infectious material from naturally infected Chilean Coho salmon was injected into various salmon species to reproduce the disease. The organism was confirmed by Koch's postulates to be the agent causing SRS (Cvitanich *et al.*, 1991). This study indicated that the organism is an obligate intracellular pathogen found within membrane-bound vacuoles, which is characteristic of chlamydiae and some rickettsiae. However, the organism does not follow the characteristic chlamydial developmental cycle, which includes formation of small infectious elementary bodies and larger noninfectious reticulate bodies. Also, the organism did not react with a genus-specific chlamydial antibody, suggesting that the organism does not belong to the order Chlamydiale (Weiss and Moulder, 1984).

Sections of kidneys, livers and spleens from naturally infected and experimentally infected fish, showed numerous enlarged vacuolated cells containing basophilic granules. Electron microscopy of sections of the liver revealed coccoid organisms, enclosed within

membrane-bound cytoplasmic vacuoles. Externally they appeared to have an undulating outer membrane and a second inner membrane. In the order Rickettsiale, membrane-bound vacuoles and rippled cell walls are commonly observed. The organism was sensitive to a wide range of antibiotics including chloramphenicol, tetracycline, erythromycin, and oxytetracycline, but resistant to penicillin G, which suggests a rickettsial origin. The agent was not associated with erythrocytes, therefore, placing it in the family *Rickettsiaceae*.

Fryer went on to place the organism taxonomically, although this was difficult as the organism was morphologically similar to organisms in the tribe *Ehrlichieae* of the family *Rickettsiaceae*. It has the most common feature of this group, replication within membrane-bound cytoplasmic inclusions in host cells, although host specificity and serological characteristics suggest that it does not fit into any of the established genera. The previously characterised ehrlichial agents are pathogens of mammals; although this agent is a pathogen of poikilotherms and replicates optimally in fish cell cultures incubated between 15-18^{o}C, replication does not occur at temperatures greater than 25^{o}C. There appears to be no common antigens between the organism and ehrlichial agents examined, although a very low level reaction occurred when the type strain, LF-89, was tested with canine granulocytic ehrlichia antiserum by indirect immunofluorescence. No reaction was apparent with antisera against *E. equi, E. canis* or *N. helinthoeca*. (Lannan *et al.*, 1991). Fryer *et al.*, (1992) proposed that the organism should belong in the order Rickettsiale for the following two reasons : -

It is an obligate intracellular bacterium, and various attempts at primary isolation or passage of cell culture isolates on bacteriological media were unsuccessful, and

It has a morphology similar to some rickettsiae, for example *Ehrlichia*.

Fryer subsequently determined the sequence of the LF-89 16S rRNA gene, the secondary structure of which conformed to the gamma sub-division of the Proteobacteria. The 16S rRNA gene sequence from this salmonid pathogen showed no specific relationship to any of the 450 bacterial 16S rRNA sequences in the rDNA Data Base project. Comparison with the sequences of *R. rickettsii, R. typhi., R. prowazekii, E.*

risticii, *R. quintana*, *W. persica* and *C. burnetii* indicated that LF-89 was more closely related to *W. persica* and *C. burnetii* (which also belong in the gamma sub-division of the Proteobacteria) than to members of the genera *Rickettsia*, *Ehrlichia* and *Rochalimea* (which are in the alpha sub-division), Figure 1. The rickettsial agent was not a specific relative of any of these intracellular bacteria (Fryer *et al.*, 1992; Shankarappa *et al.*, 1992). LF-89 has a unique 16S rRNA sequence; along with the temperature requirements, host range, and serological characteristics this pathogen warrants its classification as a new genus and species in the family Rickettsiaceae. Fryer suggested the establishment of a new genus, *Piscirickettsia*, and a new species name *Piscirickettsia salmonis* (rickettsial organism affecting salmon) (Fryer *et al.*, 1992). Figure 1 shows the relatedness by virtue of their 16S rRNA sequence of various members of the Rickettsiaceae family, fish pathogens and a selection of bacteria that do not fit into either category.

1.6 Description of the genus Piscirickettsia

The properties of *P. salmonis* are summarised in Table 2. Replication of the organism within vacuoles in host cells is characteristic of some rickettsial species, (see Figure 3, p68). *P. salmonis* can be cultivated in several fish cell cultures, including CHSE-214, CHH-1, CSE-119 and RTG-2 cell lines (Lannan *et al.*, 1984; Plumb and Walk, 1971), but not in cell-free media.

TABLE 2 Characteristics of *Piscirickettsia salmonis*

Pleomorphic organisms, predominantly coccoid, diameter 0.5-1.5µm
Gram negative, non-motile organism, visualised by staining with Giemsa stain
Obligate intracellular parasite
Optimum growth temperature range 15-18^{o}C
Does not replicate on bacteriological media
Divides by binary fission
Visible in cytoplasmic vacuoles by light or electron microscopy
Replication inhibited by antibiotics (chloramphenicol, tetracycline, erythromycin)

Figure 1 16SrRNA relationship among rickettsia and selected bacteria
Accession numbers of 16SrRNA genes from the following bacteria taken from EMBL databases and their abbreviated codes used in the figure.

Erhlichia sennetsu	**ES**	M73219
Erhlichia risticii	**ER**	M21290
Erhlichia canis	**EC**	M73221
Rickettsia prowazekii	**RP**	M21789
Rickettsia typhi	**RT**	M20499
Rickettsia rickettsii	**RR**	M21293
Bartonella bacilliformis	**BB**	M65249
Coxiella burnetii	**CB**	M21291
Piscirickettsia salmonis	**PS**	X60783
Escherichia coli	**EC**	J01695
Legionella pneumophila	**LP**	M59157
Vibrio anguillarum	**VA**	X16895
Aeromonas salmonicida	**AS**	X60405
Renibacterium salmoninarum	**RS**	X51601
Wolbachia persica	**WP**	M21292
Cowdria ruminantium	**CR**	U03777
Anaplasma marginale	**AM**	M60313
Rochalimea quintana	**RQ**	U28268
Brucella abortus	**BA**	X13695
Salmonella typhi	**ST**	U88545
Yersinia enterocolitica	**YE**	M59292
Wolbachia persica	**WP**	M21292
Listeria monocytogenes	**LM**	M58822
Chlamydia trachomatis	**CT**	D89067

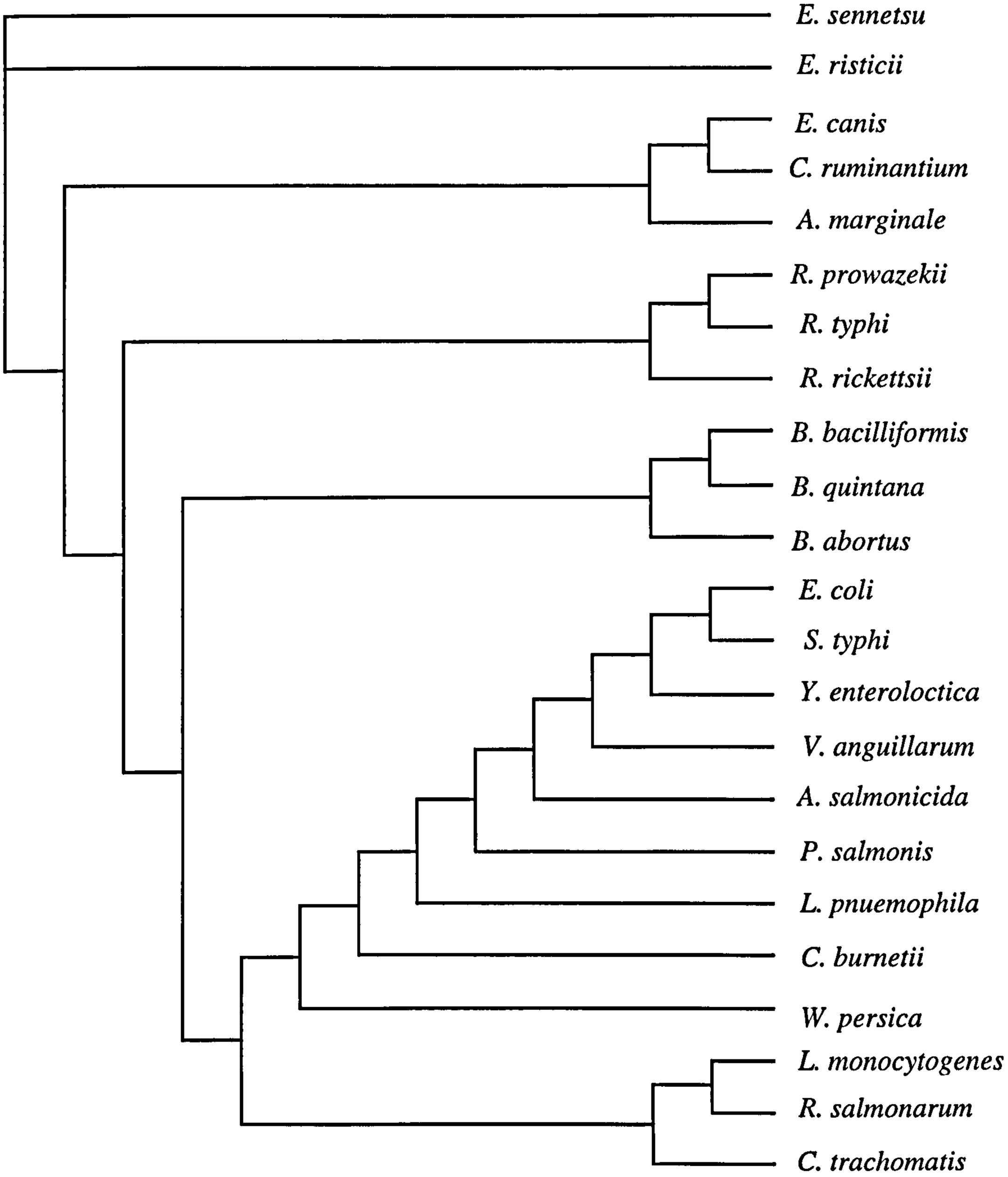


Figure 1 16SrRNA relationship among rickettsia and selected bacteria

Performed using PAUP analysis to determine relationship between the above 16S sequences taken from EMBL database. The tree is heuristic based on minimum distance.

P.salmonis is now known to infect four species of salmonids, *O. kisutch*, *O. tshawytscha*, *O. mykiss* and *S. salar*, and it is probable that other fish species are also susceptible. The disease has been induced experimentally in *O. kisutch* and *S. salar* by intraperitoneal injection of fluids from infected cell cultures (Garces *et al.*, 1991), but the method of natural transmission has not yet been determined.

The type strain, LF-89 was isolated from Chile in 1989, and was deposited with the American Type Culture Collection as ATCC (R) strain VR1361.

1.7 Transmission of *P. salmonis*

In rickettsial diseases of mammals, an arthropod vector is almost always required but no such agent has yet been shown to be involved in SRS. The external environments of fish and mammals are very different, so it is possible that in this case a vector is not required. Q fever, a rickettsial infection of cattle and man, caused by *Coxiella burnetii*, can be transmitted by a vector but infection can also occur by direct contact with contaminated material, such as placenta or milk. Therefore, it is possible that in Coho salmon transfer could occur merely through the water.

The presence of skin lesions on the majority of the diseased fish could indicate the existence of a vector, as this is seen in most mammalian diseases caused by *Rickettsiae*. Although a rickettsia has been designated as the causative agent of the disease, it may be that other factors are required to precipitate the massive fish losses which occur, e.g. inadequate nutrition or stress caused by environmental changes such as fluctuating temperature. On some of the affected farms in Chile severe storms have occurred before the outbreak of the disease, and there was also a preceding algal bloom and fluctuating sea temperature in initial incidents of disease (Branson and Diaz-Munoz, 1991).

As the affected fish originated from various freshwater sources, and the fish had been at sea for 2-3 months prior to the disease, it has been proposed that the organism may have originated from a local marine source and that this organism, together with the factors mentioned above, has been the cause of the large losses experienced.

1.8 Rickettsia-like organisms

Rickettsia-like organisms (RLOs) have been reported in non-salmonid fish, shellfish and crustaceans (Fryer and Lannan, 1994); therefore, it is possible that these could act as reservoirs of infection for rickettsia infecting animals in the marine environment as these could move freely through net pens with large mesh sizes. Epitheliocystis is a widely reported infection of fish caused by a chlamydia. Chlamydia-like infections of fish are widespread, but RLOs in fish have been less frequently reported. Mohamed (1939) cited by Fyer & Lannan, (1994), noted a coccoid RLO within monocytes and in the plasma of one dead tetrodontid fish in Egypt. No further reports of this organism or of other fish rickettsia occurred until Ozell (1975), cited by Fryer & Lannan, (1994), detected a rod-shaped RLO in tissues of rainbow trout collected from a freshwater source in Europe. The agent was isolated and passaged in fish cell cultures but was not characterised beyond morphological description, nor was it maintained for further study. Viral haemorrhagic septicaemia (VHS) virus was also isolated from the affected trout, so although mortality was observed in the trout population, the role of the RLO was undetermined. In 1986 Davies reported a coccoid or elongate RLO in tissues of the dragonet, a marine fish collected in Cardigan Bay in Wales. The above information is reviewed by Fryer and Lannan, (1994). In 1994 Chen reported outbreaks of a disease caused by RLO in cultured tilapia in Taiwan. Also in this year an RLO was reported in the gill epithelial cells of Pacific Oyster (Renault, 1994).

A summary of the rickettsia and RLO identified in fish is shown in Table 3.

1.9 Survival of *Piscirickettsia salmonis* in sea water

Experiments on extracellular survival carried out by Lannan and Fryer, (1994) on the LF-89 strain concluded that *P. salmonis* survived in seawater for at least one week, but did not survive in freshwater. The extended survival time of the rickettsia in seawater at temperature permissive for piscirickettsiosis makes direct transmission a possibility in the marine environment. However, the rapid inactivation of the rickettsia in freshwater limits the opportunity for direct transmission under these conditions.

TABLE 3 Rickettsial and rickettsial-like infections of fish

HOST	REGION	REFERENCE
CALLIONYMIDAE		
Callionymus lyra (Dragonet)	Cardigan Bay	Davies, (1986)
SALMONIDAE		
Oncorhynchus kisutch	Southern Coast	Fryer *et al.*, (1990, 1992)
(Coho salmon)	Chile	Branson *et al.*, (1991)
		Cvitanich *et al.*, (1991)
		Garces *et al.*, (1991)
		Lannan *et al.*, (1991)
		Fryer *et al.*, (1992)
Oncorhynchus mykiss	Germany	Ozel *et al.*, (1975)
(Rainbow trout)	Southern Coast	Cvitanich *et al.*, (1991)
	Chile	Garces *et al.*, (1991)
		Fryer *et al.*,(1992)
Oncorhynchus tshawytscha	Southern Coast	Cvitanich *et al.*, (1991)
	Chile	Garces *et al.*, (1991)
		Fryer *et al.*, (1992)
	Pacific Coast, Canada	Evelyn (1992)
Oncorhynchus gorbuscha	Pacific Coast	Evelyn (1992)
(Pink salmon)	Canada	
Salmo salar	Southern Coast	Cvitanich *et al.*, (1991)
(Atlantic salmon)	Chile	Garces *et al.*, (1991)
		Fryer *et al.*, (1992)
	Pacific Coast, Canada	Evelyn (1992)
TETRAODONITIDAE		
Unknown	Egypt	Mohamed (1939)

Table adapted from Fryer and Lannan (1994).

This may explain why the disease is rarely observed at freshwater sites, even though it has been demonstrated that piscirickettsiosis develops in freshwater in fish experimentally infected via intraperitoneal injection (Garces *et al.*, 1991).

1.10 Isolation of *P. salmonis* in other countries

Since *P. salmonis* was characterised, several morphologically similar agents have been detected in salmonid fish at widely spaced locations throughout the world. One of these organisms was identified in 1991 in diseased Atlantic salmon collected from a sea water farm in Canada (Brocklebank *et al.*, 1992). A similar, but unidentified, organism reportedly induced mortalities as early as 1970 in pink salmon held in seawater tanks in the region. The agent observed in Atlantic salmon shares one or more common antigens with *P. salmonis*, but it is apparently less virulent than the type-strain LF-89. Another rickettsial organism was detected in Norway in histological sections of liver from Atlantic salmon experiencing a disease termed necrotising hepatitis. An organism of this type was also observed in electron micrographs of tissues of Atlantic salmon reared in Ireland (Rodger and Drinan, 1993). All of these RLOs produce pathology similar to that associated with piscirickettsiosis in Chilean salmon, and they are closely related to *P. salmonis*.

1.11 Further work required on *P. salmonis*

The rickettsia isolated from Coho salmon is clearly a serious pathogen of potential concern to the entire salmonid culture industry. Methods for rapid detection of the organism in fish are required to control the spread of the disease, and to identify the source of infection in sea water and its mode of transmission. A further major requirement is the development of vaccines for preventing the disease. This thesis is concerned with a molecular study of the organism with the long term aim of developing a vaccine against *P. salmonis*. Therefore, the properties of other rickettsia and relevant molecular biology studies are reviewed.

1.12 Rickettsiaceae

Rickettsia are small pleomorphic organisms, mostly intracellular parasites with a wide natural host range which includes arthropods, birds, and mammals. Rickettsiaceae

are one of three families of the order Rickettsiales, the others being Bartonellaceae and Anaplasmaceae (Drancourt, 1994) which are not discussed here. Within the family Rickettsiaceae are three tribes, consisting of Rickettsieae, Ehrlichieae and Wolbachieae. The tribe Rickettsieae contains three genera of organisms that have successfully adapted to a parasitic existence in man: *Rickettsia, Rochalimaea,* and *Coxiella* (Weisburg *et al.*, 1989), Figure 2. The classification of *Rickettsia* is under review as new information is discovered. For example, *Coxiella burnetii* was recently removed from the order Rickettsiales due to the advent of 16S rDNA sequencing from which it was concluded that greatest similarity was to the gamma subgroup of the Proteobacteria. *Rochalimaea* has also been moved, on the basis of 16S rRNA sequence analysis, to the Bartonellaceae, having greater similarity to the alpha 2 subgroup of Proteobacteria (Weisburg *et al.*, 1989). Classification of intracellular bacteria is not as standardised as with other microorganisms, partly due to the problems encountered when growing and successfully isolating these organisms from cell material. The advent of molecular biological techniques, especially in the use of the polymerase chain reaction (PCR) has facilitated identification of new rickettsiae or rickettsia-like organisms. There are 14 known rickettsioses affecting man; six of these organisms and their diseases have only been identified in the last 12 years. The taxonomy of rickettsiae is therefore emerging rapidly due to advances in classification and identification techniques.

The genus Rickettsia is divided into three separate groups; the typhus group includes *Rickettsia typhi,* causative agent of Murine Typhus, and *Rickettsia prowazekii* which causes Epidemic Typhus. The Spotted Fever Group contains approximately 20 organisms, including *Rickettsia rickettsii* which causes the disease Rocky Mountain Spotted Fever (RMSF), and *Rickettsia sibirica* , which causes the disease Siberian Tick Typhus. The last group is the Scrub Typhus group containing *Rickettsia tsutsugamushi* , causative agent of Scrub Typus or Chigger-borne Typhus. This organism was recently placed in a new genus, *Orientia tsutsugamushi,* due to rRNA gene analysis. Their ecology involves a complex interaction of arthropod vectors and higher animals including humans. Infection of humans and animals is normally via an arthropod vector, with the exception

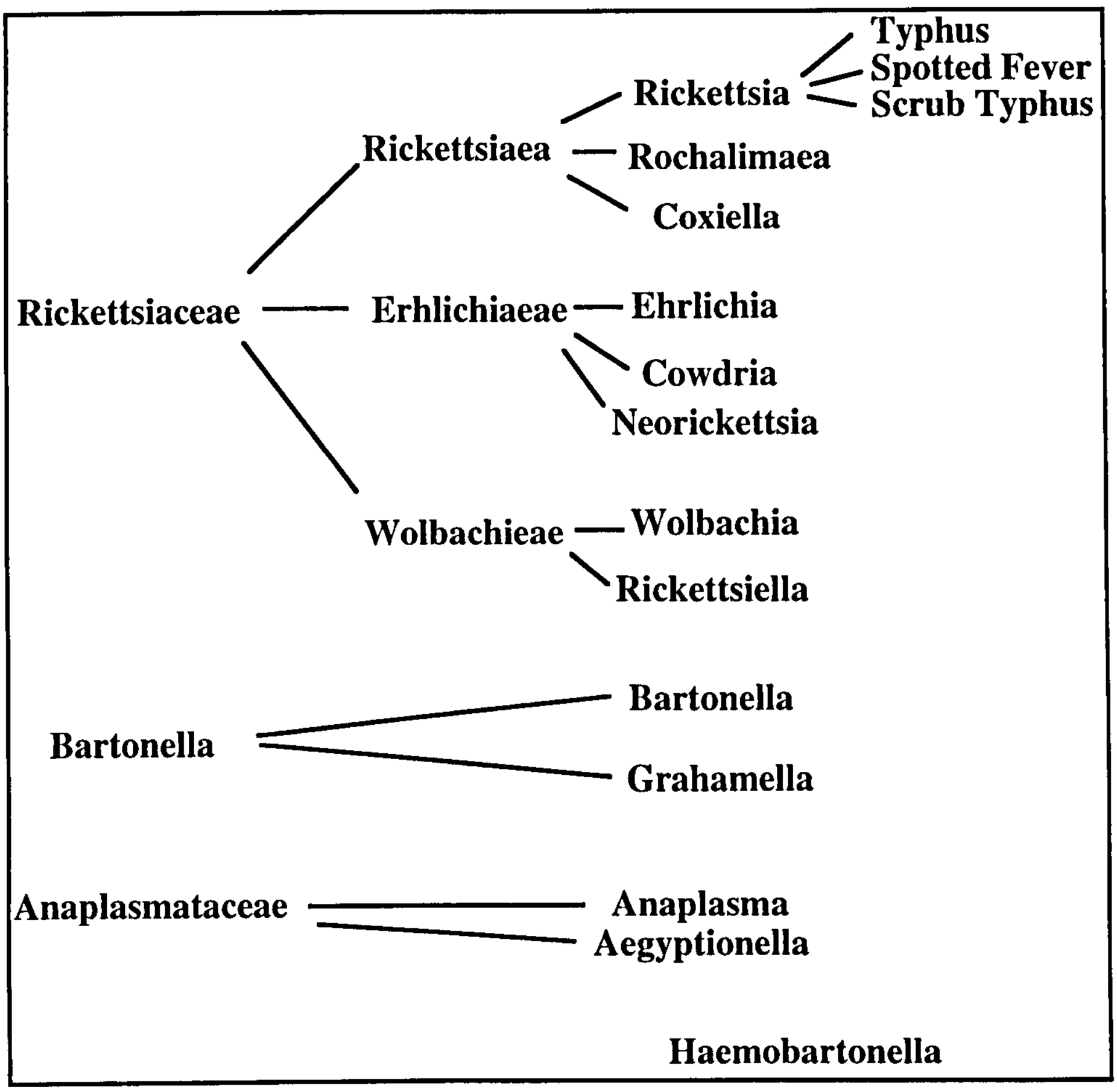


Figure 2 Family Rickettsiaceae, Bartonellaceae and Anaplasmataceae

The above table shows an updated account of the three families.

of *Coxiella burnetii.* This organism may be transmitted by aerosols or by the ingestion of contaminated material, such as milk. Natural reservoirs of infection may be maintained by transovarial passage in arthropod vectors (for example *R. tsutsugamushi* in mites and *R. rickettsii* in ticks) or by infection of rodents *(R. typhi)* or humans (*R. prowazekii* and *Rochalimaea quintana)* (Weiss and Moulder, 1984).

1.13 History of rickettsial research

The first microbiological description of rickettsiae was in the first decade of this century by Howard Ricketts, who described the etiologic agent of Rocky Mountain Spotted Fever. The agent was cultivated in small laboratory animals, from which the natural ecology and epidemiology were deduced. Other investigators used similar experimental approaches to understand several of the related diseases. The family name Rickettsiaceae honours Ricketts who, with a number of other early rickettsiologists, were eventually killed by the disease agents they were studying.

Rickettsial infections are distributed worldwide and have been significant factors in history, with epidemic typhus being associated with war, famine, and human suffering as reported in the classic book by Zinsser in 1935; a more recent review of the history of rickettsiae is also provided by Zinsser in 1988, as cited by Raoult & Roux (1997). Once the importance of the arthropod vector was appreciated outbreaks of typhus fever were prevented by using vector control measures. The use of tetracycline and chloramphenicol antibiotics has also brought the rickettsioses under control (Williams and Winkler, 1984).

1.14 Structure of rickettsiae

Rickettsiae are pleomorphic, rod-shaped or coccoid organisms that range in size from 0.3-0.6μm in width by 0.8-2.0μm in length (Weiss, 1982). They stain poorly with Gram's stain, however, their chemical composition and ultrastructure resembles those of Gram-negative bacteria (Anderson, 1965). A trilaminar cell membrane consisting of a cytoplasmic membrane and a double leaflet cell wall has been observed by electron microscopy (Hase, 1985). There are subtle differences between cell membranes of different rickettsiae, e.g. typhus and spotted fever group rickettsiae have a thick inner cell wall leaflet, whereas scrub typhus rickettsiae have a thick outer leaflet (Smith, 1979).

Rickettsiae also lack flagella and pili (Anderson, 1965). The capsular material which they produce can be stained with ruthenium red and silver methenamine, and it is, therefore, thought to be a polysaccharide in nature (Weiss, 1982). This slime layer is much thicker around rickettsiae within the cells of fed ticks than in starved ticks. This is perhaps an indication that it may play a role in the biology of these organisms, because when the ticks are starved, their rickettsiae lose virulence for guinea pigs, reviewed by Weiss (1982) . The slime layer is easily lost upon purification, and is thus difficult to characterise; its relationship to virulence remains an unanswered question. Reviews on rickettsial morphology have been provided by Moulder (1985), Ormsbee (1985) and Winkler (1990). Ultrastructural examination of rickettsiae within the cytoplasm of their host cell reveals a prominent clear layer surrounding rickettsiae of all species examined (Silverman *et al.,* 1992). Moulder (1985) and Winkler (1990) give details of some early experiments involving rickettsiae. The slime layer of *R. rickettsii* was examined in epoxy-embedded, sectioned cells and in unembedded, unsectioned infected cells using both conventional transmission electron microscopy and high voltage electron microscopy, respectively. Epoxy-embedded infected sectioned cells clearly showed a transparent slime layer, with no ultrastructural features. However, in whole infected cells examined under high voltage electron microscopy, fine structural features within the slime layer were clearly observed. Also visible at high magnifications were long extensions of rickettsial outer membranes, microfilament structures attached to the outer membrane and extensive attachments between adjacent rickettsiae. These electron-dense structures within the slime layer can also be seen in polyethylene glycol-embedded specimens when viewed under conventional transmission electron microscopy. The microfilament attachments are thought to be important in the movement of rickettsiae into the cytoplasm. Members of the Spotted Fever Group have been observed in the nucleus and cytoplasm of infected host cells and movement within the cell is due to the polymerisation of actin (Burgdorfer *et al.*, 1968, reviewed by Burgdorfer, 1981: Heinzen *et al.*, 1993 and Teysseire *et al.*, 1992). Typhus Group rickettsia have been identified within the cytoplasm only, although in experiments conducted by Teysseire and co-workers they were able to show that both Spotted Fever

Group and Typhus Group rickettsia (*R. conorii* and *R. typhi* respectively) stained positive for actin with NBD-phallacidin. Differences were apparent in the length and the number of organisms with actin tails. *R. conorii* had the longest tails, with between 20 and 68% of organisms examined displaying an actin tail. For *R. typhi* , less than 1% of the sample organisms had an actin tail and these were considerably shorter than those of *R. conori.* It was therefore postulated that the genes responsible for actin polymerisation are present throughout the genus but only expressed within closely related groups. Rickettsia have traditionally been classified on their location within the cell- Spotted Fever Group are present in the cytoplasm and nucleus, and Typhus Group are found in the cytoplasm only, their optimal growth temperatures are 32^{o}C for the Spotted Fever Group versus 35^{o}C for the Typhus Group. It has been shown that the Typhus Group rickettsiae have lipopolysaccharide (LPS), but in Scrub Typhus there is no evidence of LPS . The LPS from the spotted fever group of rickettsiae has antigenic cross-reactivity with the LPS from *Proteus* species (Amano *et al.,* 1993) and this is the basis of the Weil-Felix reaction used in diagnosis of rickettsial infection.

New Spotted Fever Group rickettsia are identified using an indirect immunofluorescence test and restriction fragment length polymorphism (RFLP) of PCR-amplified products (Anacker *et al.*, 1987; Eremeeva *et al.*, 1993; Obijeski *et al.*, 1974; Regnery *et al.*, 1991 and Philip *et al.*, 1978 which are all cited in Raoult and Roux, 1997).

1.15 Coxiella

Coxiella burnetii is the only species of the Genus Coxiella, and 16S rRNA sequencing placed *C. burnetii, Wolbachia persica* and *Legionella pneumophila* in the gamma subdivision of Proteobacteria. *Rickettsia, Ehrlichia* and *Bartonella spp.* were placed in the alpha subgroup (Weisburg *et al.*, 1989). *Coxiella burnetii* causes the disease Q fever which is prevalent worldwide; transmission usually occurs via aerosols arising from contaminated material and transmission to humans via a tick bite is extremely unusual. In contrast to *Rickettsia* and *Rochalimea, Coxiella* grows preferentially within the host membrane-bound vacuoles containing lysosomal enzymes (phagolysosomes) (Ormsbee, 1985). The outer layers of *Coxiella burnetii* is composed of outer membrane

proteins (OMPs), LPS and A-1-gamma type peptidogycan (Mege *et al.*, 1997). Mutational variation in *Coxiella burnetii* LPS can occur due to phase variation. In Phase I, the LPS is smooth and the organisms are highly virulent when recovered from infected humans and animals. Phase II results from serial passages of Phase I organisms, and these organisms have reduced virulence and rough LPS. The smooth to rough transition in the LPS is due to changes in the sugar composition; in Phase I organisms sugars such as L-virenose, dihydrohydroxystreptose and galactosamine can be found, but these sugars are not present in Phase II (Amano *et al.*, 1993). *Coxiella burnetii* forms different sized variants and produces spores under specific environmental conditions (Mege *et al.*, 1997). The bacterial genome contains 1600 kb DNA. Genes from *C. burnetii* which have been cloned and expressed in *E. coli* include gltA, citrate synthase gene (Heinzen *et al.*, 1991), the superoxide dismutase gene, heat shock proteins *htpA* and *htpB* (Vodkin, 1988) and also the 27kDa surface antigen (Hendrix, 1993). This organism also contains plasmids designated QpHI, QpHR and QpDG, although controversy still surrounds their actual function .

1.16 Ehrlichieae

The genus *Ehrlichia* parasitises circulating leukocytes of humans and a variety of wild and domestic animals. *Ehrlichia sennetsu* has a predilection for mononuclear leukocytes as does the closely related species, *E. canis*. (Rkihisa *et al.*, 1994). Both exist within the cytoplasm in membrane-bound vacuoles that form inclusions containing a variable number of organisms. Three developmental stages are observed microscopically: the earliest forms are termed elementary bodies and they in turn become the slightly larger initial bodies, which give rise to even larger bodies (Weiss, 1982).

1.17 Metabolism of rickettsiae

The intracellular location of rickettsiae in the host cell cytoplasm provides them with an extensive array of preformed metabolites (Weiss, 1982; Winkler, 1990). The metabolism of rickettsiae is very primitive, in that they oxidise only one amino acid, glutamate, by means of the tricarboxcylic acid cycle, and they cannot oxidise glucose. In

exploitation of their intracytoplasmic niche, rickettsiae have evolved carrier-mediated transport systems for key phosphorylated compounds (Moulder, 1985)

ATP generated by the aerobic oxidation of glutamate appears to be the chief source of energy. Winkler (1990) has shown that rickettsiae have a carrier-mediated transport system for ATP and ADP, which is functionally similar to that used by mitochondria. The ATP/ADP exchange system provides the exchange of extracellular and intracellular adenine nucleotides on a one-for-one basis. It does not catalyse the net transport of nucleotides, and is highly specific for ATP and ADP. Rickettsiae can therefore generate their own ATP by oxidative phosphorylation via the TCA cycle, or can take up ATP from the host cytoplasm, by means of their ATP/ADP transport system. In summary, rickettsiae can generate their own energy, or obtain ATP from the host cell. Exogenous ATP is reduced when the rickettsiae are internalised; evidence for this process is that *R. tsutsugamushi* and *R. prowazekii* are dependent on a constant supply of glutamate, which is oxidised with the concomitant phosphorylation of ATP. Host cell entry is one of these activities; when either scrub or louse-borne typhus agent is deprived of glutamate, its ability to enter cells is drastically reduced. These observations fit the conclusion that rickettsiae enter host cells by induced phagocytosis (Moulder, 1985) and (Weiss, 1982).

1.18 Entry of rickettsiae into cells

At present the mode of rickettsial entry into host cells is not entirely clear. Cohn *et al.*, (1959) as cited in Osterman, (1985) exposed cultured mouse lymphoblasts in complete tissue culture medium to partially-purified rickettsiae and showed by light microscopy that 90% of cells were infected within 30 minutes and nearly 100% in 120 minutes. They also found that rickettsiae rendered nonviable by treatment with either heat, UV irradiation or formalin did not penetrate into the host cells and that the penetration of viable rickettsiae was enhanced by the presence of L-glutamate, the principal energy source of rickettsiae, in the medium.

In their natural hosts, rickettsiae preferentially enter endothelial cells lining the small blood vessels, and most of the host damage incurred in rickettsial infections stem from the infection of these cells (Walker, 1984). *R. tsutsugamushi* and *R. prowazekii* readily

enter professional and non-professional phagocytes both *in vivo* and *in vitro* by a mechanism resembling phagocytosis. They are internalised within a phagosomal membrane from which they quickly escape (Walker, 1984); this is thought to be due to phospholipase A_2 activity weakening or perforating the host cell membranes (Manor *et al.* , 1994; Silverman *et al.*, 1992; Winkler and Daugherty, 1989). Rickettsial infection of cells has also been demonstrated to be associated with actin polymerisation (Heinzen *et al.*, 1993; Teysseire *et al.*, 1992).

The nature of the adherence step, the rickettsial adhesin, and the host cell receptor are still unknown. *R. prowazekii* can infect cells ranging from the epithelial cells of the louse gut, through avian and rodent cells all the way to human fibroblasts and endothelial cells, suggesting that the receptor may not be highly specific, and may have a relatively low affinity . It has been suggested that the receptor is cholesterol (Ramm and Winkler, 1976; Wisseman, 1982 as cited by Osterman 1985).

1.19 Intracellular Release

Release of rickettsiae from host cells is a rare example of divergence among species that are likely to share a comparatively recent evolutionary ancestor. Studies with *R. prowazekii* and chick embryo fibroblasts have shown that 72-96 hours after infection the cells are packed with rickettsiae (Moulder, 1985). After this time the cells suddenly break open and release continues for many hours with the surrounding cells being infected. Cytopathic changes are only apparent late in infection, when host cells have clearly begun to disintegrate (Khavkin, 1981, cited by Osterman 1985). Lysis may be due to overburdening the host cells with large numbers of rickettsiae or it may result from the action of phospholipase A, which has been firmly implicated in the entry and exit of *R. prowazekii* into host cells (Winkler and Daugherty, 1989).

The interaction of *R. rickettsii* with L-cells or chick embryo fibroblasts is similar to that of *R. prowazekii*. Although as early as 10 hours after infection intact host cells begin to release infectious material, there is a substantial build up of rickettsiae in the medium and a rapid spread of infection to uninfected host cells. Electron microscopy studies show a loss in plasma membrane integrity, but only late in infection after extensive

shedding of rickettsiae has occurred. Early release of *R. rickettsii* from host cells may be responsible for the rapid spread of infection characteristic of spotted fever.

R. tsutsugamushi offers a different pattern of release. By electron microscopy and light microscopy studies it has been shown that it is extruded in projections from the surface of intact cells (Rhikhisa, 1981 reviewed by Burgdorfer). In peritoneal mesothelial cells from infected mice, rickettsiae are seen at the free cell surface within envaginations of the plasma membrane, sometimes connected by a stalk to the host cell. They are released still surrounded by host cell membranes. These membrane-bound rickettsiae infect other host cells, but rickettsiae without membranes do not (Moulder 1985; Winkler 1990).

As Rickettsiae do not make powerful exotoxins, it is most likely that they cause damage by directly killing the cells that harbour them; lysis of endothelial cells leads to rupture of capillaries and small vessels. *Rickettsia* and *Coxiella* injure host cells in varying degrees depending on the nature of the host cell, the particular species and strain of parasite, and the multiplicity of infection (Winkler, 1990).

1.20 Antigenic structure

Differences in the antigenic composition of pathogenic rickettsiae facilitated their classification into genera, groups and species. A recent review by Drancourt (1994), shows the most recent taxonomic positions of rickettsiae, and Weisburg *et al.*, (1989) covers the phylogenetic diversity of rickettsiae. Table 4 lists the order Rickettsiales and the family, tribes and genera encompassed within this order. There is no common antigen for all members of the family Rickettsiaceae.

Two major types of antigen have been detected in rickettsiae; these are either soluble group-specific antigens representing shed capsular material, or type-specific antigens associated with the cell wall (Dasch, 1981; Hanson, 1985; Anderson and Tziabos, 1989; Oaks *et al* ., 1989; Anderson *et al.*, 1990). Antigenic differences permit further speciation within the first two groups (Xuejie *et al.*, 1990). Additional methods of separating various biotypes include the cross-protection test in laboratory animals, DNA base ratio analysis and reactivity to monoclonal antibodies (Oaks *et al.*, 1989). The essential protective antigens of *R. prowazekii* and *R. typhi* are species-specific and heat labile (Dasch *et al.*,

1981). An homologous, water-soluble, 120kDa polypeptide constitutes the microcapsular protein layer lying outside the rickettsial outer membrane. This 120kDa antigen and a second surface protein of 155kDa have been identified as major protective antigens in the humoral response to experimental infection.

Members of the spotted fever and typhus group rickettsiae all contain a 17kDa antigenic protein. Genes encoding this protein from various pathogenic species have been cloned, sequenced and compared and all exhibit a high degree of sequence homology. However, selected sequences of the genes of the 17kDa antigens are unique to the spotted fever group and have been used to synthesise primers for use in the PCR (Amano *et al.*, 1993).

As noted above, phase variation analogous to smooth to rough variation in bacteria has been observed in *C. burnetii*, but in no other rickettsiae. In *C. burnetii* there are two main surface antigens; the presence or absence of phase I antigen causes the phase variation. Organisms isolated from natural infections are in phase I, but after repeated passage in the yolk sac, the LPS antigen appears, and the organisms move into phase II. Transition between the two phases may occur when one or more carbohydrates from the LPS moiety are no longer synthesised. Since the polysaccharide phase I antigen interferes with immune phagocytosis and antibody production it may serve to conceal the organism from the immune system of its natural host and permit inapparent infection of long duration (Valkova and Kazar, 1995).

1.21 Molecular genetics

The study of rickettsial genes stems from early investigations on the composition and size of the genome, and the circularity and size of the *R. prowazekii* genome have been determined by pulsed field electrophoresis.

The molecular genetics of rickettsiae has been reviewed by Winkler (1990). A wide selection of rickettsial genes have been cloned (see Aliabadi *et al.*, 1993; Aniskovich *et al.*, 1991; Mahan *et al.*, 1994; Scheunke and Walker, 1994; Hendrix, 1993; Hickman, 1991; Crocquet-Valdes *et al.*, 1994; Emelyanov, 1993; Gilmore *et al.*, 1991; Hahn *et al.*,

1993; Shaw and Wood 1994; Stover *et al.*, 1990; Ohashi *et al.*, 1990), these are summarised in Table 4.

The first cloning and expression of a rickettsial gene in *E. coli* was by Wood *et al.*, (1983), and the protein for citrate synthase, an enzyme which had previously been purified, was expressed in *E. coli*. The deduced protein sequence of the *R. prowazekii* citrate synthase shows that the enzyme has a weight of 49kDa, comparable in size with both *E. coli* and pig heart monomers (Wood *et al.*, 1987), and comparison of the sequence of the *E. coli* and rickettsial citrate synthase enzymes shows 59% homology of amino acid sequences. This contrasts with only 20% homology between the rickettsial and pig heart enzymes, and these comparisons are intriguing since the rickettsial citrate synthase shares the regulatory properties of the pig heart enzyme rather than those of the *E. coli* enzyme (Phibbs, 1982 cited by Winkler 1990). The sequence of the ATP/ADP translocase gene shows that it codes for a membrane protein with a weight of 57kDa. The rickettsial translocase bears little resemblance to the mitochondrial ATP/ADP translocase. While both translocases are hydrophobic, basic membrane proteins, there is no homology between the translocases, even at the amino acid level; the mitochondrial translocase is much smaller and is functionally a dimer.

The *recA* gene from *R. prowazekii* has also been isolated and characterised by Dunkin and Wood (1994). A gene bank of *R. prowazekii* strain E was prepared in λ EMBL4 in *E. coli* where 37, 16 and 14 kDa antigens were expressed (Aniskovich *et al.* 1991). Three OMPs were also cloned and expressed in *E. coli* by Emelanov *et al.*, (1993). Genes for protein antigens from *R. prowazekii* (52kDa, Krause *et al.*, 1985 as cited by Mallavia, 1991), *R. rickettsii* (17kDA and 155kDa, McDonald *et al.* 1987) and *R. tsutsugamushi* (56 and 110 kDa, Oaks *et al.*, 1989) have also been cloned and expressed in *E. coli*. The genes for the 17kDa antigen from *R. conorii*, *R. prowazekii* and *R. typhi* have been cloned, sequenced and compared to the equivalent gene in *R. rickettsii* (Smith 1979).

Gene	Organism	Reference
tlc ATP/ADP translocase	*R. prowazekii*	Krause *et al* ., 1985 Williamson *et al* ., 1989
glt A citrate synthase	*R. prowazekii* *C. burnetii*	Wood *et al*., 1983 Wood *et al*., 1987 Heinzen *et al*., 1987
sod superoxide dismutase	*C. burnetii*	unpublished
51 kDa surface Antigen	*R. prowazekii*	Krause *et al*., 1985
17 kDa surface Antigen	*R. rickettsii* *R. conorii* *R. prowazekii* *R. typhi*	Anderson *et al*., 1997 Anderson *et al*., 1989
120 kDa surface antigen	*R. rickettsii*	Gilmore *et al*., 1989
155 kDa surface antigen	*R. rickettsii*	Policastro *et al*., 1990
190 kDa surface antigen	*R. rickettsii*	Anderson *et al*., 1990
sta 56 surface antigen	*R. tsutsugamushi*	Stover *et al*., 1990
sta 58 heat stress protein	*R. tsutsugamushi*	Stover *et al*., 1990
stp 11	*R. tsutsugamushi*	Stover *et al*., 1990
Sta 150, 110, 72, 56, 49, 27 & 20 kDa surface Ags	*R. tsutsugamushi*	Oaks *et al*., 1989 Oaks *et al*., 1987
*htp*A, *hrp*B heat shock proteins	*C. burnetii*	Stover *et al*., 1990

Table 4 Selection of genes cloned from Rickettsiae Genetics of Rickettsiae is reviewed by Mallavia, (1991) and cites the above references.

Differences in the nucleotide sequence of the gene encoding the 58kDa antigen of *R. tsutsugamushi* have been shown by restriction mapping of DNA in a comparison between eleven *R. tsutsugamushi* isolates. The results indicate that significant genetic differences occur within this species. The transcription of rickettsial genes in *E. coli* requires that promoters on the rickettsial gene be recognised by the *E. coli* RNA polymerase. Upstream promoter sequences in the four 17kDa antigen genes show great similarity to the consensus sequence of *E. coli* promoters. The rickettsial genome is greater than 70% A-T rich, therefore, promoter-like sequences are easily found.

1.22 Vaccines

A vaccine has been developed for the 155 kDa genus-common antigen that successfully protected mice against a normally lethal dose of *R. rickettsii* , and this was also used to vaccinate guinea pigs in which a dose-dependent immune response was elicited. Transformants containing the gene for the 155kDa antigen produced both heat-sensitive and heat-resistant epitopes recognised by monoclonal antibodies when sonic lysates from recombinant *E. coli* containing this subclone were used (McDonald *et al.*, 1987). Also a recombinant *R. conorii* vaccine has been successfully used to protect guinea pigs against the disease, Rocky Mountain Spotted Fever and a formalin-killed vaccine of *R. rickettsii* was used to successfully protect human volunteers against this organism (Dumler *et al.*, 1992). The 56 kDa antigen of *R. tsutsugamushi* has been suggested as a vaccine candidate against scrub typhus (Seong *et al.*, 1997), and rOmpA has been suggested as an ideal candidate as a subunit vaccine for Spotted Fever Group rickettsia due to its conservation across several pathogenic Spotted Fever Group organisms (Crocquet-Valdes *et al.*, 1994). The rOmpA protein has also been employed in a baculovirus-expressed vaccine and this was successfully used to immunise guinea pigs inducing antibodies which reacted with *R. rickettsii* and which protected them against a second challenge with virulent organisms (Sumner *et al.*, 1995).

1.23 Fish vaccines

The first commercially used fish vaccine was a killed vibriosis bacterin which was very effective and simple to produce. Killed bacterial vaccines for *Vibrio anguillarum* (vibriosis), *Vibrio ordalii* (vibriosis) and *Vibrio salmoninarum* (Hitra) have all proved simple to produce and they can be administered by immersion bath; the protective antigen in each case is LPS O-polysaccharide (Lillehaug, 1990). Bacterin vaccines have also been developed for *Aeromonas salmonicida* (Middtyling, 1997) although these require incorporation of an oil adjuvant to be effective. Live vaccines provide a desirable development as they can often be administered more easily than the alternative killed vaccines. A potential drawback with live vaccines is the reversion to virulence, although the use of suitably engineered mutants of *A. salmonicida* should avoid this problem (Vaughan 1997).

OBJECT OF RESEARCH

This project was directed towards the long term aim of producing a recombinant vaccine for use in control of piscirickettsiosis in salmonids.

The specific aims were to:

1. grow *P. salmonis* in tissue culture cell lines in the laboratory;

2. purify the organism from cellular material in sufficient quantities for extraction of DNA;

3. prepare a *P. salmonis* recombinant library in a phage lambda vector;

4. characterise the recombinant library and prepare probes for screening the library to identify clones for further analysis.

In addition, since a new isolate of *P. salmonis* was discovered during the project: -

5. to characterise the rickettsial agent isolated form an outbreak of disease in Atlantic salmon in Scotland.

The first section involved culture of cell lines in antibiotic-free media, and monitoring the growth of *P. salmonis* in cell culture.

The second section involved scaling up the production of infected cell cultures to provide sufficient bacteria for purification by differential and density gradient centrifugation. Purified bacteria were used in section 3 for extraction of DNA and preparation of a library in lambda ExCell, some characteristics of which were analysed in section 4, namely, release of inserts from the library, determination of their size, partial characterisation of some inserts by sequence determination.

The last section arose following the discovery of an outbreak of disease in Atlantic salmon at two sites in Scotland. The organism had the characteristics of a rickettsia and its morphology, and replication in tissue cultures were studied. So that the relationship between the organism and existing piscirickettsia could be determined the 16SrRNA sequence was analysed. Antiserum was also raised to purified bacteria and preliminary characterisation carried out by ELISA and immunoblotting. Finally, DNA was extracted form purified bacteria and used to prepare a recombinant library in lambda Fix II (λFixII).

MATERIALS AND METHODS

2.1 Source of reagents

Unless otherwise stated, all chemicals were supplied by Sigma Chemicals, and all restriction and modifying enzymes were supplied by GIBCO, Life Technologies Ltd.

2.2 Bacteria and conditions of culture

2.2.1. Bacterial strains

The bacterial strains used or constructed in this study, with the exception of *P. salmonis* (see below) are shown in Table 5. Strains of *E. coli* were maintained on LB plates, supplemented where required with the appropriate antibiotics; long-term storage was at -70°C in LB supplemented with 50% glycerol, or on PROTECT beads supplied by Technical Service Consultants Ltd.

2.2.2 Bacterial growth media

The composition of media is given in Appendix I. All media were sterilised by autoclaving at 108 kPa for 20 min or by filtration through a sterile 0.22 µm Millipore filter. Glassware was sterilised by heating to 160°C for 2 hr. When required, antibiotics were added after sterilisation of the medium to the following final concentrations: ampicillin, 50 µg/ml; carbenicillin, 50 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 12.5 µg/ml; tetracycline, 10 µg/ml; spectinomycin, 50 µg/ml, and streptomycin 10 µg/ml;. When required, X-gal (5-bromo-4 chloro-3-indolyl-ß-D-galactoside) in DMF (dimethylformamide) and IPTG (isopropyl-thiogalactoside) were added to final concentrations of 40 µg/ml and 2 µg/ml respectively after sterilisation.

2.3 Bacteriophages and plasmids

2.3.1 Bacteriophages

The phages and plasmids used in this study are described in Table 6. Phage lysates were maintained in the dark at 4°C in SM buffer with one drop of $CHCl_3$ added; long term storage was at -20°C.

Table 5 Bacterial strains used in this study and their sources

Strain	Properties
Escherichia coli	
XL1-Blue	*sup* E44 *hsd* R17 *rec* A1 *end* A1 *gyr* A46 *thi rel* A1 *lac* - F' [*pro* AB+ *lac*Iq *lac* Z (Δ M15 Tn10(*tet* r)] (Bullock *et al.*, 1987) supplied by Stratagene.
XL1-Blue MRA	Δ(mcrA) 183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi1,gyrA96, relA1, lac
XL1-Blue MRA (P2)	XL1-Blue MRA (P2 lysogen) supplied by Stratagene
NM522	[F'laclqZ(M15], proA+B+/*sup*E, *thi*-1,((lac-proAB)((*hsd*MS), (*mcr*B)5(rk-, mk-mcrBC-), supplied by Pharmacia.
LE392	*sup* E44*sup* F58*hsd* R514*gal* K2*gal* T22*met* B1*trp*R55*lac* Y1 (Borck *et al.*, 1976; Murray *et al.*, 1977) supplied by Pharmacia.
TG1	*sup*E *hsd* Δ5 *thi* Δ (*lac -pro*AB)F' [*tra*D36, *pro*AB+lacIq*lac* Z ΔM15] (Gibson, 1984)
NP66	*thr*, *leu*, *pro*, *thi*, *bio*, cl857 *int*+*xis*+, (HI, lacZM15, cmr,/pJN13[(80 repressor, pACY184 replicon] /pXis[xis+replicon]/pXis[xis+, strr, pSC101 replicon] Supplied by Pharmacia
Legionella pneumophila	Knoxville serogroup 1 grown on yeast charcoal agar supplied by Dr M. Djebarra, Glasgow Univ.

Antibiotic sensitivities : cm, chloramphenicol; str, streptomycin; spec, spectinomycin.

Table 6 Phages and plasmids used in this study

Phage	Supplier
λExCell	Pharmacia
λFixII	Stratagene
λcI1857Sam7 -wtl	Stratagene
M13KO7	Helper phage, GIBCO

Plasmid	Supplier
pUC18	GIBCO, Life Technologies
pExCell	Pharmacia
pAG16S1	pUC18 with *P. salmonis* 16S gene this study
pAGMIP	pUC18 with *L. pneumophila* MIP gene this study
pAGB41.21	pUC18 with 3.5 kb insert (B41.21) from *P. salmonis* λ/ExCell, this study

2.3.1.2 Titration of phage suspensions

Phage stocks were titred using methods described by Silhavy *et al.* (1984). Dilutions of stock lysates, from 10^{-1} to 10^{-6}, were prepared in SM buffer for each titration. Titres were calculated using the formulae: -

$$\text{phage concentration (pfu/ml)} = \frac{\text{no. of plaques x diln. factor}}{\text{vol. of extract plated}}$$

$$\text{recombinants/mg} = \frac{\text{total pfu}}{\text{total mg DNA packaged}}$$

total pfu = titre x total extract vol.

2.3.2 Plasmids

All plasmids used or constructed are described in Table 6.

2.4 Culture of *Piscirickettsia salmonis*

2.4.1 Bacteria

Piscirickettsia salmonis was supplied by Marine Harvest Chile (labelled isolates A, B and C), Marine Harvest McConnell, Lochailort (Ardintoul strain), and the type strain LF-89, was obtained from ATCC.

2.4.2 Tissue culture

2.4.2.1 Tissue culture cell lines and their maintenance

The fish cell lines CHSE-214 (Chinook salmon embryo derived) and RTG-2 (rainbow trout gonad derived) and the frog derived cell line, XTC originating from *Xenopus leavis,* South American clawed toad were maintained in Minimal Essential Medium (MEM) with Earle's salts, supplemented with 10% foetal calf serum (FCS) and 1% L-Glutamine. in the absence of antibiotics at 15°C. All media were obtained from Gibco Life Technologies Ltd., Paisley.

Cell lines were maintained until a confluent monolayer was visible by light microscopy, taking approximately 2-5 days depending on the size of flask and inoculum. The monolayer was rinsed with 5 ml trypsin/EDTA solution, which was removed and replaced with 1ml trypsin/EDTAsolution until cells were visibly sloughing off the flask

base. The cells were resuspended in growth medium, divided into flasks at a ratio of 1:2 and incubated as above.

2.4.2.2 Storage of cells in liquid nitrogen

Medium was decanted from a confluent monolayer of cells and 1 ml of Trypsin/EDTA solution was used per flask to rinse the monolayer. After incubation for a few minutes, when the cells had begun to slough off the flask surface, 5 ml of growth medium was added, the cells washed off the surface of the flask and collected in a sterile universal bottle. The cells were collected by centrifugation at 1000 rpm for 2 min, and resuspended in 2 ml of FCS with 10% DMSO. The suspension was distributed into Nunc tubes (1 ml each) which were placed in a container (Jencon) of isopropanol; the whole tub was incubated at 4°C for 1 hr, then -20°C overnight followed by -70°C for 1 h before final transfer to liquid nitrogen.

When thawing, the tubes were removed from liquid nitrogen and immediately warmed to 37°C in a water bath, the cells resuspended in warmed FCS, briefly centrifuged and resuspended in growth medium before addition to a culture flask.

2.4.3 Growth of *P. salmonis* in tissue cultures

P. salmonis LF89 was received as a culture frozen in dry ice and was stored at -70°C until required. After thawing, it was used to infect a 25cm^2 flask of CHSE cells. The culture supernatant fluid obtained after 3 weeks was used to infect 70% confluent monolayers of cells. Infection resulted in cytopathic effects within approximately 7 days, and the complete monolayer was lysed within 14 - 17 days. Other strains of *P. salmonis* were cultured similarly.

Large scale culture of *P. salmonis* was carried out using either several (typically 12) 150cm^2 tissue culture flasks, or 2L roller bottles.

2.4.4 Purification of *P. salmonis*

Infected supernatant of *P. salmonis* supplied by Chile, approximately 500 ml was divided between three 250 ml centrifuge tubes and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was removed and the pellet resuspended in 10 ml MEM with 0.1% Tween20. The sample was homogenised and centrifuged at 1000 rpm for 10 min

at 4°C. The supernatant was filtered through an AP25 filter and centrifuged at 10,000 g for 10 min. The supernatant was discarded, the pellet resuspended in 1 ml MEM and layered onto a 50% isotonic Percoll gradient prepared in growth media. The gradients were centrifuged at 30,000 g for 60 min at 4°C.The gradient produced two distinct bands, which were removed and resuspended in PBS and centrifuged at 10,000 g for 30 min, this was repeated three times, the pellet was resuspended in 1 ml of PBS. Samples, 10 µl were removed for smears by fixing in formol saline, stained with acridine orange and Giemsa at various dilutions.

2.5 Antisera

2.5.1 Rabbit anti-*P. salmonis* antiserum

High titre serum was supplied by Marine Harvest Chile in three Eppendorf tubes labelled; PHAROS B95006: RABBIT No. SB51: Ag RICKETTSIA: 9.11.96

The tubes were stored at -20°C.

2.5.2 Sheep anti-*P. salmonis* antiserum

Rickettsiae were cultured in CHSE cells, purified by Percoll density gradient centrifugation, washed in PBS and inactivated by incubation overnight at 4°C with formalin (final concentration 5% v/v). Cells were collected by centrifugation at 15000 x g for 10 min at 4°C, washed twice in PBS, resuspended in PBS and the protein concentration (Bradford assay) adjusted to 115 mg/ml.

Immunisation of a sheep was carried out by the Scottish Antibody Production Unit, Carluke, Lanarkshire. After obtaining pre-immunisation control serum, the sheep was immunised with 1 ml of the above antigen preparation emulsified in Freund's Incomplete Adjuvant on Day 1, and after 4, 8, 12 and 35 weeks. Blood samples were taken one week after each booster immunisation, serum obtained and stored at -20°C until required for use.

2.5.3 Fish antisera to *P. salmonis*

Sera from fish immunised with inactivated *P. salmonis* were supplied by Dr. Enrique Madrid, Marine Harvest, Chile.

2.5.4 Horseradish peroxidase-labelled antisera

Horseradish peroxidase (HRP)-labelled sheep-anti-rabbit IgG and HRP-labelled goat-anti-sheep IgG were obtained from SAPU; they were stored at -20°C and were used at a final dilution of 1/1000.

2.6 Construction of genomic libraries of *P. salmonis* DNA

2.6.1 Construction of a DNA library of *P. salmonis* LF89 isolate in λExCell

2.6.1.1 Preparation of rickettsial DNA

P.salmonis purified from tissue culture was supplied by Marine Harvest, Chile stored in 5 ml of ethanol. The entire sample was transferred to a 50 ml centrifuge tube and centrifuged at 10,000 x g for 30 min at 4°C. The pelletted bacteria were resuspended in 1 ml sterile TE, pH8.0. The resuspended bacteria were incubated for 20 min at 37°C with 1 mg/ml lysozyme; 0.5% SDS was added and 100 mg/ml Proteinase K, and the mixture incubated for 2 hr at 50°C to ensure lysis complete. The DNA was then extracted twice with equal volumes of phenol:chloroform: isoamyl alcohol (25:24:1), then extracted with chloroform and precipitated with two volumes of ice-cold 100% ethanol, and 0.1 vol. of 3M NaAc. After incubation for 15 min at -70°C the precipitated DNA was collected by centrifugation at 12,000 x g for 15 min at 4°C and washed twice with 70% ethanol. The DNA was then air dried on the bench and resuspended in dH_2O. The addition of 10 µg of yeast tRNA supplied by Sigma was required due to the anticipated low DNA concentration, tRNA acts as a carrier to improve the recovery of small amounts of DNA. tRNA was prepared by dissolving 10 mg/ml in sterile TE (pH7.6) containing 0.1 M NaCl. The solution was extracted twice with phenol equilibriated with Tris, pH7.6 and twice with chloroform. The RNA was precipitated with 2.5 volumes of ethanol at room temperature and recovered by centrifugation at 12,000 rpm for 15 min at 4 °C. Redisssolved at a concentration of 10 mg/ml in sterile TE, aliquoted and stored at -20 °C.

2.6.1.2 Partial digest of *P. salmonis* DNA

The purified DNA (10 ng) was partially digested with *EcoRI* to produce compatible ends for ligation into the λ ExCell vector. To determine appropriate conditions for partial digestion, control reactions were set up using *Aeromonas salmonicida* DNA and *Pasteurella haemolytica* DNA; these experiments showed that digestion with 5 units of *EcoRI* for 30 min at 37°C produced a high proportion of fragments in the size range 0 to 6 kb, the size range recommended for the cloning vector. The partially digested *P. salmonis* DNA was extracted with phenol:chloroform and ethanol precipitated to remove buffer and enzyme which could interfere with ligation reactions.

2.6.1.3 Ligation of insert DNA to vector

The λExCell vector (Promega) was supplied digested with *EcoRI* and dephosphorylated to minimise religation of vector to itself. Ligation was carried out with 10 ng of insert DNA and 2 µg of λExCell vector at 16°C for 4.5 h.

2.6.1.4 Packaging ligated vector plus insert

The packaging extracts were prepared as in the manufacturer's instructions and the ligated vector plus insert DNA was packaged to form infective lambda recombinants. A control packaging reaction was also set up using an extract of *E. coli* strain LE392 with control lambda DNA supplied by the manufacturer. Part of the packaging mixture was immediately titred and the remaining material stored at 4°C. The results of the phage titrations are shown in Table 8.

2.6.1.5 Preparation of host cells

Strain NM522 is an *E. coli* K12 derivative which is restriction minus and modification minus. The lyophilised culture was resuspended in 1 ml of LB and grown overnight at 37°C with shaking before plating onto M9 minimal medium containing 1 mg/ml thiamine. The strain must be maintained on minimal medium to ensure retention of the F' episome which is required for infection by phage and complementation of ß-galactosidase. NM522 was grown in LB or 2 x YT media immediately prior to infection by phage. For long term storage a stationary phase culture, grown in either LB or M9

MM containing thiamine, was mixed with an equal volume of glycerol and stored at -70°C.

Strain NP66: The lyophilised culture was resuspended in 1 ml of LB and grown overnight at 32°C with shaking and subcultured onto LB plates containing 50 µg/ml spectinomycin and 30 µg/ml chloramphenicol. The plates were incubated at 32°C. The strain was maintained on media containing the above antibiotics to ensure the presence of the plasmid pXis which is essential for *in vivo* release of pExCell. For long term storage, the above *E. coli* culture with appropriate antibiotics was mixed with an equal volume of glycerol and stored at -70°C.

2.6.1.6 Lawn cell preparation

5 ml of broth inoculated with a loop of NM522 was incubated overnight at 37°C with shaking; 5 ml of LB broth with 0.2% maltose was inoculated with 50 µl of the overnight culture and grown until the A_{600} reached 1.0, when the cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C. The pellet was resuspended in 10 mM $MgSO_4$ to a final A_{600} = 2.0. The cells were then ready for use but could be stored at 4°C for up to one week.

2.6.1.7 Blue/white screening with ExCell and pExCell

For blue/white screening 2 µl of 1M IPTG and 50 µl of 10% X-gal (in DMF) were incorporated into the plating mixture or top agar. The increased concentration of X-gal compensates for the reduced activity of ß-galactosidase caused by insertion of the multi-cloning site into the *lacZ* gene.

2.6.1.8 *In vivo* release of phagemid pExCell from individual clones.

The phage was obtained by taking a core from single fresh plaque and releasing the phage into 300 µl of SM buffer by allowing diffusion for a minimum of 1 hour.

The *E. coli* NP66 cell preparation was obtained by culture of the organism, overnight at 32°C with shaking, in 5 ml of 2 x YT broth containing 50 µg/ml spectinomycin, 30 µg/ml chloramphenicol and 0.2 % maltose. From the overnight culture 50 µl was used to inoculate 5 ml of 2 x YT broth containing 50 µg/ml spectinomycin,30 µg/ml chloramphenicol and 0.2 % maltose, which was grown until an A_{600} of 0.5-0.8 was

reached. The cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C and the pellet resuspended in NZCYM containing 50 µg/ml spectinomycin to a final A_{600} of 2.0. The cells must be used within 1 hour.

2.6.1.9 Release of pExCell

100 µl of the above freshly prepared NP66 cell was incubated at 39°C for 20 min to allow expression of the proteins required for site-specific recombination between the attL and attR sites. 100 µl of SM solution containing the cored plaque was added to the NP66 cells and incubated for a further 20 min at 39°C. To terminate infection of NP66 with λExCell 200 µl of 1 M sodium citrate solution (room temperature) was added and the mixture transferred to 5 ml of prewarmed (32°C) 2 x YT broth containing 50 µg/ml spectinomycin. The culture was incubated at 32°C with moderate shaking for 1.5 h, and 50 µl of the released culture was used to inoculate 5 ml of LB + ampicillin (100 µg/ml) and incubated overnight at 37°C with shaking, the released culture was also plated onto LB plates + ampicillin, from which DNA can be prepared by any of the usual protocols for plasmid DNA preparation.

2.6.1.10 Production of single stranded pExCell DNA

A colony of NM522 containing pExCell was transferred from an MM plate to 2 x YT broth supplemented with 0.001%thiamine and 100 µg/ml ampicillin, and this culture was grown to an A_{660} of 0.5-0.8 at 37°C; 2 ml of the culture was infected with the helper phage M13K07 at a multiplicity of infection of 10. The culture was shaken at 300 rpm for 1 h in a 50 ml tube and 400 µl of the infected cells removed, mixed with 10 ml of 2 x YT media containing 70 µg/ml kanamycin and grown at 37°C for 14-18 hours in a shaking incubator at 300 rpm. Cells were removed by centrifugation at 4000 rpm for 15 min at 4°C and the centrifugation step repeated until a cell pellet was no longer obtained, leaving a supernatant containing the pExCell SS-DNA phage. To precipitate the SS-DNA, 0.25 vol. of 3.5 M ammonium acetate/20% PEG solution added was added and the solution mixed by repeated inversion. The solution was placed on ice for 30 min, centrifuged for 15-30 min at 11000 x g to collect the precipitated phage, the supernatant carefully removed and the tubes drained. The pellet

was resuspended in 400 ml TE with gentle vortexing and transferred to a microcentrifuge tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, the solution vortexed for 30 sec and then centrifuged for 1 min to separate the two phases. The upper phase was carefully transferred to a fresh tube where it was re-extracted, prior to a final extraction with chloroform. Ethanol precipitation was used to recover the DNA which was then air dried, dissolved in 10 µl of dH_2O and stored at -20°C. For DNA sequencing an $A_{260/280}$ ratio of at least 1.7 is required.

2.6.2 Construction of a DNA library of *P. salmonis* Ardintoul strain in Lambda FixII

2.6.2.1 Preparation of genomic DNA from *P. salmonis* Ardintoul strain using CTAB

This protocol was adapted from that of Ausubel *et al.* , (1987). Purified *P. salmonis* was prepared from infected tissue culture cells as described elsewhere; 1.5 ml of the bacterial suspension was centrifuged in a microcentrifuge at full speed to produce a compact pellet, the supernatant was discarded and the pellet was resuspended in 567 µl TE buffer. Then, 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added to give a final concentration of 100 µg/ml proteinase K in 0.5% SDS. The solution was mixed thoroughly and incubated for 1 hr at 37°C, during which time the solution became viscous as the detergent lysed the cell wall; 100 µl of 5M NaCl was added and mixed, then 80 µl of CTAB/NaCl solution was added, mixed and incubated for 10 min at 65°C. An equal volume (700-800 µl) of chloroform:isoamyl alcohol (24:1) was added, mixed and centrifuged for 5 min in a microcentrifuge. The aqueous supernatant was then transferred to a fresh microfuge tube leaving behind the white interface material. After adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), mixing thoroughly and centrifugation as above, the clear supernatant was again transferred to a fresh tube where 0.6 vol. of isopropanol was used to precipitate the DNA. At this stage it was not necessary to add salt to aid the precipitation since the NaCl concentration was already high. The DNA pellet was precipitated by

centrifugation at full speed (12000 rpm) in a microcentrifuge for 15 min at 4°C. The DNA appeared as a white/clear precipitate along the side of the tube and was carefully washed with 70% ethanol and centrifuged again for 5 min; the supernatant was then carefully removed and the residual DNA pellet air dried on the bench. The DNA was resuspended in dH_2O and stored at 4°C.

2.6.2.2 Preparation of the insert

Prior to ligation to the lambda FixII vector arms the insert DNA was first partially digested with *Sau3AI* , by digesting approximately 0.7 µg Ardintoul isolate CTAB-processed genomic DNA for three diferent incubation times, 5, 10 and 20 min, at 37°C. The three digests were pooled.

2.6.2.3 Extraction of partially digested *Sau3AI* Ard DNA

The pooled digests were extracted with an equal volume (30 µl) of phenol:chloroform: isoamyl alcohol (25:24:1), inverted for mixing and centrifuged in a microcentrifuge at 12,000 rpm for 5 min at room temperature. The upper, aqueous, phase was again extracted with an equal volume of chloroform:isoamyl alcohol (24:1) as above, and the upper phase transferred to a fresh Eppendorf for ethanol precipitation of the DNA which was finally dissolved in 10 µl dH_2O.

2.6.2.4 Partial fill-in of the insert Ard DNA

The first two nucleotides generated by the restriction digestion were filled in. To the digested *Sau3AI* insert DNA (10 µl) 5 µl of 10X fill-in buffer, 5 µl of 10 mM dGTP, 5 µl of 10 mM dATP and 10 U of Klenow enzyme (2U/ µl) were added. The mixture was incubated at room temperature for 20 min, before addition of 33 µl of 10x STE and 100 µl of 1X STE, followed by two extractions with phenol:chloroform, ethanol precipitation and final dissolution in 5 µl of dH_2O.

2.6.2.5 Ligation of the filled-in insert DNA to Lambda FixII arms

To 2 µl of the above DNA preparation (approximately 1 µg DNA), 1 µl Lambda FixII vector, 1 µl of T4 DNA ligase buffer (5X, GIBCO) and 2U of T4 DNA ligase, 2 µl, were added and the ligation mixture incubated overnight at 4°C.

2.6.2.6 Ligation of pMF test insert

To the pMF test insert (0.3 µl contained 0.3 µg) was added 1 µl of lambda FixII vector (1 µg), 1 µl of T4 DNA ligase buffer (5X, GIBCO), 2 U of T4 DNA ligase (2 µl) and 0.4 µl of dH_2O to achieve a volume 5 µl; the ligation mixture was incubated as above.

2.6.2.7 Control ligation - no insert

To 1 µl of Lambda FixII vector was added 1 µl of T4 DNA ligase buffer (5X, GIBCO), 2 µl of T4 DNA ligase (2U) and 2 µl of dH_2O. The reaction mixture was incubated as above.

2.6.2.8 Packaging the λFixII ligate with Gigapack II

The required number of packaging extracts were removed from storage at -70°C and placed on ice; each extract was individually warmed between the fingers until it began to thaw, and immediately the DNA was then added (1 µl of each ligation). After gentle pipetting and brief centrifugation for 3-5 sec, the reaction mixtures were incubated at room temperature for 2 h (n.b. this must not be exceeded); 500 µl of SM buffer and 20 µl of chloroform were added, the mixtures centrifuged briefly to remove debris and the supernatant transferred to a new Eppendorf tube for storage at 4°C until subsequent titration

2.6.2.9 Positive control for the Gigapack II packaging extract

The above procedure was followed substituting 1 µl of wt lambda c1857DNA in the ligation mixture.

2.6.2.10 Host cells for λFixII

Host cells were *E. coli* XL1-blue MRA and MRA(P2) which were cultured at 37°C in LB broth. For infection by lambda the host bacteria were grown for 4 to 6 h with shaking at 37°C in the appropriate medium supplemented with $MgSO_4$ and maltose until the A_{600} reached 1.0. The bacteria were harvested and resuspended in 10 mM $MgSO_4$ to A_{600} = 0.5 and stored for 48 h at 4°C.

2.7 General DNA methods

2.7.1 Plasmid DNA preparations

2.7.1.1 Promega Wizard mini preparations

An overnight culture (5 ml) of bacteria containg the plasmid of interest was centrifuged for 10 min at 4000xg, the pellet resuspended in 200 µl of Cell Resuspension Solution (Appendix), and transferred to an Eppendorf tube; 200 µl of Cell Lysis Solution was added and gently mixed by inverting the tube several times. Mixing was continued until the suspension had cleared, when 200 µl of Neutralization Solution was added and the tube inverted several times before centrifugation at full speed (12000 rpm) in a microcentrifuge for 5 min.

One ml of Wizard Minipreps DNA Purification resin was added to the supernatant and a Wizard minicolumn was prepared for each sample by removing the plunger from a 3 ml disposable syringe and attaching the syringe barrel to the Luer-lock system on each minicolumn. The Resin/DNA mixture was pipetted into the syringe barrel, the syringe plunger was inserted and gently pushed forcing the slurry through the minicolumn. The syringe was detached from the minicolumn, the plunger removed and the minicolumn washed with 2 ml of Column Wash Solution. The syringe barrel was removed and the minicolumn transferred to an Eppendorf tube where it was centrifuged at full speed for 2 min to remove any residual column wash solution. The minicolumn was transferred to a new tube and 50 µl of sterile dH_2O was added; after waiting 1 min the DNA was eluted and centrifuged at top speed in a microfuge for 30 sec. The DNA was stored at 4°C or -20°C.

2.7.1.2 Promega Wizard plus SV miniprep plasmid purification system

An overnight bacterial culture (5 ml) containing the plasmid of interest was centrifuged at 4000 rpm in a sterile plastic Universal bottle for 10 min at 4°C. The supernatant was removed and the tube was inverted on to a paper towel to remove residual supernatant; 250 µl of Wizard SV Plus Cell Resuspension Solution was added and the pellet was resuspended by pipetting. The resuspended cells were transferred to a sterile microfuge tube, 250 µl of Wizard SV Plus Cell Lysis Solution was added and the tube inverted four times. The solution was incubated for 5 min at room temperature until the cell suspension had cleared; 10 µl of Alkaline Protease Solution was added and again the contents were mixed by inverting the tube four times. After incubation at room

temperature for 5 min (n.b. the 5 min incubation time should not be exceeded as nicking of the DNA can occur), 350 µl of Wizard Neutralization Solution was added and the tube immediately inverted four times. The bacterial lysate was then centrifuged at 12000 rpm (microfuge) for 10 min at room temperature. Plasmid DNA purification units were prepared by inserting one Wizard Plus SV Minipreps Spin Column into one 2 ml Collection tube for each sample. The cleared lysate was then transferred to a Wizard SV Plus Miniprep Spin Column, the supernatant was centrifuged on a bench centrifuge for 1 min, the Wizard SV Plus Miniprep Spin Column was removed and the flowthrough discarded. The column was washed by the addition of 750 µl of Wizard SV Plus Minipreps Column Wash solution, and centrifuged on the bench centrifuge for 1 min. The column was removed and the flowthrough discarded, the column reinserted into the collection tube and 250 µl of Wizard SV Plus Minipreps Column Wash solution added and centrifuged on a bench centrifuge for 2 min. The column was then transferred to a new sterile microcentrifuge tube where the plasmid DNA was eluted by adding 100 µl of nuclease-free water to the column and centrifugation for 1 min in a bench centrifuge. The DNA was stored at either 4°C or -20°C.

2.7.1.3 Promega Wizard PLUS SV maxiprep plasmid DNA purification system

For larger scale plasmid preparation the cells from 100-500 ml of an overnight *E. coli* culture grown with shaking at 37°C were collected by centrifugation at 5000 rpm for 10 min at room temperature and the resultant pellet resuspended in 15 ml of Cell Resuspension Solution. Then, 15 ml of Cell Lysis Solution was added and mixed gently until cell lysis was judged to be complete, when the solution became clear and viscous; this process took up to 20 min. Neutralization Solution (30 ml) was added and the tube contents immediately mixed by inverting several times and incubation at room temperature for 10 min. The suspension was centrifuged at 12000 rpm for 15 min at 20°C, and the cleared supernatant was transferred to a centrifuge bottle. Isopropanol (0.5 vol, room temperature) was added, mixed by inverting the bottle and centrifuged

for 15 min at 12000 rpm at room temperature. The supernatant was discarded and the DNA pellet resuspended in 2 ml of dH_2O.

2.7.1.4 Preparation of plasmids by alkaline lysis

Bacterial cells from an overnight culture (1.5 ml) were harvested in a sterile microfuge tube and washed once with sterile saline. The pellet was then resuspended in 100 µl of alkaline lysis buffer and incubated for 45 min at 60°C. The DNA was extracted using phenol:chloroform (1:1) and the aqueous layer analysed on an agarose gel with appropriate standards of undigested plasmids compared with a known plasmid profile. A smaller volume was also used to analyse colonies, in which case the colony was resuspended in 25 µl of lysis buffer and the above protocol followed as normal.

2.7.1.5 Colony lysis method to prepare plasmids

Rapid disruption of bacterial colonies to test the size of plasmids (Sambrook *et al.*, 1989), based on the method by Barnes (1977), produces sufficient yield of DNA to analyse on an agarose gel. Briefly, bacterial colonies grown on LB agar containing the appropriate antibiotic until they were approximately 1 mm in diameter were picked off the agar surface using a sterile toothpick or wire loop, streaked onto a master agar plate with the appropriate antibiotic and grown for a few hours, then stored at 4°C until required to recover the necessary colonies. The remainder of each colony from the original plate was resuspended in a microfuge tube containing 50 µl of sterile 10 mM EDTA (pH8.0); 50 µl of freshly made 0.2 N NaOH, 0.5% SDS, 20% sucrose was added and the solution vortexed for 30 sec. The mixture was incubated for 5 min at 70°C and then allowed to cool to room temperature. 1.5 µl of 4 M KCl was added and the mixture vortexed for 30 sec, before incubation for 15 min on ice. The bacterial debris was centrifuged at 12000 rpm for 3 min at 4°C in a microcentrifuge, and 10 µl of the supernatant was added to 3 µl of gel loading dye and loaded onto a 0.8% agarose gel. Uncut plasmid DNA (i.e. supercoiled DNA) was used as a standard. Plasmids without inserts have the same profile as control plasmid DNA and plasmids containing insets run more slowly than the control. The original colony was used to generate greater quantities of plasmid DNA for further analysis.

2.7.2 Preparation of transformation-competent cells

2.7.2.1 Cells for electroporation

An overnight 10 ml culture of the strain to be electroporated was grown in 2 x YT medium with shaking at 37°C. The culture was added to 1L of 2 x YT broth and grown with shaking until A_{600} = 0.5 was reached. The culture was incubated on ice for 30 min and the cells pelleted by centrifugation at 6000 rpm at 4°C for 20 min. The pellet was resuspended in 1L of ice-cold sterile 1M Hepes buffer, pH7, again pelleted as above and resuspended in 500 ml of ice-cold sterile 1M Hepes buffer, centrifuged again and resuspended in 20 ml of ice-cold sterile 1M Hepes buffer containing 10% glycerol. After centrifugation at 5000 rpm for 20 min at 4°C the cells were resuspended for a final time in 2-3 ml of 10% glycerol in LB; the suspension was dispensed into Eppendorf tubes, snap frozen by dropping the tubes into liquid nitrogen and stored at -70°C.

2.7.2.2 Cells for heat shock transformation

An overnight 10 ml culture of the strain to be transformed was grown in 2 x YT broth with shaking overnight at 37°C. A 500 µl volume of the overnight culture was inoculated into 50 ml prewarmed 2 x YT broth and cultured with shaking at 37°C until A_{600} = 0.5 was reached. The cells were chilled on ice for 10 min and centrifuged at 4000 rpm for 15 min at 4°C. The supernatant fluid was decanted and the pellet resuspended in 25 ml ice-cold sterile 100 mM $CaCl_2$. Again, the cell suspension was chilled on ice and centrifuged as above, resuspended in 5 ml ice-cold 100 mM $CaCl_2$ and kept on ice until required (incubation on ice up to overnight can increase competence of the cells for transformation). For storage, an equal volume of sterile glycerol was added to the cell suspension which was dispensed into sterile Eppendorf tubes; these were snap frozen by immersion in liquid nitrogen and stored at -70°C.

2.7.2.3 Transformation by electroporation

Competent cells were gently thawed on ice. DNA, 1-5 µl at a concentration of 10 µg/µl) in sterile water, was placed in a prechilled (-20°C) 0.2 cm sterile electroporation cuvette, supplied by Equibio Ltd., and 20 µl of thawed, competent cell suspension was added. After mixing, the suspension was shaken to the bottom of the cuvette and immediately placed in a prechilled safety chamber slide. The BIORAD Gene Pulser was set at 25 mF and 2.0 kV and the Pulse Controller at 200 ohms. The cuvette was pulsed once, giving a time constant of 4-5 msec. Immediately, 1 ml of SOC medium (preheated to 37°C) was added to the cuvette and, after transfer to a Universal bottle, the culture was incubated with shaking for 1 hr at 37°C. Samples of 100 µl were plated directly on to selective medium and the plates incubated overnight at 37°C.

2.7.2.4 Transformation by heat shock

Competent cells were thawed gently on ice. DNA, 10 µl at a concentration of 10 µg/µl in distilled water, and 100 µl of thawed competent cells were mixed in an Eppendorf tube on ice and incubated on ice for 1 hr. The solution was mixed again and subjected to heat shock at 45°C for 90 sec, before being returned to ice briefly before addition of 1 ml prewarmed LB plus 10 mM $MgSO_4$; incubation was continued for 1 hr with shaking at 37°C before samples of 100 µl were plated directly on to selective medium and the plates incubated overnight at 37°C.

2.7.3 Preparation of lambda DNA

2.7.3.1 Plate lysate method

A fresh culture of the appropriate *E. coli* host strain was set up in 5 ml of LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$. This was shaken overnight at 37°C. For each 90 mm diameter plate, approximately 1 x 10^5 pfu of bacteriophage were mixed with 100 µl of the fresh overnight culture. Alternatively, an agar plug containing phage from a cored plaque was eluted. This was done using a Pasteur pipette to pick a single plaque from an agar plate. The agarose plug was gently expelled into an Eppendorf containing 1 ml of SM buffer and incubated for 1 h at room

temperature. 100 µl of eluted phage was mixed with 100 µl of a fresh *E. coli* overnight culture and incubated for 30 min at 37°C.

2.5 ml of melted (45°C) top agarose was added to the infected cells, mixed by shaking gently and poured onto an LB plate prewarmed to 37°C. When the top agarose hardened, the plates were inverted and incubated at 37°C for 6 h or until the plaques became confluent. The plates were allowed to cool for 30 min at 4°C, then overlaid with 10 ml of SM buffer and allowed to stand for 2 h. The buffer containing eluted phage was centrifuged at 4000 rpm for 10 min at 4°C to remove bacterial cells. The supernatant was carefully transferred to a fresh tube, and chloroform was added to a final concentration of 0.3% (v/v) for storage at 4°C for up to 6 months.

2.7.3.2 Phage miniprep procedure

RNaseA and DNase I were added to the above phage lysate to a final concentration of 1 µg/ml and the mixture was incubated for 30 min at 37°C. 5.8g of NaCl and 9.3g of PEG were added per 100 ml of lysate and mixed until completely dissolved. The dissolved mixture was incubated on ice overnight. The precipitated phage particles were recovered by centrifuging at 10000xg for 20 min at 4°C. The supernatant was removed and the tube drained by inverting on a paper towel. 1 ml of SM buffer per 10 ml of starting lysate was added and the bacteriophage particles were resuspended by vortexing. Centrifugation at 8000 x g for 2 min removed debris and the supernatant was transferred to a new tube and 100 µl of 10% SDS (per 100 µl of lysate) and 100 µl of 0.5 M EDTA pH8.0 was added and incubated at 68°C for 15 min. 1 vol of phenol:chloroform:isoamylalcohol (25:24:1) was added and the mixture vortexed gently. Separation of the two phases was achieved by centrifugation at 12000 x g for 5 min, and the upper phase was transferred to a new tube and the extraction repeated. The upper aqueous layer was transferred to a fresh Eppendorf tube, and an equal volume of isopropanol was added, mixed and incubated for 1 h at -20°C. The precipitated DNA was collected by centrifugation at 12000 x g for 15 min at 4°C; the supernatant was carefully removed and the DNA washed with 70% ethanol. The pellet

was air dried and resuspended in 20 µl of dH_2O for each 10 ml of starting lysate. The lambda DNA was stored at 4°C.

5-10 µg of recombinant lambda DNA should recovered from 2 x 10^{11} pfu of starting material.

2.7.3.3 Purification of lambda DNA: modified Wizard method

A single plaque was cored into 1 ml of SM buffer and left overnight at 4°C, 0.1 ml was (approx. 10^5-10^6 pfu/ml) used to infect 300 µl of A_{600} = 0.6, of bacterial suspension. The phage and bacteria were mixed and incubated for 30 min at 37°C, 0.7% agarose was added to the mixture of phage and bacteria and poured onto prewarmed LB plates. When the plates were dry they were inverted and incubated at 37°C until confluent lysis occurred (approx. 6 h). 10 µl of 0.01% gelatin in SM buffer was added to the plates and left overnight at 4°C with shaking. The supernatant was collected and the plate surface was washed with SM buffer and the volume made up to 10 µl with SM buffer. 0.1 ml of $CHCl_3$ was added to the solution and mixed by inverting. Debris was removed by centrifugation at 3000 rpm for 30 min at 4°C. The supernatant was collected in 15 ml centrifuge tubes and 50 µl of 0.1% bromophenol blue and 5 µl of 50% suspension of Whatman DE52 equilibrated in SM buffer was added. Mixing was continued until the dye bound to the charged material, which took approx. 5-10 min. The charged material is comprised of lambda proteins and bacterial debris, etc., and this was collected by centrifugation at 3000 rpm for 5 min at room temperature. 0.8g of guanidine thiocyanate was added per ml of supernatant, along with 1 ml of Wizard DNA Clean-up Resin and mixed until homogenous, taking 15 min in total. The resin was concentrated by centrifugation at 1000 rpm, the supernatant removed down to 5 ml and this mixture was resuspended and put through a syringe to collect it on a Wizard column; the column was washed with 80% isopropanol, and centrifuged in a microfuge for 20 sec to remove the alcohol. The DNA was eluted by adding 100 µl dH_2O at 80°C, the column microfuged for 20 sec and the eluate collected in an Eppendorf tube. This step was repeated.

2.7.4 Endonuclease restriction of DNA

Chromosomal, plasmid and phage DNA were digested using endonucleases supplied by GIBCO, Life Technologies Ltd. Digestion was carried out in a total volume of 10 µl and in the presence of 0.1 vol. of 10x reaction buffer and 1U of the appropriate endonuclease(s). Incubations were generally carried out for 1 h and the reaction stopped by transfer to -20°C or by analysis on a 0.8% agarose gel.

2.7.5 Purification of nucleic acids

2.7.5.1 Extraction with phenol:chloroform

An equal volume of phenol:chloroform solution (1:1) equilibrated in TE buffer (Sigma) was added to the nucleic acid sample in an Eppendorf tube and mixed until the contents formed an emulsion. The mixture was centrifuged for 1 min at 12, 000 rpm at room temperature so that the organic and aqueous phases, were well separated. The upper aqueous phase was rtransferred to a new Eppendorf tube and the phenol:chloroform extraction was repeated until no layer of denatured protein was visible at the organic/aqueous interface. The DNA was recovered by ethanol precipitation.

2.7.5.2 Ethanol precipitation of DNA in microfuge tubes

The method described by Sambrook *et al.* (1989) was used. To 400µl (or less) of solution containing the DNA was added 2 volumes of ice-cold 100% ethanol. After mixing by inversion 0.1 vol. of 3M sodium acetate solution was added, and the solution again mixed before incubation at -70°C for 15 min or -20°C for 30 min. The solution was centrifuged at 12000 rpm in a microcentrifuge for 15 min at 4°C, the supernatant was carefully removed and the precipitated DNA was washed carefully with 70% ethanol, and centrifuged again for 5 min as above. The ethanol was removed and the DNA air dried on the bench until no more liquid was visible; this took up to 30 min, after which the DNA was dissolved in 10 µl dH_2O and stored at either 4°C or -20°C.

2.7.5.3 DNA agarose gel electrophoresis

For separation and identification of DNA fragments, 0.8% (w/v) agarose (Promega molecular biology grade) gels, made up and run at 5 V/cm in 0.5 x TBE, were used.

For isolation of large (>10 kb) or small (<1 kb) DNA molecules, 0.5% and 1.0 % agarose gels, respectively, were used. Ethidium bromide was included in the gels at a final concentration of 5 μg/μl.

2.7.5.4 Isolation and purification of DNA from agarose gels

After digestion with the appropriate restriction enzyme(s), desired fragments were separated by electrophoresis on an agarose gel. Using minimal UV exposure the desired bands were identified and excised in the smallest possible volume of agarose. DNA was extracted from the agarose using either a Geneclean II Kit or Sephaglas Kit (Pharmacia).

Geneclean Kit: The weight of the gel slice was determined by placing the gel slice in a preweighed microfuge tube and reweighing. From the weight of the slice the volume was estimated by assuming a density of 1g ml^{-1}, and 4.5 vol. of NaI and 0.5 vol. of TBE Modifier were added. The contents of the tube were heated for 10 min at 55°C, with periodic mixing before addition of 5 μl of GLASSMILK. The mixture was incubated at room temperature for 5 min with shaking every minute before collecting the glass pellet by centrifugation for 30 sec in a microfuge. The pellet was washed three times in 500 μl of New Wash solution (3:1 v/v ethanol:H_2O) and resuspended in 5 μl sterile dH_2O by very gentle agitation. The eluted DNA was separated from the glass by centrifugation in a microfuge, and the elution step was repeated with a further 5 μl of sterile dH_2O. The two samples were combined to give a total volume of 10 μl, and the concentration of the eluted DNA was determined by analysing a small volume on an agarose gel by comparison with known standards (*HindIII* digest of λ DNA) .

2.7.5.5 Sephaglas kit (Pharmacia)

The weight of the agarose slice was determined as above; if less than 250 mg the normal protocol was followed but if greater than 250 mg the volumes were scaled up accordingly. To the gel slice 250 μl of Gel Solubilizer was added and the mixture vortexed vigorously before incubation at 60°C for 10 min or until the agarose had dissolved. The Sephaglas BP suspension of "glass milk" was shaken until uniform and 5 μl was added to the dissolved agarose solution. After gentle vortexing the tube was

incubated for 5 min at room temperature with vortexing every minute to resuspend the Sephaglas. High speed centrifugation for 1 min in a microcentrifuge was used to sediment the Sephaglas and the supernatant was carefully removed without disturbing the pellet. 40 µl of wash buffer was added, and the pellet was resuspended by vortexing the tube gently. The centrifugation and washing step was repeated 3 times, after which the tube was inverted at room temperature on the benchtop for 10 min to dry the DNA. After air-drying 10 µl of Elution Buffer was added and the pellet resuspended by gentle vortexing. After incubation for 5 min at room temperature to allow the DNA to elute from the glass, the supernatant containing the DNA was recovered after centrifugation for 1 min in a microfuge. The elution step was repeated to give a total elution of 10 µl, and the yield of the DNA was estimated by agarose gel electrophoresis as described above.

2.7.6 Spectrophotometric determination of DNA concentration

For estimating DNA concentrations the optical density of the solution was measured at 280nm and 260nm.The concentration of nucleic acid in the sample was calculated from the relationship OD_{260nm} = 1 for approximately 50 µg/ml DSDNA, 40 µg/ml for SS DNA and 20 µg/ml for SS oligonucleotides. The ratio of the readings at 260 nm and 280 nm provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have 260/280 ratios of 1.8, and contamination with phenol leads to a decrease in the ratio.

2.7.7 Ligation of DNA fragments

2.7.7.1 Ligations incorporating cohesive termini

Ligations were carried out using 1:1 and 3:1 insert:vector molar ratios of DNA in a total volume of 10 µl. Dephosphorylated vector DNA was used where necessary. Vector and insert DNA were mixed in an Eppendorf tube

2.7.7.2 Ligation of blunt-ended DNA

Ligations of blunt-ended DNA was carried out essentially as described above but insert:vector molar ratios were in the range 3:1 to 5:1. ATP at a concentration of 0.5

mM, and 0.5 Weiss units of bacteriophage T4 ligase were incorporated in the ligation mixture.

2.7.8 TOPO TA cloning® (Invitrogen)

This kit allows quick and efficient cloning of PCR products of 400bp to 1 kb. After performing a PCR reaction and confirming size of product the fragment can then be cloned. Typically 0.5 to 2 µl of a PCR sample was used (10 ng/µl). To the PCR product 1 µl of the pCR®TOPO vector was added and the volume brought up to 5 µl with dH_2O. The contents were gently mixed and incubated for 10 min at room temperature (approx. 25°C). The tube was incubated on ice whilst the One Shot™ Transformation was performed. The One Shot™ Transformation reaction vial was removed from -70°C and allowed to thaw on ice, 2 µl of 0.5 M ß-mercaptoethanol was added and mixed by stirring (the mixture should not be pipetted). After addition of 2 µl TOPO-Cloning reaction the tube was incubated for 15 min on ice. The cells were heat shocked for 30 sec at 42°C and 250 µl of SOC medium was added, mixed and incubated for 30 min at 37°C with shaking. 100 µl of each transformation was spread onto a prewarmed plate and incubated overnight at 37°C. Recombinant colonies appear small and cream and are screened to determine the presence of an insert.

2.7.9 Southern blotting

After fractionation by gel electrophoresis, the DNA was transferred to nylon membranes (Hybond-N+, Amersham) by capillary action under alkaline conditions as described by Sambrook *et al.* (1989). The gel was depurinated in 25 mM HCl for no longer than 10 min, before soaking in denaturation solution (1M NaCl, 0.5M NaOH) with constant agitation for two 15 min periods, with an intermediate wash with sterile dH_2O. It was then soaked for two 15 min periods in neutralization solution (1M Tris, 1.5 M NaCl, pH 8), with agitation and rinsing in between with sterile dH_2O. The gel was transferred (DNA side up) onto a piece of 3MM Whatman filter paper on a glass support soaked in 2 x SSC transfer buffer. A piece of Hybond N+ nylon membrane, about 1 mm larger than the gel in each direction, was cut and placed on top of the gel, followed by a piece of Whatman 3MM filter paper of the same size. After smoothing

out any air bubbles between the membrane and the filter paper, the gel was surrounded with Nescofilm, covered with a stack of paper towels and compressed with a ~500 g weight. Transfer was allowed to occur overnight. The membrane was washed briefly in 2 x SSC, and the DNA fixed on both sides by exposure to UV radiation in a UV crosslinker oven The filter was stored at 4^{o}C in an acetate bag until screening.

2.7.10.1 In situ plaque hybridisation

Hybond nylon membrane was placed onto the agar surface and its position marked for future orientation using three small triangles cut out of the membrane and marked on the plate. After 1 min the membrane was removed and placed, plaque side up, in a petri dish containing denaturation solution. It was then placed in a petri dish of Neutralization solution. The membrane was briefly washed for 30 sec in 2 x SSC and air dried plaque side up. Fixation was carried out by UV crosslinking for the optimum time.

2.7.10.2 Immunoblotting

From the genomic library nitrocellulose lifts were made in the same way as for the nylon membranes for transfer of DNA, thus allowing transfer of proteins to the nitrocellulose. Agar plates containg several hundred plaques from the lambda libraries of *P. salmonis* were incubated at 4°C for 1 h before addition of the nitrocellulose membranes. After 30 min the membranes were washed three times with TBS for 10 min at room temperature, then blocked for 1 hr at 4^{o}C with 2% bovine serum albumin solution in TBS. Membranes were again washed as above before addition of a 1/60 dilution of anti-*P. salmonis* antiserum. After overnight incubation at 4^{o}C the membrane was washed three times with TTS, for 10 min each time, at room temperature. HRP-labelled sheep anti-rabbit IgG (SAPU) was added at a dilution of 1/500 and the membrane incubated with shaking for 90 min at room temperature. After three TTS washes as above the filters were developed using 4-chloronaphthol solution. Positive reactions were indicated by blue/purple dots occurring at positions equivalent to particular plaques.

2.7.10.3 Isolation of plaques

Individual plaques of interest were cored using a sterile glass Pasteur and ejected into an Eppendorf tube containing 1 ml of SM buffer, plus 2 drops of chloroform and stored at 4°C.

2.7.11 Non-radioactive labelling of DNA probes by random priming

DNA fragments were isolated and purified as previously described. Labelling was carried out by incorporation of DIG-labelled deoxyuridine-triphosphate (Boehringer Mannheim kit). The fragment, in a volume of 10 µl was denatured by boiling for 10 min and immediately placed on ice. 2 µl of hexanucleotide mix, 2 µl of 1 mM dNTP labelling mix (including DIG-dUTP) and 1 µl of 2 U/ml Klenow enzyme were added and incubated overnight at 37°C. The reaction was stopped by addition of 2 µl of 0.2 M EDTA (pH8) and the DNA precipitated for 30 min at -70°C with 3 µl of 3 M LiCl and 60 µl cold ethanol.

After centrifugation for 15 min in a microfuge at 12000 x g, the pellet was washed with 70 % ethanol, dried and resuspended at 4°C in 10 µl sterile dH_2O.

2.7.12 DNA-DNA hybridisation

DNA-DNA hybridisations were normally carried out either at 55°C (low stringency conditions), or at 68°C (high stringency conditions). Salt conditions are described below.

2.7.12.1 Non-radioactive hybridisations (chemidetection)

The hybridisation procedure was carried out following the manufacturer's instructions (Boehringer Mannheim) for the DIG Detection Kit. All volumes refer to a membrane size of 100 cm^2 and volumes were adjusted accordingly for other sizes of membrane.

The membrane, in a hybridisation tube at the appropriate temperature, was prehybridised with at least 20 ml of hybridisation solution containing 5xSSC, 1% (w/v) blocking reagent (0.1 % N-lauroylsarcosine, 0.02% (w/v) SDS made up in distilled H_2O) for approximately 4 h. This was replaced with 2.5 ml of hybridisation solution containing denatured (as described above for labelling) DIG-labelled probe and hybridised overnight. The membrane was washed with 2 x SSC, 0.1 % SDS for two 5

min periods and then with two 15 min washes with 0.1% SDS, 0.1 x SSC; both washes were carried out at the hybridisation temperature. A final wash was carried out at room temperature in washing buffer (0.1 M maleic acid, 0.15 M NaCl pH7.5; 0.3% Tween 20) and incubated for 30 min at room temperature with agitation in blocking solution (1% blocking solution in maleic acid buffer). It was then incubated for 30 min in anti-DIG-Alkaline-Peroxidase conjugate, prepared in blocking solution, with agitation at room temperature. The membrane was washed for two 15 min periods in washing buffer, before equilibration in detection buffer (0.1M Tris/HCl, 0.1M NaCl, 50mM $MgCl_2$, pH 9.5) for 5 min with the membrane sealed between two acetate sheets; 5 ml CSPD® (Disodium 3-(4-methoxyspiro{1,2, dioxetane-3,2-(5'-chloro) tricyclo[3.3.1.1] decan}-4-yl phenyl phosphate -chemilluminescent substrate) in detection buffer was added and the membrane incubated for 5 min at room temperature. The excess CSPD® was removed and the membrane within the acetate bag was incubated at 37°C for 15 min to enhance the chemiluminescence. The membrane was exposed to X-ray film for 30 min. The exposure time was subsequently reduced or increased to give an optimum signal.

2.7.12.2 Chemical detection

The above procedure was followed to the stage of equilibration in detection buffer, after which 10 ml of freshly prepared colour substrate solution (45 µl NBT-solution (vial 4) and 35 µl X-phosphate solution (vial 5) added to 10 ml of detection buffer) was added in the dark. The solution was not agitated. The colour precipitate began to form within a few minutes, and when the desired intensity of staining of spots or bands was detected the reaction was terminated by washing with dH_20.

The results were recorded by photocopying or photography.

2.8 DNA sequencing

Manual sequencing was carried out using a Bio-Rad Sequi-Gen Nucleic Acid Sequencing Cell (38 cm wide; 100 cm long) and a Sequenase Version 2.0 kit (United States BioChemical) which included universal primers. Automated sequencing was

carried out using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin & Elmer) on the Applied Biosystems 377 Sequencer. Sequence assembly and analysis were performed using the programs of the GCG Software package, Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wis. Database searches were performed with the FASTA algorithm (Pearson & Lipman, 1988) and BLAST (Altschul, 1990; Altschul *et al.*, 1997).

2.8.1 Denaturation of double stranded DNA

Double stranded plasmid DNA (5 µg) was denatured in 300 µl of 0.2M NaOH, 0.2 mM EDTA for 30 min at 37°C. The mixture was neutralised by adding 0.1 vol. of 3M CH_3COONa (pH5.5) and the DNA was precipitated with 2 vol. of 100% ethanol at -70°C for 30 min. After centrifugation for 15 min in a microfuge at 12000 x g, the pellet was washed in 70% ethanol, dried and resuspended in 7 µl of sterile dH_2O.

2.8.2 Sequencing reactions

After addition of 2 µl of Sequenase reaction buffer and 1 µl of primer (0.5 pmol) to the template DNA (approximately 1 µg), the volume was brought up to 10 µl with sterile dH_2O. Annealing was carried out at 65°C for 2 min and the mixture cooled to <35°C over 15-30 min. The solution was placed on ice and the following were added: 1 µl 0.1M DTT, 2 µl of 1 x labelling mix (containing 7.5 mM dGTP, dCTP, and dTTP), 0.5 µl Redivue{α-^{35}S} dATP, and 2 µl of Sequenase Version 2.0 diluted 1:8 in ice cold Enzyme Dilution Buffer. The solutions were mixed and incubated for 2 min at room temperature. After labelling, 3.5 µl of the mix was transferred into four prewarmed Eppendorf tubes (37°C) containing 2.5 µl of either ddGTP, ddTTP, or ddCTP termination mixes. (Termination mixes also contained 80 mM dGTP, dATP, dCTP, dTTP and 50 mM NaCl). After mixing, the tubes were incubated for 5 min at 37°C. Reactions were stopped by addition of 4 µl of Stop Solution, the contents were mixed and stored at -20°C until required for loading on to the gel. Samples were denatured for 2 min at 75°C immediately before loading onto the sequencing gel. All incubations were carried out in either a water bath or a thermal cycler (Techne).

2.8.3 Polyacrylamide gel electrophoresis-Sequencing

Glass plates, combs and spacers were thoroughly washed in warm detergent solution, followed by distilled H_2O and a final rinse with ethanol. One surface was treated with Gel-Slick (silicone solution) and the plates were assembled according to the manufacturer's instructions. The gels were cast at a final concentration of 6% Acrylamide/bisacrylamide gel (ratio 19:1, 7 M Urea, 1 x TBE) EASI-GEL supplied by Scotlab, TEMED (N, N, N, N- tetra-methylenediamine) and ammonium persulphate (APS) in the following amounts;

Easi gel	70 ml
TEMED	70 µl
APS	140 µl (0.125 g/500 µl water prepared directly before use).

The gel was poured between two sealed 42 x 33 cm plates, and the flat side of a shark's tooth comb was inserted ~0.5 cm into the gel solution. Combs were clamped into position using bulldog clips until the gel polymerised (~2 h). The combs were removed and reinserted into the gel, teeth down. The gel was mounted in a Model S2 Sequencing apparatus from Life Technologies with power supplied by a GIBCO BRL electrophoesis power supply. After a pre-run for 30 min at 70-80W, 3 µl of each sequencing reaction was loaded onto the gel and it was run at 55°C in 1 x TBE. Running conditions were set at 300 mA/ 80W or 280 mA/ 70W for 2.5 - 3h. At the end of the run, the mould plates were separated and the gel transferred onto a piece of 3MM Whatman paper by placing it on top of the gel and applying gentle pressure so that the gel became attached to the paper. The 3MM paper was then peeled away from the glass support with the gel attached. The gel was covered with Saran Wrap and was dried under vacuum for at least 1 h at 80°C. The Saran Wrap was removed and the gel was exposed to X-ray film for 16-24 h at room temperature. Exposure time was increased as necessary to give an optimum signal.

2.8.4 Automated sequencing

DS-DNA from plasmids was prepared for automated sequencing by centrifugation through a Sephacryl S-200 minicolumn to remove dNTPs, primers, etc. Not all

plasmids were prepared in this way, as those purified with the Wizard Plus SV system did not require further purification. PCR products were also treated with Sephacryl S-200 columns as above or used directly. The amount of DS-DNA used was 250-500 ng per reaction, and PCR products were used at 30 -90 ng per reaction. Thin-walled PCR 0.5 ml microfuge tubes were used in the PCR thermal cycler for the first step, in which 8.0 µl of Terminator Ready Reaction Mix (Applied Biosystems) was added to template DNA, primers were used at a constant amount of 3.2 pmol, and dH_20 was used to bring the reaction volume to 20 µl. The cycle sequencing protocol was : 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min repeated for 25 cycles using a Perkin and Elmer Cycle Sequencer. Excess terminators were removed by ethanol precipitation, by adding 2 µl of 2 M sodium acetate, pH 4.6, and 50 µl of 100% ethanol (Analar grade); the mixtures were incubated on ice at room temperature for 10 min, centrifuged at 12000 rpm in a microcentrifuge for 15 min at 4°C and the ethanol carefully removed and replaced with 250 µl of 70% ethanol. This was carefully removed and the tubes drained of remaining ethanol and allowed to air dry on the bench. Tubes were then returned to the Molecular Biology Support Unit where they are stored at -20°C before analysis by Applied Biosystems 377 Sequencer.

2.9 PCR

2.9.1 Standard PCR reactions

Template DNA was used at various concentrations, the other constituents remained constant at the following concentrations, unless otherwise stated; 200 µM of each dNTP mix, 300 µM of each primer, 1 x Taq polymerase buffer, 1.5 mM $MgCl_2$ and 1.25 units of Taq Polymerase in 50 µl volume. The enzyme, buffer and $MgCl_2$ were all supplied by Gibco, Life Technologies. A summary of the primers used in this study are shown in Table 7. The Thermal Cycler used was the Omm-E Thermal Cycler (Hybaid Ltd.), unless otherwise stated, with the following programme:-

1X	94°C	5 min
	94°C	1 min
30X	57°C	1 min
	72°C	1 min
1X	72°C	5 min

The PCR products were visualised by agarose gel electrophoresis as described earlier.

2.9.2 Optimisation of conditions for PCR

A kit produced by Invitrogen was used to determine the optimal conditions for amplification of particular templates with specific primers by use of a range of buffers with varying concentrations of salt and at different pH. All buffers were supplied as 5X concentrates in 300 mM Tris.HCl, 75 mM ammonium sulphate. DMSO was also supplied with the kit.

The 16 5X buffers with varying pH and salt concentrations were : -

BUFFER A	7.5 mM $MgCl_2$, pH 8.5	BUFFER B	10 mM $MgCl_2$, pH 8.5
BUFFER C	12.5 mM $MgCl_2$, pH 8.5	BUFFER D	17.5 mM $MgCl_2$, pH 8.5
BUFFER E	7.5 mM $MgCl_2$, pH 9.0	BUFFER F	10 mM $MgCl_2$, pH 9.0
BUFFER G	12.5 mM $MgCl_2$, pH 9.0	BUFFER H	17.5 mM $MgCl_2$, pH 9.0
BUFFER I	7.5 mM $MgCl_2$, pH 9.5	BUFFER J	10 mM $MgCl_2$, pH 9.5
BUFFER K	12.5 mM $MgCl_2$, pH 9.5	BUFFER L	17.5 mM $MgCl_2$, pH 9.5
BUFFER M	7.5 mM $MgCl_2$, pH 10	BUFFER N	10 mM $MgCl_2$, pH 10
BUFFER O	12.5 mM $MgCl_2$, pH 10	BUFFER P	17.5 mM $MgCl_2$, pH 10

PCR reactions were set up using these buffers to determine optimum salt and pH conditions. Two control templates (800 bp and 2 kb) were supplied with control primers to produce a PCR product with any of the 16 buffers.

The suggested protocol was to mix all ingredients (except for dNTPs) in a tube, add the tube to the thermal cycler at 80°C and then to add dNTPs. The cycling parameters specified for the controls were:

Table 7 Primers used in this study

Name	Sequence	Source
SP6	5' CAA GCT ATT TAG GTG ACA CTA TAG 3'	GIBCO
T7	5' TAA TAC GAC TCA CTA TAG GGA GA 3'	GLA UNI
SP6	5' CAT ACG ATT TAG GTG ACA CTA TAG 3'	GLA UNI
16S EUBACTERIAL		
1525r	5' AAG GAG GTG ATC CAA CC 3'	GLA UNI
926f	5' AAA CTC(T) AAA T(G)GA ATT GAC GG3'	GLA UNI
519r	5' GT(A)A TTA CCG CGG CG(T)G CTG 3'	GLA UNI
907r	5' CCG TCA ATT CC(A)T TTA(G) AGT TT 3'	GLA UNI
1100r	5' GGG TTG CGC TCG TTG 3'	GLA UNI
1114f	5' GCA ACG AGC GCA ACC 3'	GLA UNI
530f	5' GTG CCA GCC(A) GCC GCG G 3'	GLA UNI
27f	5' AGA GTT TGA TCC(A) TGG CTC AG	GLA UNI
926f	5' AAA CTC AAA TGA ATT GAC GG	OSWEL
1525r	5' AAG GAG GTG TTC CAG CC 3'	OSWEL
pExCell primer reverse 2343-2325		
P2325 (Y1144)	5' ATC GCC ATT CGC CAT TCA C 3'	OSWEL
pExCell primer forward 1841-1860		
P1841 (Y1143)	5' CAA TAC GCA AAC CGC CTC TC 3'	OSWEL
M13U (4172)	5' GTA AAA CGA CGG CCA GT 3'	GLA UNI
M13 -20 primer forward		
MIPR1 (P6107)	5' TAA GGA TCC CGT CGC AAG CAC TGA 3'	OSWEL
MIPF1 (P6106)	5' GTA TGA GCT CTT AAG TGT AAG ACT A 3'	OSWEL
T3 (Z1722)	5' AAT TAA CCC TCA CTA AAG GG 3'	OSWEL
T7 (Z1723)	5' GTA ATA CGA CTC ACT ATA GGC 3'	OSWEL

Primers suppied by Glasgow University, Molecular Biology Support Unit, Anderson College, University of Glasgow, Glasgow, G12 8QQ, and Oswel Reasearch Products Ltd., Medical & Biological Sciences Building, University of Southhampton, Bolderwood, Bassett Crescent East, Southampton, SO16 7OX.

94°C 2 min 1 cycle

94°C 1 min}

55°C 2 min} 25-35 cycles

72°C 3 min}

72°C 7 min 1 cycle.

10 µl of product was analysed on a 0.8% agarose gel and the remainder of the PCR product was stored at -20°C.

2.9.3 PCR detection of *Legionella pneumophila* MIP gene

Legionella pneumophila DNA was extracted following the method by Starnbach *et al*., 1989. *Legionaela pneumophila* was supplied on a Charcoal Agar plate by Dr. Mourad Djebara, Division of Infection and Immunity. Bacteria were scraped off the plate and resuspended in 100 µl of digestion buffer (100 mM Tris.HCl, 1 mM EDTA pH 8.5, 2% Laureth 10, 400 µg of proteinase K per ml). The mixture was vortexed briefly and heated to 55°C for 3 hr , then incubated for 10 min at 95°C and centrifuged briefly in a bench centrifuge. The tube was placed on ice, centrifuged for 2 min in a microcentrifuge, and 10 µl aliquots used in a PCR reaction with a "PCR master mix" consisting of 10 mM Tris pH8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 200 mM each dNTP, 1.25U of *Taq* polymerase and 0.2 mM of each primer (MIP forward and MIP reverse); 40 µl of master mix was added to each tube and 10 µl of template DNA was also added. The cycle parameters were : 1 cycle at 94°C for 2 min; 30 cycles at 94°C for 1 min, followed by 57°C for 1 min and 72°C for 1 min; 1 cycle at 72°C for 5 min.
10 µl of each reaction was analysed on a 0.8% agarose gel and the remainder of the PCR product was stored at -20°C.

2.9.4 Analysis of λ recombinant clones using PCR

As well as screening recombinant clones using conventional methods of plasmid purification, PCR was used to analyse positive transformants directly . A PCR cocktail was prepared containing PCR buffer, dNTPs, primers and *Taq* polymerase in a 20 µl reaction volume. Individual colonies were picked and resuspended in the PCR cocktail; the reaction was incubated for 10 min at 94°C to inactivate nucleases, and the reaction

continued for 30 cycles of : 94°C, 1 min, 55°C, 1 min, and 72°C, 1 min. A final extension at 72°C for 10 min completed the reaction. The products were analysed on an agarose gel.

2.10 Microscopy

2.10.1 Giemsa staining of infected cells

Cells grown on glass coverslips were fixed and stained with Gram's stain and Giemsa stain. Stock Giemsa stain was diluted 1/10 with PBS, and centrifuged to remove debris. Coverslips with infected cells were fixed using 1 ml of methanol and left to air dry. Giemsa stain was then applied to cover the slide and incubated for 15 min at room temperature. The stained cells were washed in PBS and left to air dry before examining under oil immersion.

2.10.2 Fluorescence microscopy of bacteria

An overnight culture of *E.coli* was harvested and diluted to give 2 x 10^4 - 2 x 10^5 cells per ml, for use as controls for *P. salmonis* to distinguish between bacteria and tissue culture debris. The cell suspension was mixed with an equal volume of DAPI solution, (1 mg in 100 ml) and filtered through 0.2 µm Nucleopore filters, 25 mm diameter . These were prepared by soaking in a solution of Irgalan Black (0.2% in 2% acetic acid) for 24 hr then rinsing twice with sterile dH_2O. 1 ml of each cell suspension, *E. coli* and *P. salmonis* (infected tissue culture material), was filtered through the Nucleopore filters and placed on a glass slide. The bacteria were examined under the 100x oil immersion objective in a Leitz Orthoplan fluorescence microscope with Ploem illumination. Bacteria fluoresced blue/green and the nucleopore filters appeared evenly stained. The *E. coli* were visible in clumps and *P. salmonis* was visible attached to cell debris.

2.10.3 Electron microscopy

Infected supernatants were removed from lysed monolayers which exhibited cytopathic effects across the complete monolayer and culture supernatant was used for electron microscopy. Approximately 10 ml of culture fluid was centrifuged at 4000 x g and the

pellet resuspended in 1 ml cacodylate buffer containing glutaraldehyde. The sample was processed by the Electron Microscopy Unit, IBLS, University of Glasgow.

2.11 General protein methods

2.11.1 Bradford protein assay

The protein standard used was 500 µg/ml BSA. The Bradford reagent was prepared as follows; 100 mg Coomassie Brilliant Blue G250 was dissolved in 50 ml 95% ethanol, 100 ml 85% (w/v) phosphoric acid was added and the solution diluted to 1 L with distilled water. Standards containing 0-50 µg BSA/100 µl were used. To 100 µl of standard or test sample 5 ml Bradford reagent was added and throughly mixed and the absorbance at A595 nm measured within 2 min and 1 hr. A graph was plotted using the data obtained from the standards and protein concentration of the samples extrapolated from the graph.

2.11.2 SDS-PAGE electrophoresis

SDS-PAGE was carried out as described by Sambrook *et al.*, (1989) using a vertical gel electrophoresis system with 14 cm x 14 cm gels. Before use glass plates, spacers and combs were washed in warm detergent, Decon, rinsed in distilled water then ethanol, and after assembly were sealed using electrical tape. Gels were cast at a final concentration of 12% acrylamide and all reagents used were of electrophoresis grade. The resolving gel consisted of 16.6. ml distilled water , 13.4 ml acrylamide solution (30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide stock solution supplied by Flowgen), and 10 ml lower gel buffer. The mixture was degassed under vacuum before the addition of 200 µl freshly prepared 10% APS (Ammonium Persulphate) and 20 µl TEMED. After the solution was poured it was overlaid with butanol. When polymerisation had occurred (approx. 45 min at room temperature), the overlay was poured off and the top of the gel was washed several times with distilled water. The fluid was drained off and any remaining water was removed using the edge of a paper towel. The stacking gel was prepared using 6.0 ml of distilled water, 1.5 ml of acrylamide, 2.5 ml of lower gel buffer, degassed as above and 30 µl 10% APS and

20µl TEMED added. This was poured directly onto the polymerised resolving gel and a comb was inserted immediately. The gel was allowed to polymerise for approx. 30 min at room temperature and the comb was removed. The wells were washed immediately with distilled water and the gel mounted in the electrophoresis unit. Gels were run in SDS-PAGE Running Buffer using a current of 30 mA per gel. Samples were prepared at the appropriate protein concentration and added in equal volumes to solubilising buffer and boiled for 10 min in a boiling bath; the samples were cooled to room temperature before loading onto gel with a Hamilton syringe, typically 30 µl was loaded. At the end of the run, the plates were separated and stained with Coomassie or transferred to nylon for Western Blotting.

2.11.3 Coomassie Blue Staining

The gel was simultaneously fixed and stained in Coomassie Blue fixing solution (0.25 g of Coomassie Blue in 90 ml methanol:distilled water (1:1 v/v) and 10 ml glacial acetic acid) for at least 4 hr at room temperature with gentle agitation. The gel was destained with 3-4 changes of 30% methanol and 10% acetic acid solution for 4-8 h with gentle agitiation. It was then sealed between acetate sheets and photographed.

2.11.4 Western blotting

The SDS-PAGE gel was transferred to nylon (Sambrook *et al.*, 1989) and sandwiched between 3MM paper and Scotchbrite pads soaked in Transfer buffer (2.5 g Tris, 12.0 g of glycine, 166 ml of methanol and 834 ml of distilled water). The sandwich was placed in the apparatus with the membrane facing towards the positive electrode. A Biorad electrophoresis cell was used with water cooling while transfer occurred at 0.2 Amps for 4 h. The membranes were incubated in 20 ml 2% BSA in Tris/Tween/Saline solutiuon (TTS, for 100 ml, 2 ml 1M Tris pH 7.2, 1 ml Tween-20 and 97 ml of sterile saline), for 1 h at room temperature with gentle agitation. The membrane was washed three times for 5 min in TTS, then 20 ml of 1/50 anti-*P. salmonis* antiserum was added and incubated for 90 min at room temperature with gentle agitation. After three 5 min washes in TTS 1/500 HRP labelled anti-sheep immunoglobulin in TTS was added and

incubated for 90 min at room temperature with gentle agitaiton. After three 5 min washes in TBS and a final 5 min wash in 10 mM Tris pH 7.2, the substrate (60 mg of HRP colour development solution, 4-chloro-naphthol dissolved in 20 ml of methanol, and added just before use to 100 ml TBS with 20 µl of Hydrogen peroxide). The reaction was incubated until bands were visible and the reaction stopped after approx. 15 min by rinsing in sterile distilled water. The membrane was sealed in an acetate bag and recorded.

2.12 IFAT Indirect Fluorescent Antibody Test

The method was modified from that of Lannan *et al.*, (1991). Smears were prepared using 5 µl of tissue culture material infected with LF89 or Ardintoul agents when 100% CPE was visible. Smears were air dried and methanol fixed. Anti-Ardintoul antiserum (1/20 in PBS) was added to fixed slides for 10 minutes at room temperature and control smears used preimmune serum (1/20 in PBS). Slides were washed 5 times with 1 ml PBS and FITC anti-sheep IgG (1/200 in PBS) was added. after incubation for 10 minute at room temperature in the dark, slides were washed 5 times in PBS, coverslips added, before examination with a Leitz Orthox Microscope with appropriate filters for FITC. Selected images were saved to disk.

2.13 ELISA

Using a 96-well ELISA plate the titres of the anti-*P. salmonis* antisera were determined. Each antigen preparation was diluted in ELISA Coating Buffer, and 4 rows of 8 wells were coated overnight with 100 µl of the appropriate antigen solution. After removing the solution each well was blocked using 100 µl 10% skimmed milk prepared in coating buffer for 2 hr at 4°C. The first antibody solutions were added in doubling dilutions from 1/2000 to 1/128000 prepared in saline and added to seven wells (90µl) with row eight as buffer control. After incubation for 2 hr at 37°C and washing six times the second antibody SAPU HRP-anti (sheep IgG) was added at a dilution of 1/1000 to all wells. After appropriate washes the OPD (O-Phenylenediamine dihydrochloride) substrate was added and the reaction developed for 30 min in the dark. OPD was prepared by dissolving Peroxidase Substrate Sigma Fast tablets in 20

ml of distilled water. 30 µl 12.5% H_2SO_4 was added to terminate the reaction and A492 was then read in an ELISA plate reader.

2.14 OUCHTERLONY

Agarose was dissolved (10 mg/ml) in PBS by heating in a microwave oven. Molten agarose, 5 ml was removed and allowed to set on a glass slide on a level surface. Holes (6 in a circle and one in the centre) were punched in the agarose, once set, using a Pasteur pipette. Sera (10 µl) anti-IgM antiserum, Δ80 IgM was added to the two holes at the top and purified salmon IgM (10 µl) added to the bottom two holes. Incubated overnight at room temperature in a humidified box. The gel was washed with gentle agitation in dH_2O, six changes over 24 hours and dried at room temperature. The gel was stained in Coomassie Blue for 5 min. Destaining followed, by agitating in 5% methanol, 7% acetic acid until bands of precipitation were apparent.

Chapter 3: Investigations on Chilean isolates of *Piscirickettsia salmonis*

3.1 Growth of *P. salmonis* LF-89 in tissue cultures

3.1.1 Introduction

Piscirickettsia salmonis, being an intracellular pathogen, must be cultured within living cells. Chinook Salmon Embryo cells (CHSE) and Rainbow Trout Gonad cells (RTG-2) (Lannan *et al.*, 1991; Plumb & Wolf, 1971) were used as host cells in this study. When obtained, both stocks had been maintained routinely in the presence of antibiotics. Due to the antibiotic sensitivity of *P. salmonis,* the cells were subsequently grown without antibiotics, and tested for sensitivity to *P. salmonis* at each passage. Three passages were sufficient to remove residual antibiotics as rickettsiae, supplied by Marine Harvest Chile, did not survive until after the third antibiotic-free passage. After removal of antibiotics, the rickettsiae were added to 70% confluent cell lines and incubated at 17^{o}C for up to 14 days, with daily monitoring by light microscopy to detect bacterial replication within the cells. Daily photographs recorded the cell changes and representative pictures are shown in Figures 3 to 5.

Multiplication of the bacteria within vacuoles in the cell cytoplasm resulted in various morphological changes. Firstly the cells appeared to round up, vacuoles were apparent in the cytoplasm after 3-5 days, and the bacteria were visible within these vacuoles. Although, the bacteria were not motile, Brownian motion of rickettsiae within the vacuoles was clearly visible. The cells became darker in colour as the vacuoles within the cytoplasm expanded in size, eventually pushing the nucleus to one side. The number of vacuoles within a cell was variable. Eventually the vacuole caused the cell to rupture, releasing rickettsiae into the surrounding medium to initiate a further cycle of infection. Once the majority of the cell monolayer lysed, at approximately seven days in this instance but usually at about 14 days with lower multiplicities of infection, the medium containing rickettsiae was harvested for further experiments. This was repeated until 2 L of infected culture material had accumulated. Due to the small size of the bacterium, and the small size of the genome, a large number of bacteria were required for genetic manipulations.

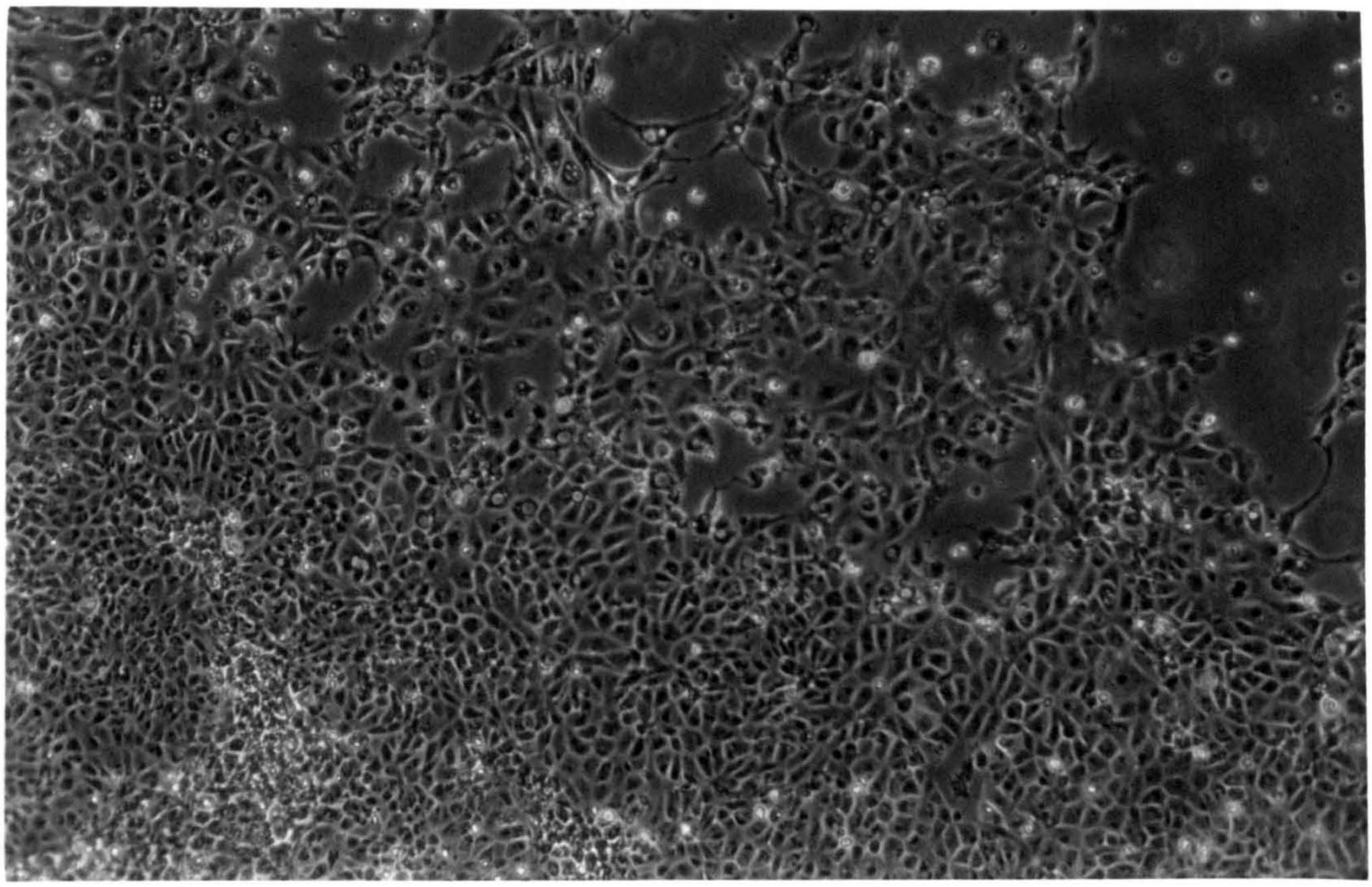


Figure 3 CHSE cells infected with *P. salmonis* LF89
CHSE-214 cells exhibiting cpe. Note clusters of rounded cells, the vacoules and monolayer degenerating. Day 5 postinfection with *P. salmonis* LF89. Phase contrast, x40 magnification.

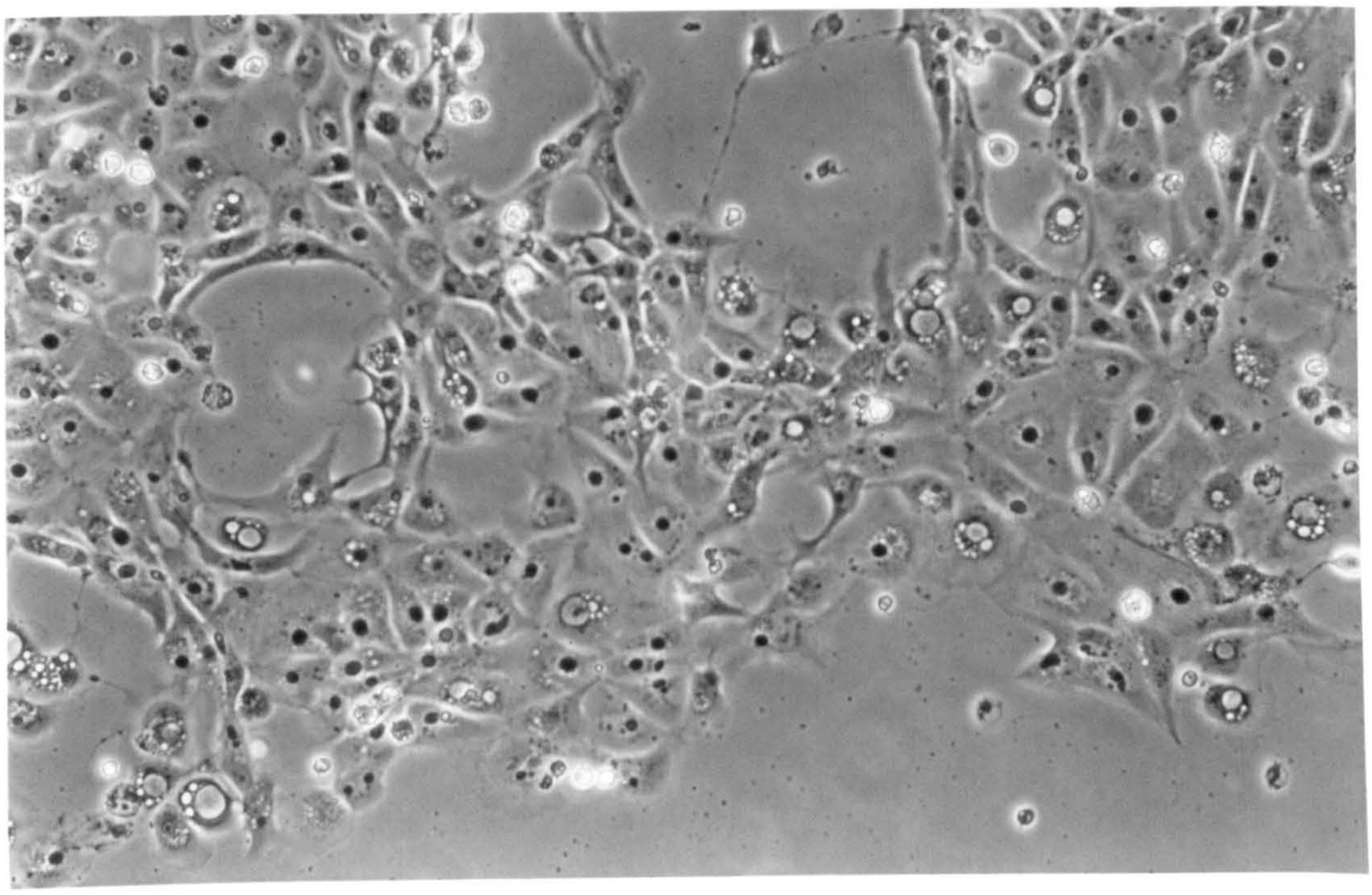


Figure 4 CHSE cells infected with *P. salmonis* LF89
CHSE-214 cells exhibiting cpe. Day 7 postinfection. Note numerous vacuoles and near destruction of the monolayer. Phase contrast, x100 magnification.

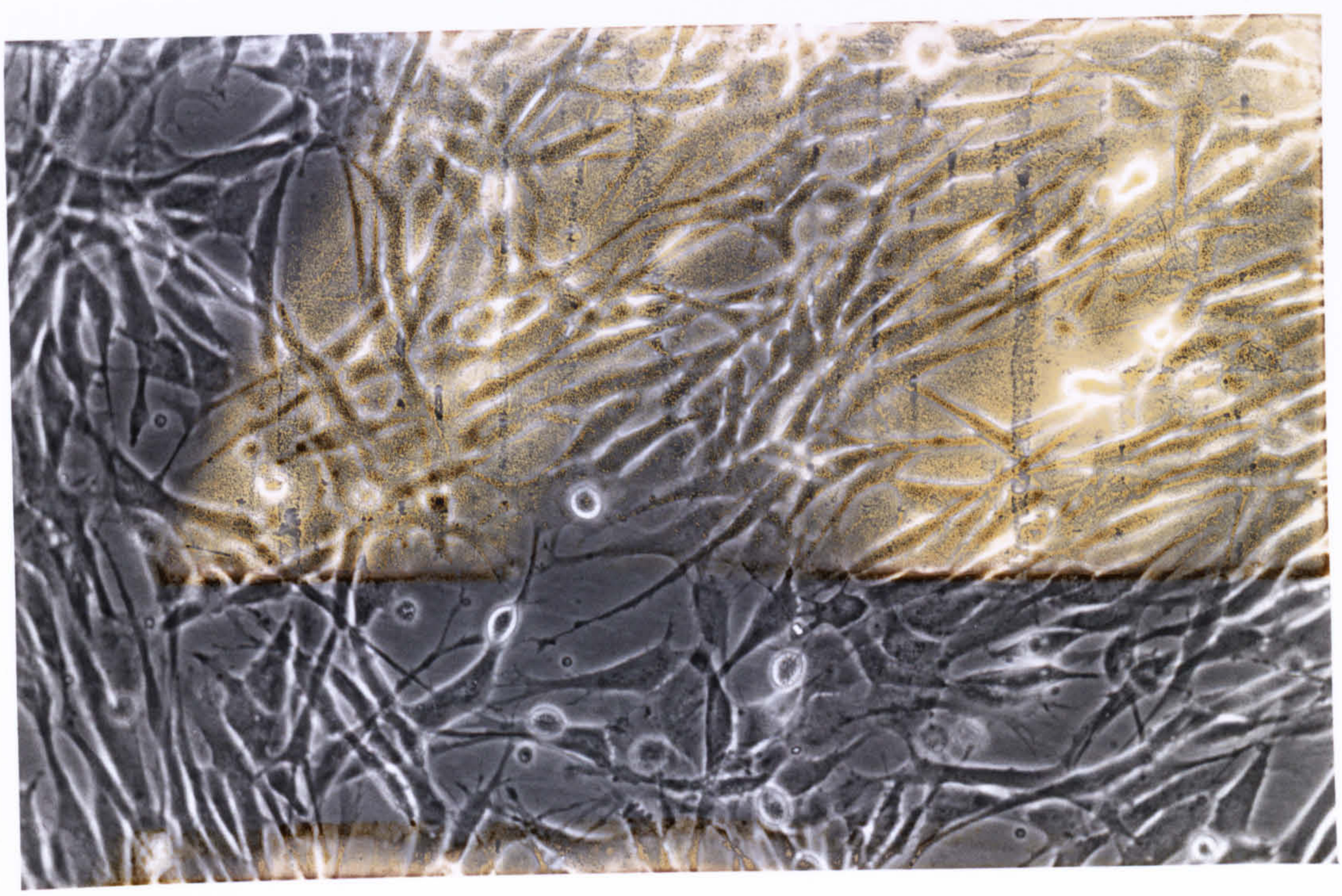


Figure 5 RTG-2 cells infected with *P. salmonis* LF89
CHSE-214 cells exhibiting cpe. Note clusters of rounded cells, the vacoules and monolayer degenerating. Day 5 postinfection with *P. salmonis* LF89. Phase contrast, x40 magnification.

3.1.2 Sterility testing

To maintain a constant supply of both cell lines, sterility checks were incorporated into the tissue culture routine. Growth media and the individual supplements were plated onto a range of growth media, both solid and liquid, and incubated at a range of temperatures. Contamination was detected by inspecting the media daily over the period of one week, for bacterial growth. The range of agars employed included Luria agar, Tryptone Soya Agar, Minimal Medium Agar and Marine agar; these were incubated at 15, 20 and 37°C. Broths used included, Luria Broth, Marine Broth, and Tryptone Soya Broth. Any contamination resulted in the batch of medium or supplement being discarded.

3.1.3 Optimal temperature for growth of tissue cultures

To determine the optimal growth temperature for the cell lines, 25 cm^2 sterile tissue culture flasks were set up with equal concentrations of cells and incubated at 15, 17 and 20°C. Growth was monitored by light microscopy on a daily basis and 17°C was chosen as the optimum temperature, as found by Garces *et al.*, (1991). The above experiment was repeated with rickettsia-infected cells, and the same temperature was found to be optimum for productive infection.

3.1.4 Detection of rickettsia by staining

Rickettsia were visualised within their infected host cells by staining with Acridine Orange or Giemsa stain. This was achieved by growing CHSE cells on 12 mm diameter coverslips in a six well tissue culture plate with five coverslips per well. The plates were set up in duplicate for both infected and uninfected cells and monitored daily for signs of infection. For this two coverslips were removed from each plate, fixed with methanol and stained with either Acridine Orange or Giemsa. Infected and uninfected were compared for up to 15 days. As shown in Figure 6, rickettsia can be seen clearly within cytoplasmic vacuoles of infected CHSE cells.

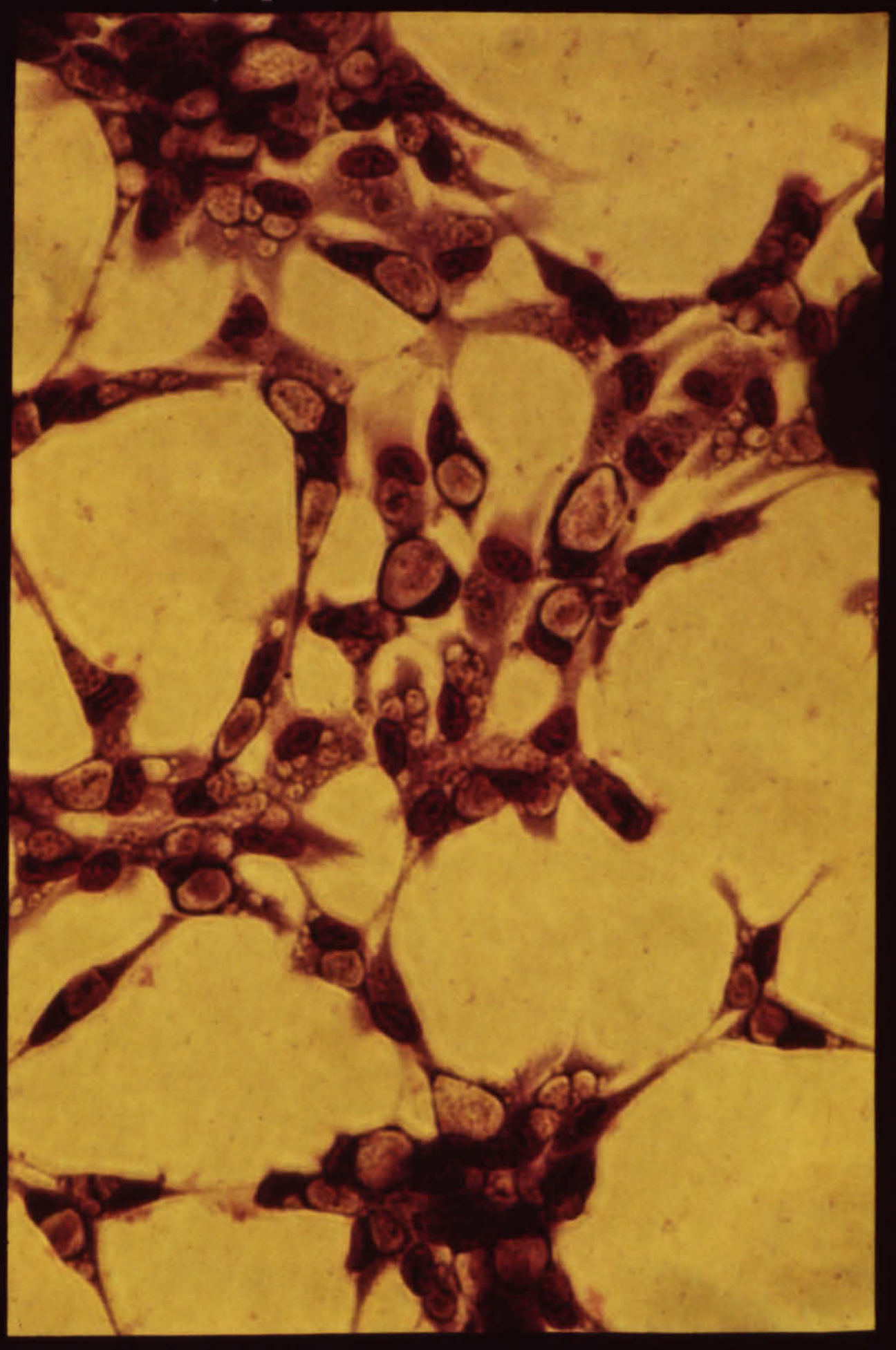


Figure 6 Giemsa stained *P. salmonis* infected CHSE cells
P. salmonis LF89 visible within cytoplasmic vacuoles of Giemsa stained CHSE-214 cells. Note the number of vacuoles varying from cell to cell and the vacuole pushing the nucleus to one side.

3.1.5 Separation of bacteria from tissue culture material

The method chosen for purification of rickettsiae from cell debris was differential centrifugation followed by density gradient centrifugation. The initial low speed centrifugation step (640 x g for 10 min) removed the larger cell debris, leaving behind the bacteria and finer tissue culture material which were separated on the Percoll gradient. To confirm that cellular and bacterial material were successfully separated, the two bands generated from the Percoll gradient were analysed by staining with Giemsa and light microscopy. This clearly showed that the upper band contained cellular debris and the lower band contained bacteria along with a small amount of cellular debris. The bacteria from the lower band were collected by centrifugation at 10000 x g for 15 min at 4 °C, to remove the Percoll and residual cell debris.

3.2 Construction of a genomic library from *P. salmonis* DNA in λ ExCell

3.2.1 Genomic DNA extraction and quantification of *P. salmonis* DNA

Genomic DNA was extracted from *P. salmonis* and the yield of DNA determined by agarose gel electrophoresis by comparison with lambda *HindIII* standards of known concentration. All of the extracted DNA was analysed, and this was estimated to be approximately 20 ng in total (Figure 7). The *P. salmonis* DNA was excised from the agarose gel and recovered using Sephaglas before concentrating by ethanol precipitation.

3.2.2 Partial digest of *P. salmonis* DNA

The purified DNA (10 ng) was partially digested with *EcoRI* to produce compatible ends for ligation into the lambda ExCell vector. To determine appropriate conditions for partial digestion, control reactions were set up using *Aeromonas salmonicida* DNA and *Pasteurella haemolytica* DNA; these experiments showed that digestion with 5 units of *EcoRI* for 30 min at 37°C produced a high proportion of fragments in the size range 0 to 6 kb, the size range recommended for the cloning vector (results not shown).

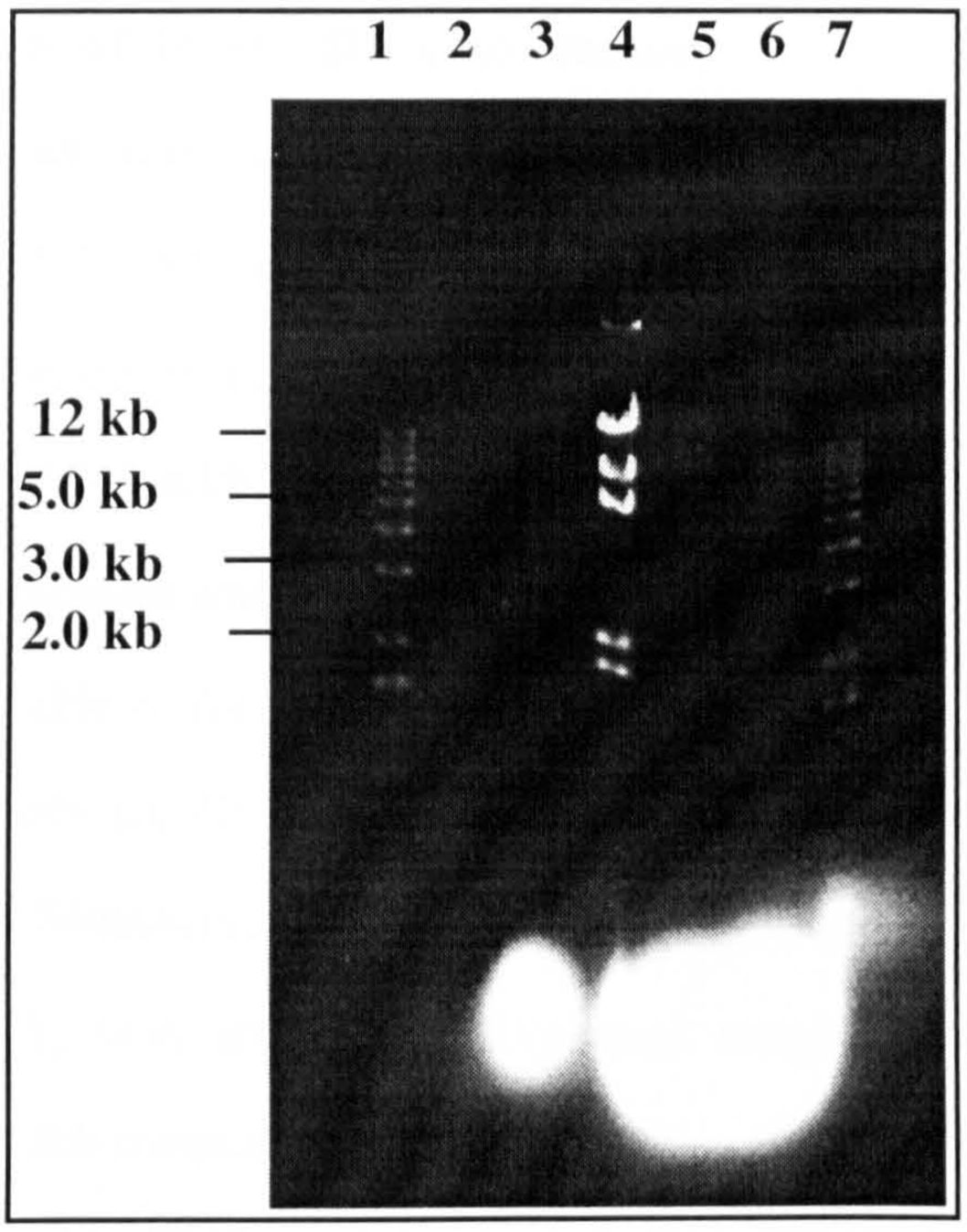


Figure 7 *P. salmonis* genomic DNA extraction

P.salmonis was separated from tissue culture material as described elsewhere. The Percoll gradient resulted in two phases, an upper and a lower band from which DNA was extracted and anlysed above on 0.7% agarose, the DNA appears very faintly in the above scanned image, it was more apparent on the transilluminator, resolution was lost in the scanning procedure. tRNA was used as a carrier, visible at bottom of gel.

Lane 1 KB ladder

Lane 2 Upper band

Lane 3 Upper band

Lane 4 λ*HindIII* standard

Lane 5 Lower -*P.salmonis* DNA

Lane 6 Lower -*P.salmonis* DNA

Lane 7 KB ladder

The partially digested *P. salmonis* DNA was extracted with phenol:chloroform and ethanol precipitated to remove buffer and enzyme, which could interfere with ligation reactions.

3.2.3 Ligation of insert DNA to vector

Ligation was carried out with 10 ng of insert DNA and 2 µg of Lambda ExCell vector at 16 °C for 4.5 h and the ligated product was packaged to form infective lambda recombinants. A control packaging reaction was also carried out using an extract of *E.coli* strain LE392 with control lambda DNA supplied by the manufacturer. Part of the packaging mixture was immediately titred and the remaining material stored at 4 °C. As shown in Table 8, the yield of the test lambda with *E.coli* LE392 was 1.6×10^9 recombinants per µg DNA, comparable to that expected from the manufacturer's specifications. Similarly, the number of background plaques, 2.1×10^4 pfu/µg *P. salmonis* DNA, was within the expected range for 4 kb insert fragments of approximately 4kb mean size. The transfection efficiency was 3.3×10^7 recombinants per mg *P. salmonis* DNA, and this was comparable with other published results from recombinant libraries from rickettsia (see Discussion). Refer to Figures 8 and 9 for representation of library as lambda plaques.

3.3 Preparation of DNA probes for screening *P. salmonis* recombinant library

3.3.1 *P. salmonis* 16S rRNA gene

To probe the *P. salmonis* recombinant library to confirm its rickettsial origin the 16S rRNA gene of *P. salmonis* was chosen as a target as this could be amplified from genomic DNA using PCR and is normally present in several copies in bacterial genomes. DNA was extracted by the method of Saris *et al.*, (1990) from three infected cultures supplied from Chile, labelled A, B and C. The conditions for ligation were refined by using different concentrations of insert and vector, and transforming different amounts of the ligation by heat shock and electroporation into fresh competent cells. From transformants obtained using the 27f and 1525r primers in the PCR, 60 plasmids were analysed, one of which, pAG16S1, contained an insert of 1.5,

Table 8 *P.salmonis* recombinant library in λExCell NM522

DILUTION	NO.OF COLONIES	
NEAT	BLUE; TMTC	WHITE; TMTC
10^{-1}	BLUE; AVE. 113.5	WHITE; AVE. 122.5
10-2	BLUE; AVE. 34	WHITE; AVE. 51.5
10-3	BLUE; CONFLUENT	WHITE; CONFLUENT
10-4	BLUE; CONFLUENT	WHITE; CONFLUENT
10-5	BLUE; CONFLUENT	WHITE; CONFLUENT
10-6	BLUE; CONFLUENT	WHITE; CONFLUENT

TRANSFECTION EFFICIENCY (WHITE)
10^{-1} Ave. 7.78×10^4
10^{-2} Ave. 1.79×10^5

BACKGROUND (BLUE)
10^{-1} Ave. 5.95×10^4
10^{-2} Ave. 1.18×10^5
Suggested transfection efficiency (white) = 3.3×10^7 recombinants/ug
Background (blue) =2.1×10^4 pfu/ug
Packaging efficiency of LE392=1.575×10^9 recombinants/ug
(10^{-6} dilution with 300 colonies)

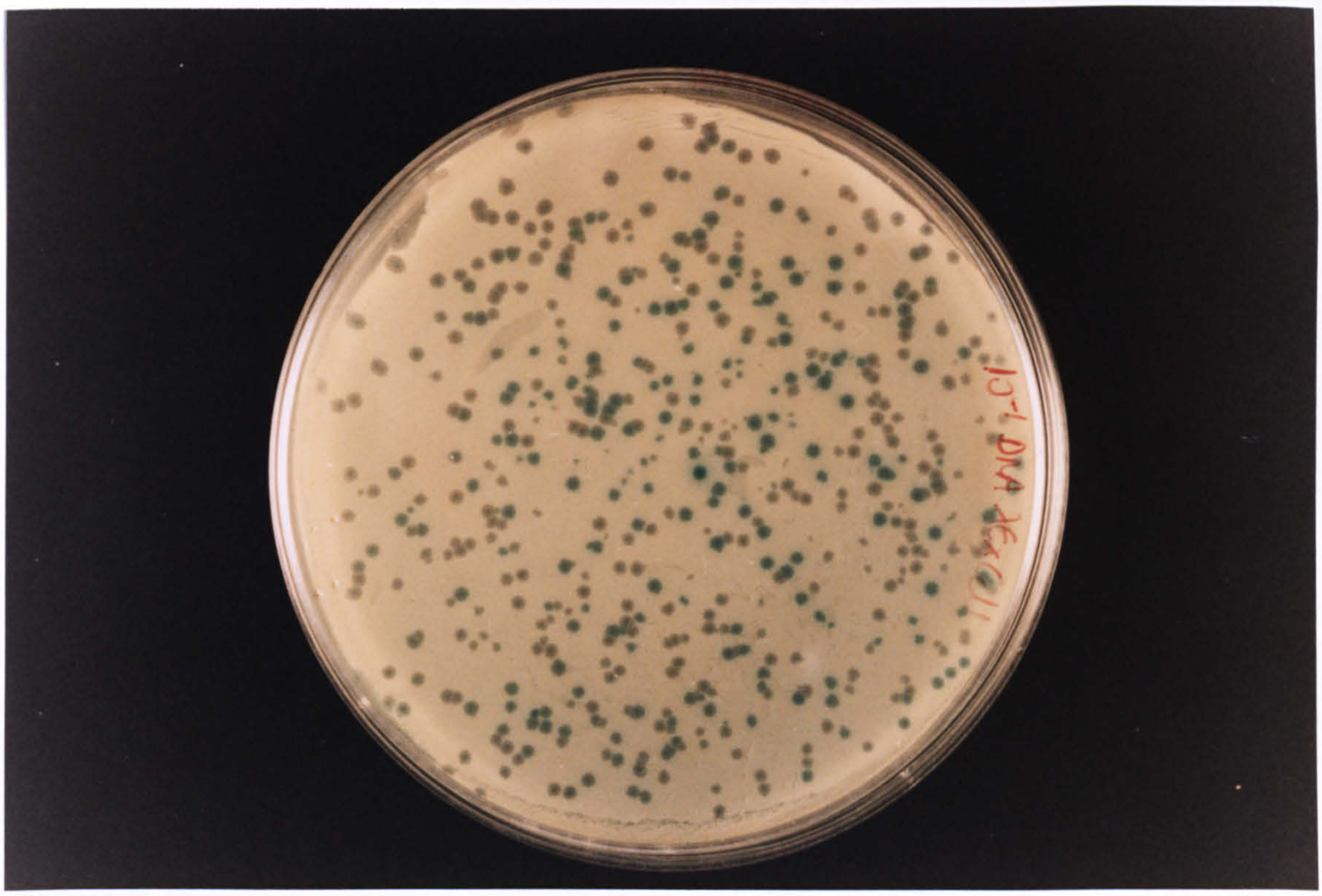


Figure 8 *P. salmonis* LF89/λExCell Library

An example of the recombinant *P. salmonis* LF89/ExCell λ plaques which constitute the recombinant library, neat dilution. Note the clear (white recombinant plaques) and the blue (non-recombinant plaques).

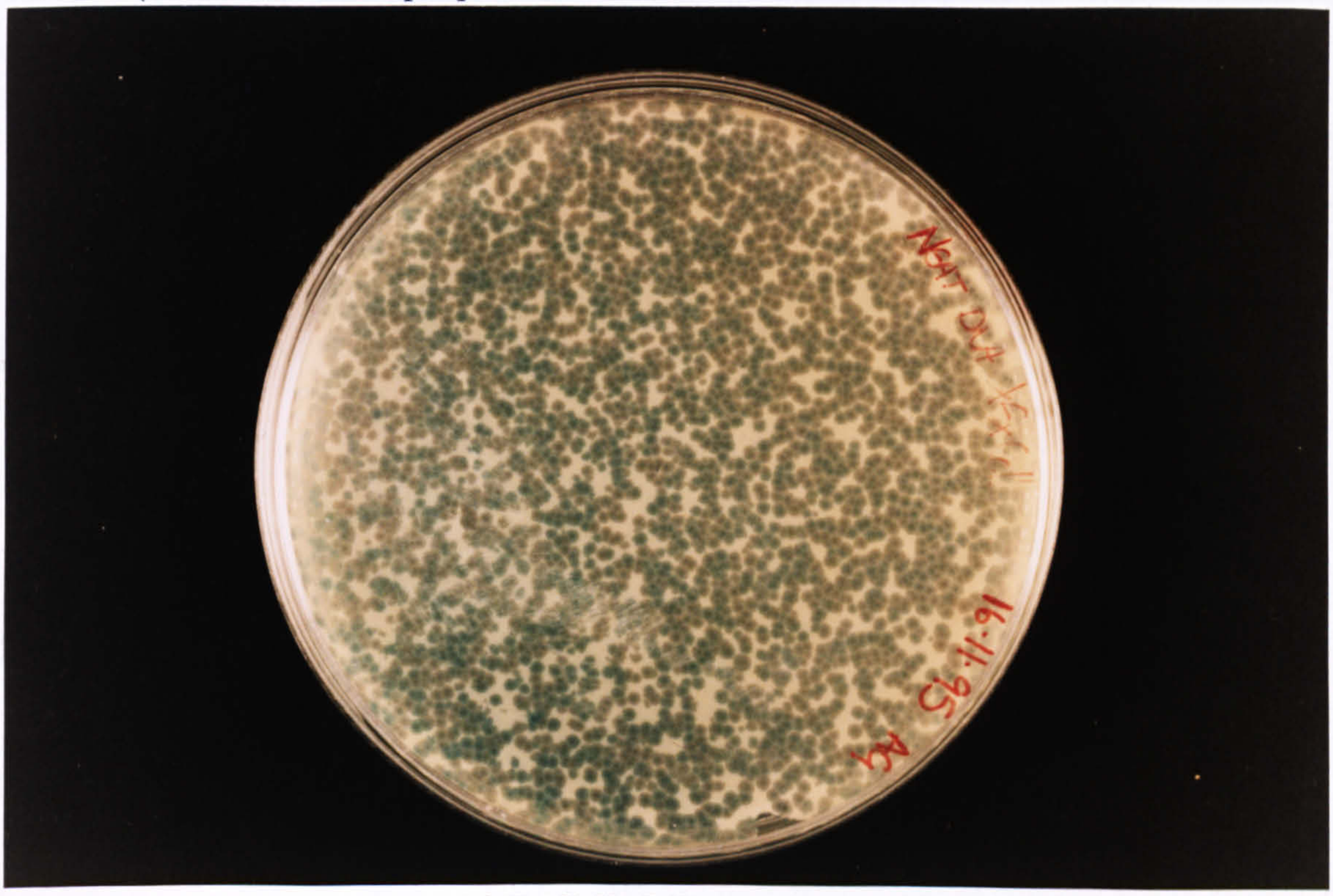


Figure 9 *P. salmonis* LF89/λExCell Library

As above at the 10^{-1} dilution.

the correct product size for the 16S rRNA gene of *P. salmonis*. Digestion of pAG16S1 with *EcoRI* resulted in the pattern expected for pUC18 containing an insert corresponding to the 16S rRNA gene, Figure 10 (for double digestion the expected result of 2.7 kb and 1.5 kb was observed). *EcoRI* digestion gave 2 bands of approximately 3 kb and 1 kb indicating the orientation of the insert. A Qiagen Maxi plasmid preparation was made from a 50 ml culture to provide substantial quantities of plasmid DNA for sequencing and for probe preparation (see later). The 16S rRNA gene from the Ardentoul strain of *P. salmonis* was prepared similarly.

3.3.2 Preparation by PCR of a probe for *Legionella pneumophila* macrophage infectivity potentiator

A second probe prepared was that for the *Legionella pneumophila* macrophage infectivity potentiator (mip), a gene which is essential for infection of macrophages and other cells (Cianciotto *et al.*, 1990). Closely related genes have been found in other intracellular pathogens, such as *Chlamydia trachomatis* (Lundemose *et al.*, 1992) and *Coxiella burnetii* (Mo *et al.*, 1995)), and the present probe was produced to determine if a homologue could be found in *P. salmonis*.

DNA extracted from the Knoxville strain of *L. pneumophila,* was used in varying concentrations as template for PCR with the primers designed by Ludwig *et al.*, (1994) (see Appendix). A 1 kb product was detected from the reaction of 10 µl of template and both concentrations of primer. The negative controls, in which the DNA or primers were replaced with dH_2O led to no product being amplified. The PCR products were separated from primers on a 0.8% agarose gel, the 1 kb band was recovered and used for DIG-labelling and cloning into pUC18 (Figure 11).

One plasmid analysed by agarose gel electrophoresis had a size approximately 1 kb greater than pUC18, and the restriction enzyme digestion pattern was that expected of the *L. pneumophila* mip gene (Figure 12).

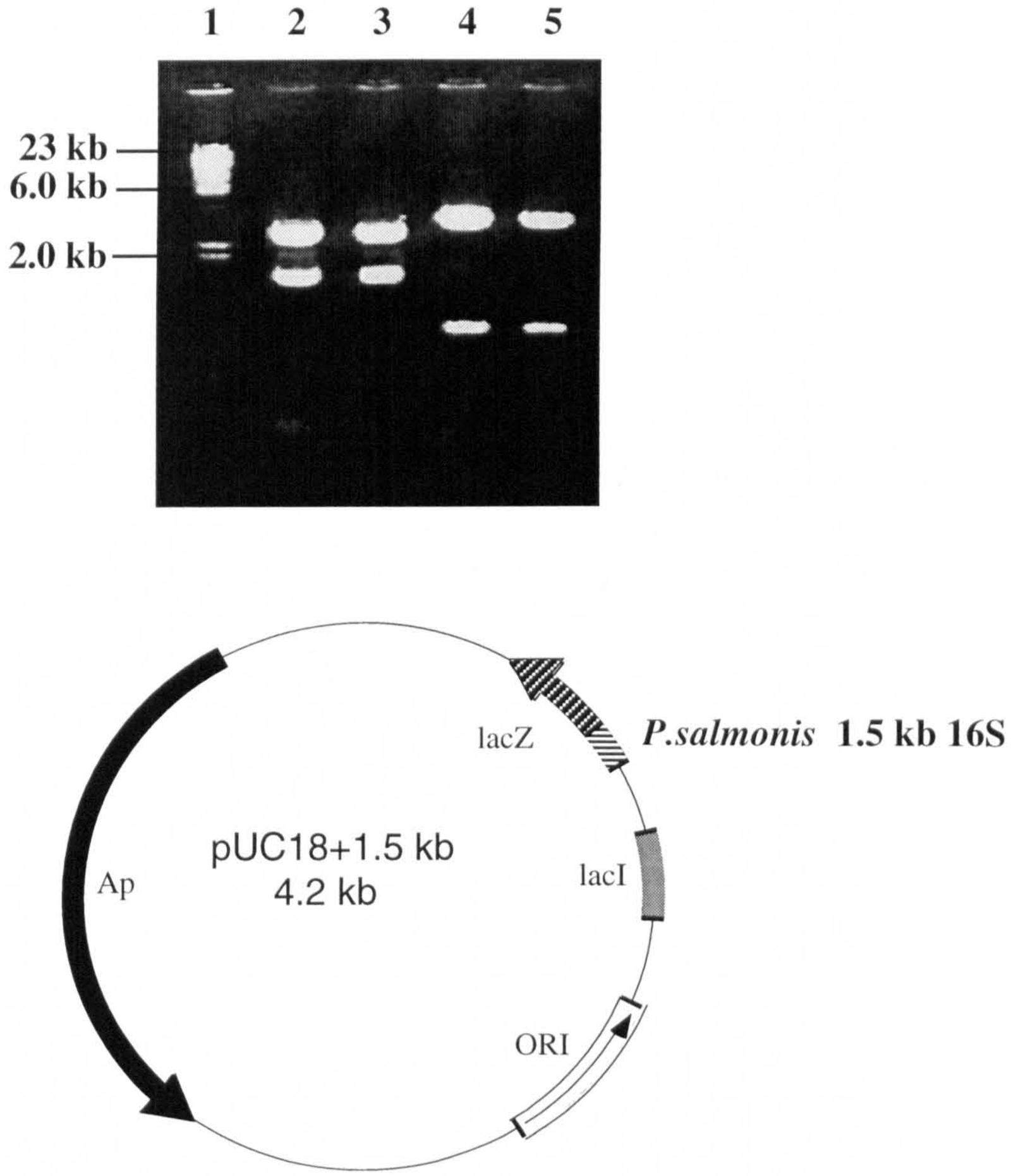


Figure 10 Restriction enzyme digestion of pAG16S1

pAG16S1, recombinant clone of 1.5 kb 16S of *P. salmonis* in pUC18 digested with *KpnI / BamHI* and *EcoRI* . To determine presence of insert and orientation.

Lane 1 λ*HindIII* digest standards

Lane 2 *KpnI / BamHI* digest

Lane 3 *KpnI / BamHI* digest

Lane 4 *EcoRI* digest

Lane 5 *EcoRI* digest

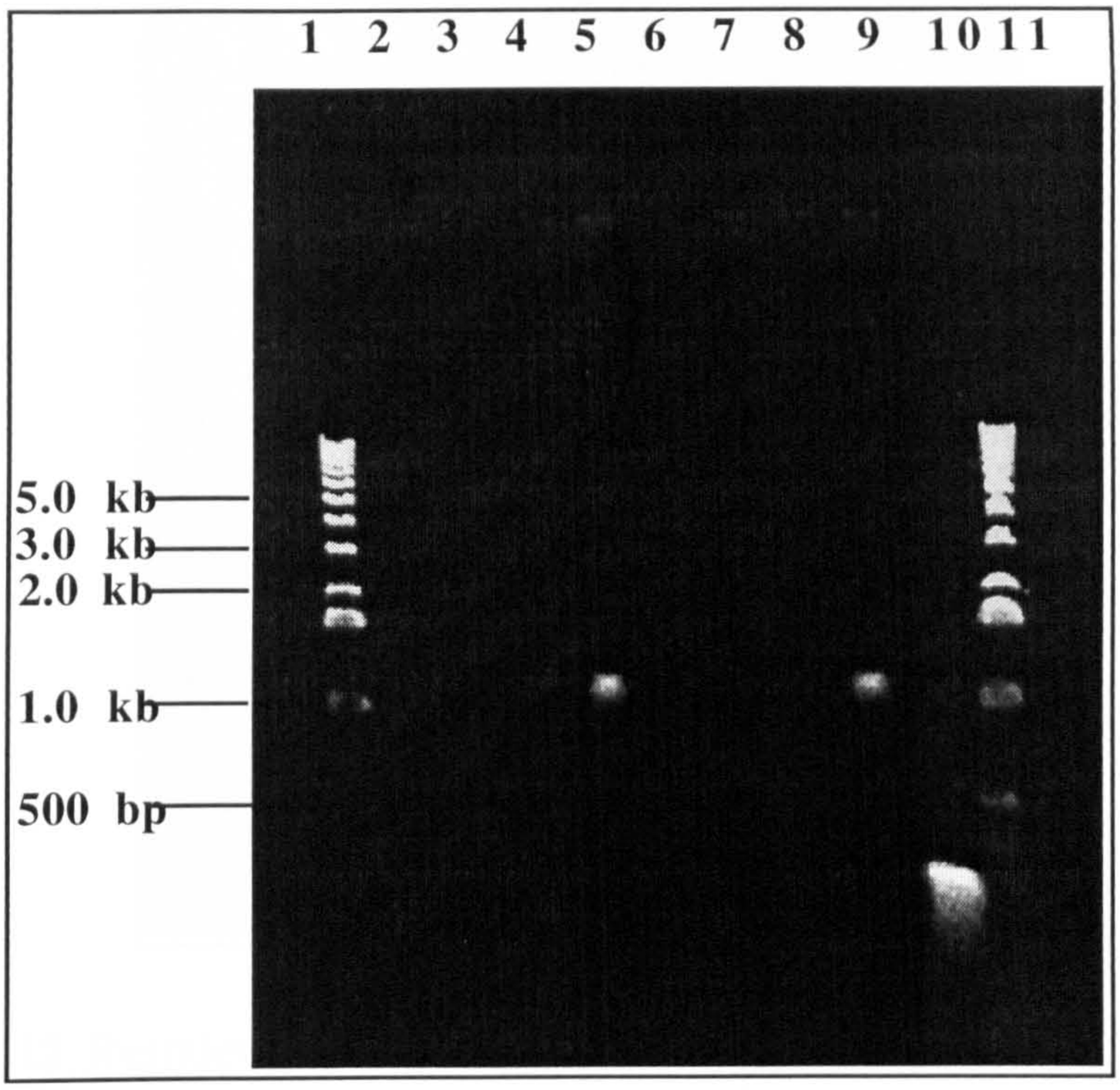


Figure 11 PCR of *Legionella pneumophila* mip gene

PCR amplification of *L. pneumophila mip* gene, as described elsewhere. A positive PCR product is visible in lanes 5 and 9, at 1 kb, the expected size of mip.

Lanes 1 & 11 KB ladder

Lane 2 Sample 1 (2 µl) negative control, no DNA, 2.5 µl of each primer.

Lane 3 Sample 2 (2 µl) negative control, no bacteria, 2.5 µl of each primer.

Lane 4 Sample 3 (2 µl) 10 µl of template DNA and 2.5 µl of each primer.

Lane 5 Sample 3 (2 µl) 10 µl of template DNA and 2.5 µl of each primer.

Lane 6 Sample 4, (1 µl) 5 µl of template DNA and 2.5 µl of each primer.

Lane 7 Sample 4, (4 µl) 5 µl of template DNA and 2.5 µl of each primer.

Lane 8 Sample 5, (4 µl) 2 µl of template and 2.5 µl of each primer.

Lane 9 Sample 6, (4 µl) 10 µl of template and 5 µl of each primer.

Lane 10 Sample 7, (4 µl) 2 µl Lp1 200 and 2.5 µl of each primer.

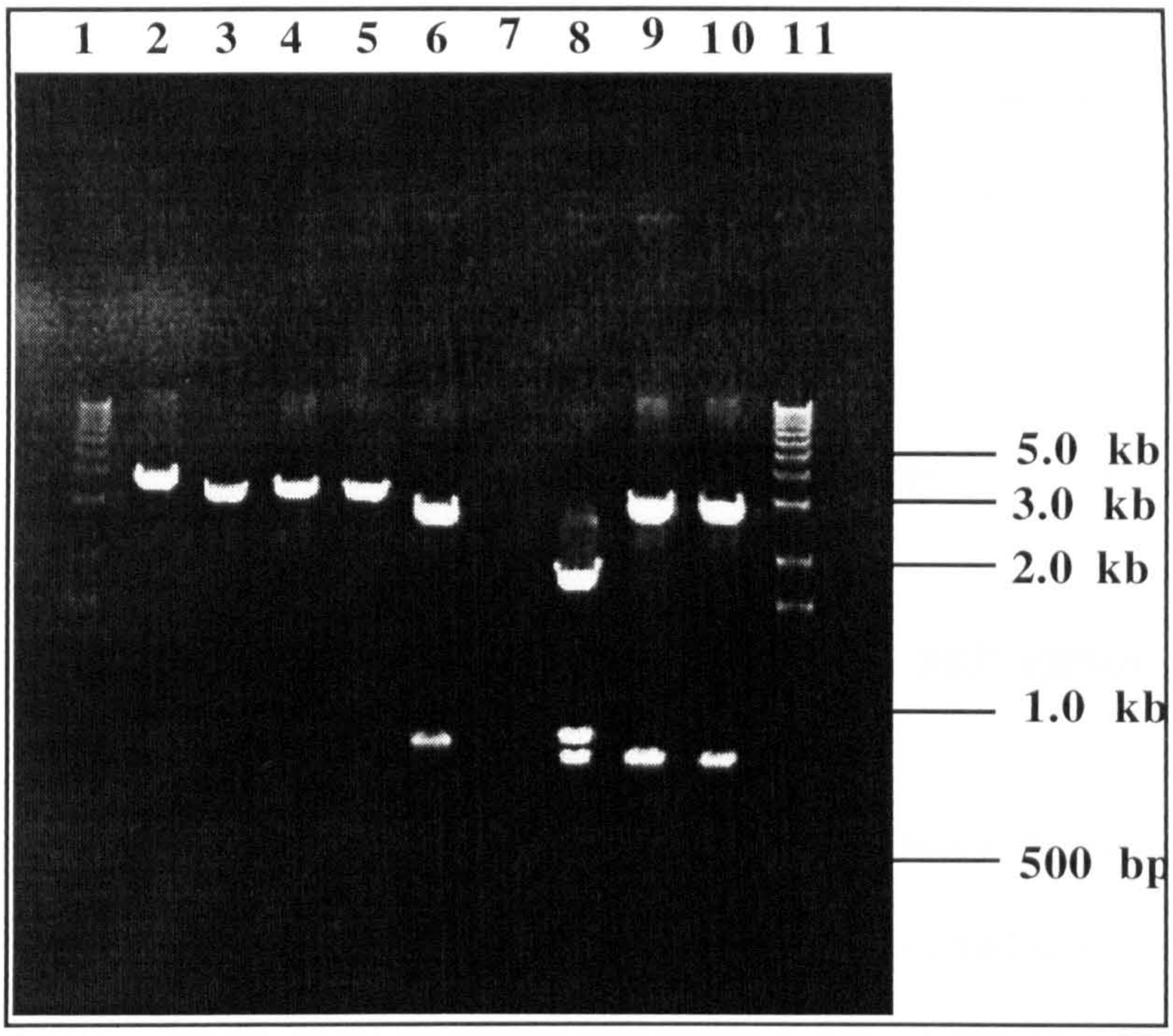


Figure 12 Restriction enzyme pattern of pAGmip1

Legionella pneumophila mip gene cloned into pUC18 (pAGmip1). Digested with the following enzymes; *KpnI, KpnI / EcoRI, PvuI, PvuI / SphI* and *SphI*

Lanes 1 & 11	5 µl of 1kb ladder standard
Lane 2	*KpnI* digest producing bands of approx. 3.5 kb (expected 3.7 kb)
Lane 3	*KpnI / EcoRI* double digest producing bands of approx. 3 kb (expected 3.2 kb + 300 bp + 200 bp).
Lane 4	*EcoRI* digest producing bands of approx. 3.3 kb (expected 3.4 kb + 300 bp).
Lane 5	*KpnI / EcoRI* double digest producing bands of approx. 3 kb (expect 3.2 kb + 300 bp + 200 bp).
Lane 6	*PvuI* digest producing bands of 2.8 kb + 900 bp (as expected).
Lane 7	BLANK
Lane 8	*PvuI / SphI* double digest producing bands of 1.8 kb + 900 bp + 800 bp (expected 1.6 kb + 900 bp + 850 bp + 150 bp).
Lane 9	*SphI* digest for 1 h, producing bands of 2.8kb + 800bp (expected 2.8 kb +900 bp).
Lane 10	*SphI* digest for 3 h producing bands of 2.8 kb + 800 bp (as expected).

3.4 Screening the *P.salmonis* λExCell library

3.4.1 Screening with a probe for the *P. salmonis* 16S rRNA gene

3.4.1.1 Preparation of DIG-labelled 16S rRNA gene probe

The plasmid pAG16S1, containing the 1.5 kb *P.salmonis* 16S gene sequence, was double digested to release the insert from pUC18; the 1.5 kb fragment was recovered, DIG-labelled (Section 3.3.1), quantified and the optimum conditions for use determined in preliminary experiments.

3.4.1.2 Screening of the library with a DIG-labelled 16S rRNA gene probe

Following plaque assay of the λExCell library the phage DNA was hybridised to Hybond N+, supplied by Amersham, and probed with the DIG-labelled 16S rRNA gene probe. The resultant screening identified all plaques as positive, the hybridisation conditions were varied resulting in no positives. This work was discontinued with the aim of returning to determine the nature of the hybridisation problem.

3.4.2 Screening with a probe to the *L. pneumophila* MIP gene

3.4.2.1 Preparation of DIG-labelled *L. pneumophila* MIP probe

DIG-labelled MIP was used to screen the Lambda ExCell *P.salmonis* library as described before. To determine optimum conditions for screening, different concentrations of MIP plasmid, pAGmip1, and *Legionella pneumophila* DNA were spotted onto filters as a preliminary experiment. The optimum concentration of MIP was DIG-labelled and used to screen the λExCell *P.salmonis* library, resulting in either all plaques reacting or none at all if the hybridisation parameters were altered. Again this work was discontinued, and screening with sera took priority, see below.

3.4.3 Screening the *P. salmonis* λExCell library with antisera

3.4.3.1 Screening with salmon convalescent antisera (December 1995)

The aim of these experiments was to detect immunologically proteins produced by recombinant phage that reacted specifically with antibodies in the antisera supplied by Marine Harvest Chile. Initially the reagents, salmon IgM, rabbit anti-salmon IgM antiserum Δ 80 and HRP-labelled goat anti-rabbit IgG were titrated to determine the

optimum concentration of the latter two reagents for use. The reactivity of the antisera and antigens was confirmed by Ouchterlony gel diffusion which showed that the anti-IgM antiserum, Δ 80 IgM, produced a single precipitin line with purified salmon IgM which gave a reaction of identity with a precipitin line formed with the salmon serum. Other fainter bands were formed between anti-IgM and the salmon serum, indicating that the antiserum contained antibodies to other serum proteins.

Filters containing proteins from the Lambda ExCell/*P. salmonis* clones were screened to detect proteins recognised by the salmon immune sera. However, 6 filters screened using the standard protocol with 1/25 salmon antiserum and 1/25 anti-IgM Δ80 gave no positive reactions.

3.4.3.2 Screening the *P. salmonis* λExCell library with salmon antisera, (March 1996)

Further anti-*P. salmonis* sera were supplied by Marine Harvest Chile in March 1996 (6 samples of 250 - 500µl) and these were used at dilutions of neat, 1/100 and 1/1000 with anti-IgM Δ 80 used at 1/50 and 1/500 diultions. Again, no positive plaques were identified with the salmon antisera.

3.4.3.3 Screening with high titre rabbit antiserum to *P. salmonis*

High titre rabbit antiserum was also supplied by Marine Harvest Chile and this was used to probe nitrocellulose membranes processed from amplified samples of the original λExCell *P. salmonis* library. Material from Bijou 11, which originated from plate 1 of the initial library plaque assay was used to prepare 5 phage assay plates and nitrocellulose imprints of these were screened with 1/60 anti-*P. salmonis* antiserum. Nine plaques gave a positive reaction with the antiserum, with 4, 1, 2, 2 and 0 positive plaques from plates 1 to 5 respectively. Each plaque was numbered, cored and resuspended in SM buffer and stored at 4°C. Plaques 1-5 were assayed at a range of dilutions, and plates containing well-separated plaques were chosen for immunoblotting, yielding a further 37 positive plaques from secondary screening (Figure 13)

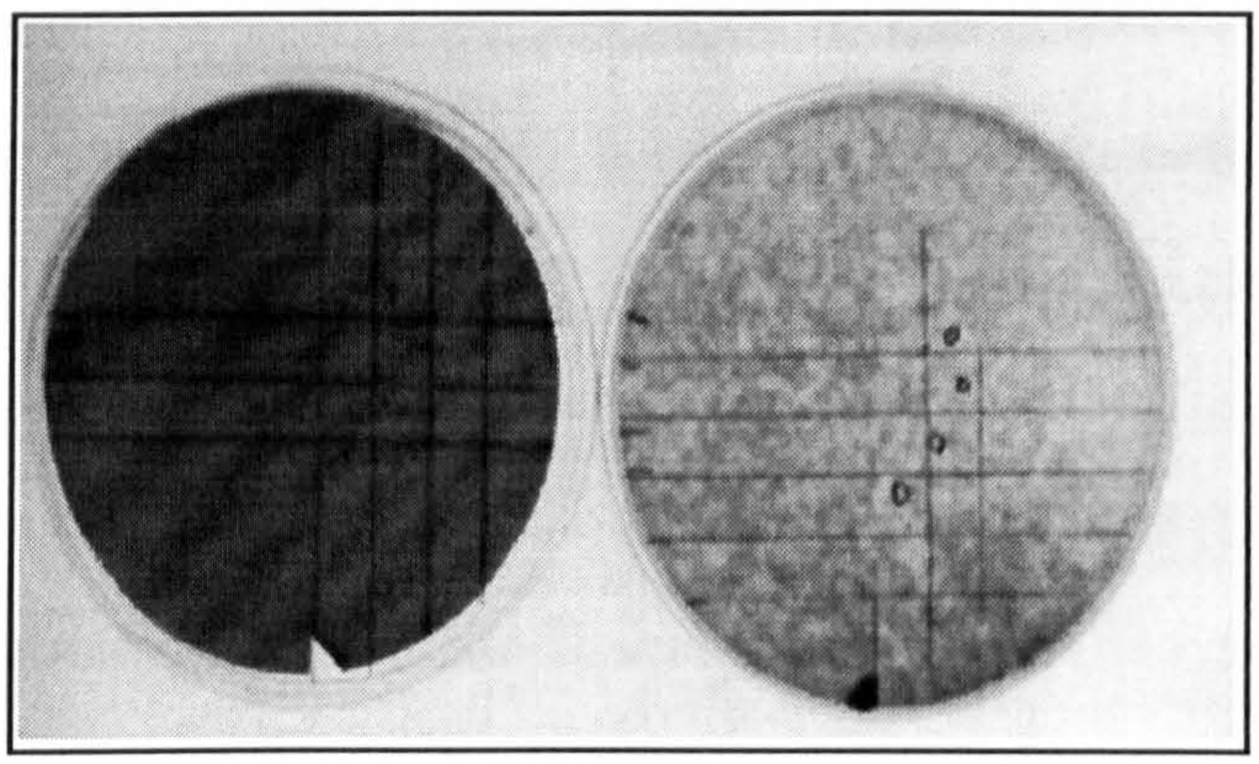


Figure 13 Identification of positives plaques from sera screening.

Plaques 1-4 identified from screening with high titre rabbit antiserum to *P. salmonis* . Identified as S1-4, aligned with corresponding plate and the plaques cored for further analysis.

3.4.3.4 Analysis of phage from plaques reacting with anti-*P. salmonis* antiserum

The initial positive phages, S1-9, were analysed to determine the size of insert carried. Phages were prepared from the plaques and pExCell phagemid release was attempted. Although S1 was not released, and did not grow in LB+Ap broth, all others were released and Wizard minipreps were performed to provide material for digestion with *BamHI* and *HindIII* restriction enzymes. Even after 3 h incubation samples were not completely digested, results not shown, and no insert was released. Thus, it appeared that the phages from plaques detected by screening with high titre antiserum did not in fact contain inserts.

A random selection of positive plaques from the screening (S1-48) was subjected to pExCell release and inserts analysed as before. In the first instance release was only obtained for 3/15 samples, and further analysis of phage from plaques 10 -34 resulted in release of 11/25 phagemids. After digestion with *HindIII* for 3 h gel analysis showed smearing of the DNA and no evidence of insert release (Figure 14).

3.4.4 Determination of mean insert size in the λExCell *P. salmonis* recombinant library

The recombinant library appeared to contain a similar number of recombinant phage to those reported in published data for other rickettisae, and it was decided to determine the mean insert size by analysis of a random selection of white and blue plaque-forming phage.

3.4.4.1 Release of pExCell

Although the release protocol given by the manufacturer was followed (see Materials and Methods), it had proved extremely difficult to obtain phagemid release for a high proportion of samples. Whereas lambda ExCell confers chloramphenicol and spectinomycin resistance, pExCell confers ampicillin resistance, and an initial problem was that only half of the phagemids analysed could confer growth in LB+Ap medium. Media and buffers were checked for contamination and found to be sterile.

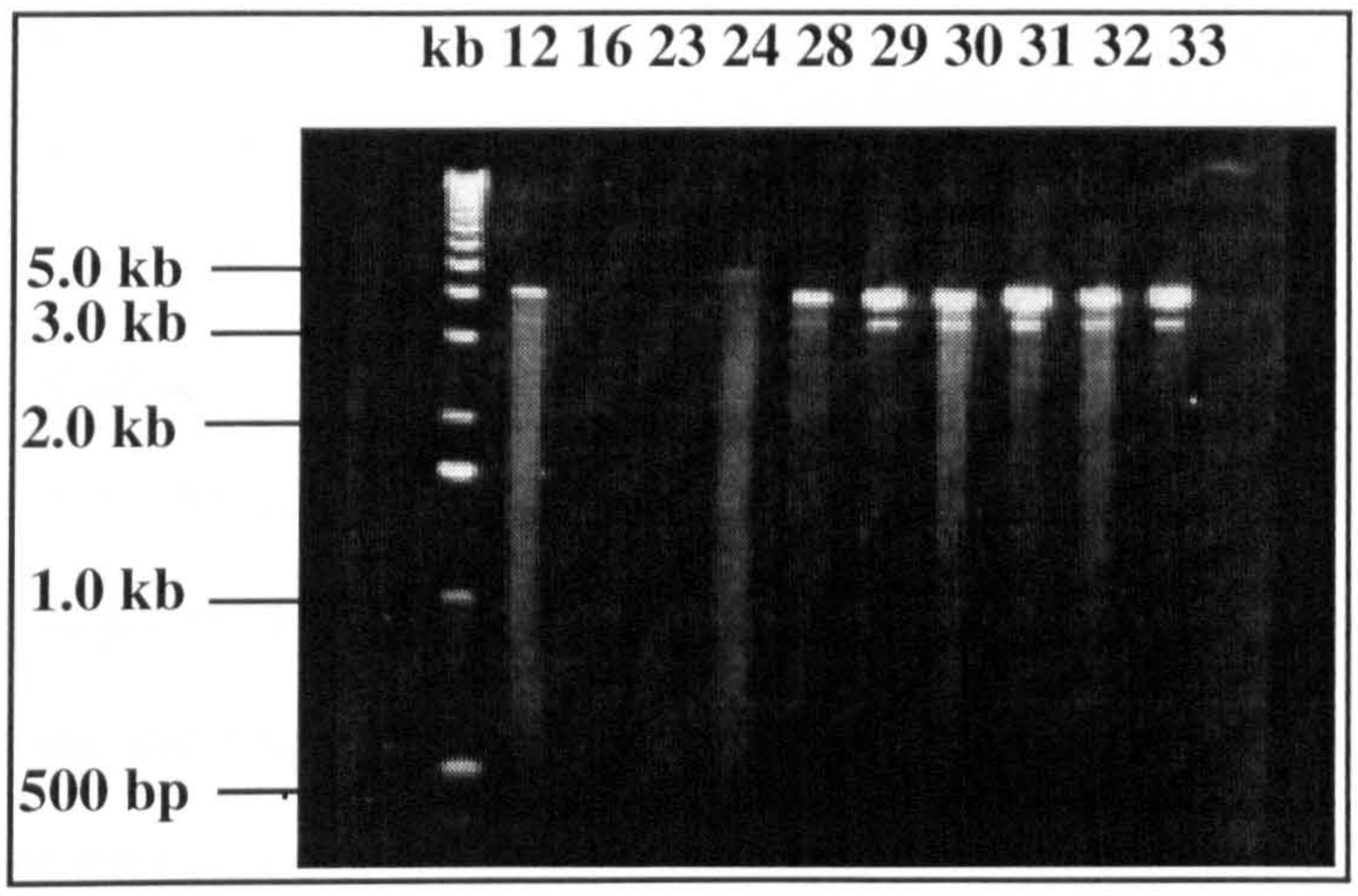


Figure 14 Plaques randomly selected from positive screening *HindIII* digestion

A selection of positive plaques identified by sera were analysed to determine the presence of insert with *HindIII* digestion for 3 h. None of the above plaques contained inserts as the phagemid is linearised at approximately 4 kb.

Lane 1 KB Ladder

Lane 2 S12 *HindIII* digest

Lane 3 S16 *HindIII* digest

Lane 4 S23 *HindIII* digest

Lane 5 S24 *HindIII* digest

Lane 6 S28 *HindIII* digest

Lane 7 S29 *HindIII* digest

Lane 8 S30 *HindIII* digest

Lane 9 S31 *HindIII* digest

Lane 10 S32 *HindIII* digest

Lane 11 S33 *HindIII* digest

After preparing fresh media and checking for contamination, the release protocol was followed precisely, and bijoux 37, 39 and 41, containing mixed phage lysates, were amplified to generate a selection of plaques. Six recombinant white plaques (nos. 1-6) and six non-recombinant blue plaques (nos. 7-12) were selected at random, cored and subjected to the release protocol. Of the six recombinant plaques only 2, numbers 1 and 5, produced bacterial growth in LB+Ap broth, indicating the presence of released phagemid in two out of six recombinants only, would normally expect six out of six, but all six of the non-recombinant plaques, numbers 7-12, were released. For analysis of the phagemid DNA, Wizard minipreps were performed from the eight released phagemids and 8 µl of each phagemid preparation was digested with *EcoRI* for 1 h, and compared by agarose gel electrophoresis with undigested sample. Figure 15 clearly shows the DNA samples to be degraded with incomplete digestion of the phagemids, when compared with undigested phagemid preparations.

3.4.4.2 Phenol:chloroform extraction of phagemid preparations

To inactivate nuclease activity in the above pExCell preparations, phenol/chloroform extraction and ethanol precipitation were carried out before re-analysis by agarose gel electrophoresis. An aliquot (8 µl) of the phenol/chloroform treated sample was analysed by agarose gel electrophoresis and no degradation was evident. After *EcoRI* digestion for 1 h at 37^{o}C, smearing was still present in each sample, and in samples 8, 11 and 12 a single product of approx. 4 kb was visible, indicating the presence of phagemid without an insert. The other samples showed only degraded DNA.

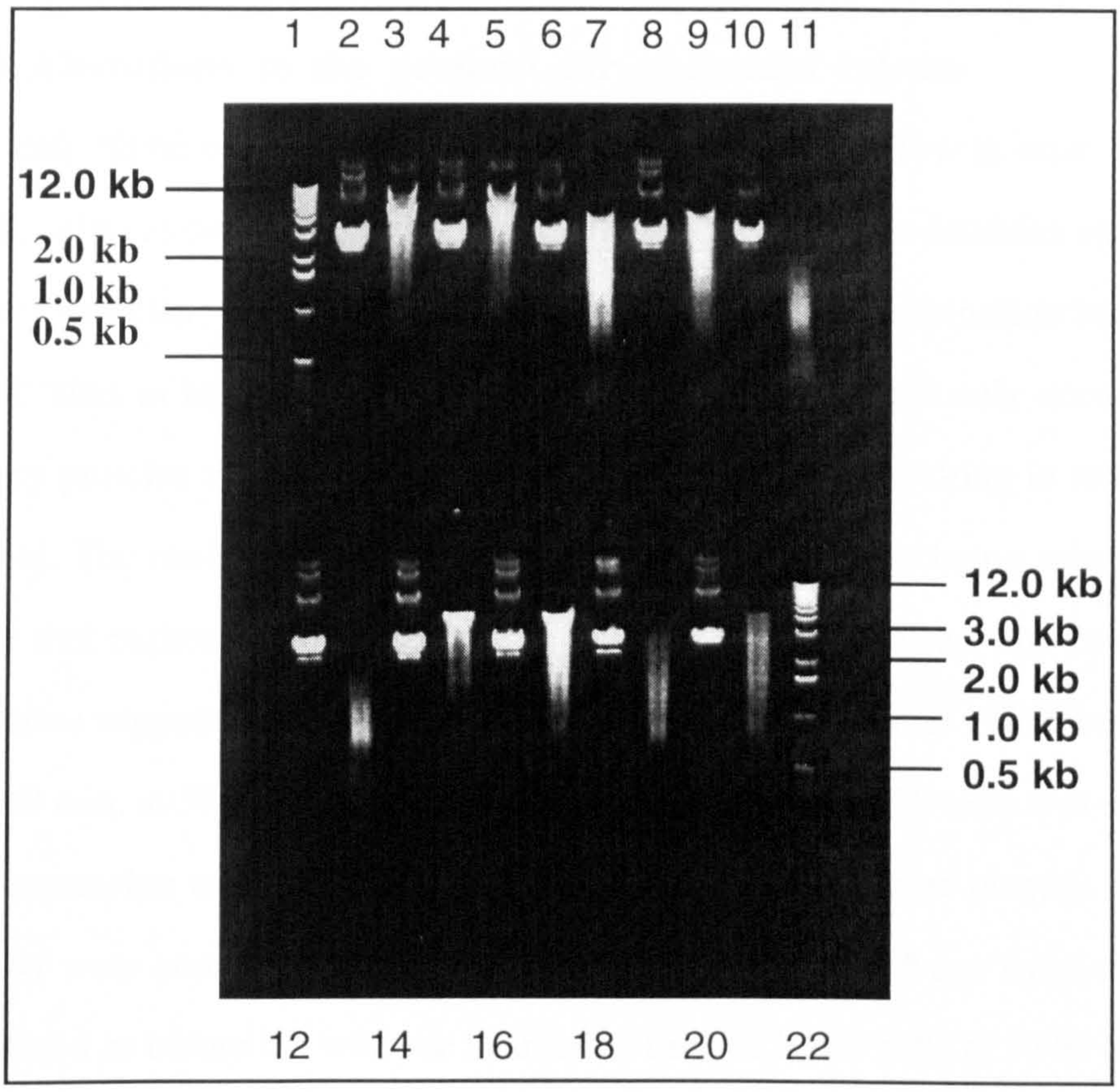


Figure 15 Comparison of undigested and digested pExCell phagemids

Randomly cored phagemids, 6 white and 6 blue (1-12) were released and phagemid DNA extracted and digested with *EcoRI* for 1h. Undigested sample was also analysed alongside.

Lane 1 KB ladder

Lane 2 1 *EcoRI* digested

Lane 3 1 uncut

Lane 4 5 *EcoRI* digested

Lane 5 5 uncut

and so on through to 12. A lot of smearing is present in the digested samples only.

The phenol/chloroform extraction step was incorporated into further plasmid preparation procedures. It was found that, alternatively, heating the preparations to 65°C for 1 h eliminated the degradation of DNA.

3.4.4.3 Alterations to the protocol for phagemid release

The *E. coli* NP66 cells are initially grown at 32°C but, to allow *in vivo* excision of pExCell, cells are then incubated at 39°C, allowing temperature-sensitive expression of accessory proteins which are necessary for site specific recombination between *attL* and *attR* sites in lambda ExCell. This recombination event can only occur when the accessory proteins are expressed in *E. coli* NP66 at 39°C, resulting in release of the phagemid. The observation that not all plaques analysed were being released, might indicate that expression of accessory proteins was not occuring. The manufacturer (Pharmacia) suggested extending the time of incubation of *E. coli* NP66 cells from 20 min to 30 min, at 39°C. Therefore, the incubation time of NP66 cells was extended to ensure expression of accessory proteins. Two white and two blue plaques from plates 39 and 41 were cored and numbered 26 to 29, respectively. Phage from each plaque was released as before but with the incubation time of NP66 cells at 39°C extended to 30 min. In further experiments, the incubation times at 39°C with NP66 plus phage were tested from 20 to 40 min. After incubation for 1.5 hr in 2 x YT broth +Sp at 32°C all cultures were slightly cloudy, indicating bacterial growth, and after overnight incubation at 37°C in LB broth +Ap, samples 26, 27 and 28 were cloudy at all incubation times. However, sample 29 was only slightly cloudy at the 20 min incubation and there was no growth, (broth clear) at the other two incubation times. Alkaline lysis plasmid preparations from samples 26, 27 and 28 showed the same band profile, and an *EcoRI* digest resulted in a major product of 4 kb for each sample, indicating the presence of phagemid only. The presence of higher molecular weight bands in low concentration indicated incomplete digestion of the phagemid. The same profile was generated when digested with *BamHI*, or double digestion with *BamHI* and *EcoRI* (Figure 16).

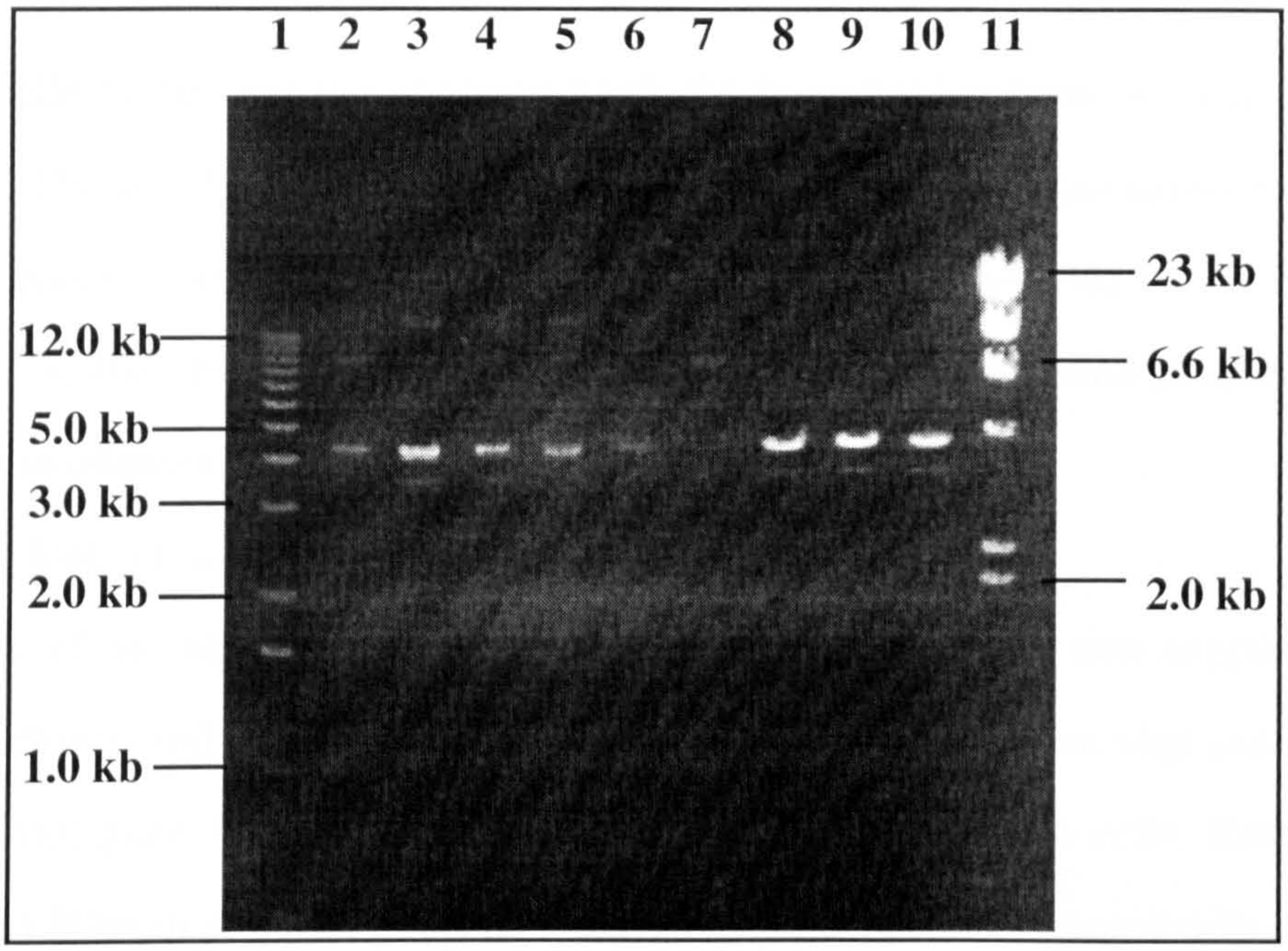


Figure 16 Alteration to phagemid release

To determine if incubation time of NP66 affects release, four plaques, 26-29 were tested at three different incubations of 20, 30 and 40 min at 39°C. Normal release protocol was followed otherwise, Plaque 29 was not released at any incubation, but the other samples 26-28 were released at all incubations. Alkaline lysis was performed on each of the released samples and 5 µl was digested with *EcoRI* for 2 hr at 37°C.

Lane 1	5 µl KB ladder
Lane 2	Plaque 26, 20 min incubation 5 µl digest analysed
Lane 3	Plaque 26, 30 min incubation 5 µl digest analysed
Lane 4	Plaque 26, 40 min incubation 5 µl digest analysed
Lane 5	Plaque 27, 20 min incubation 5 µl digest analysed
Lane 6	Plaque 27, 30 min incubation 5 µl digest analysed
Lane 7	Plaque 27, 40 min incubation 5 µl digest analysed
Lane 8	Plaque 28, 20 min incubation 5 µl digest analysed
Lane 9	Plaque 28, 30 min incubation 5 µl digest analysed
Lane 10	Plaque 28, 40 min incubation 5 µl digest analysed
Lane 11	5 µl Lambda HindIII

Conclude all have the same profile phagemid (4.2 kb) only no insert, pXis is thought to be the smaller band running directly under pExCell at 3.5 kb, described by suppliers Promega as comigrating alongwith pExCell. *EcoRI* digestion should release any inserts present.

Other experiments showed that changes in inoculation volume and incubation time did not affect release of phagemid. When plaques are individually cored they were stored in 1 ml of SM buffer with the addition of a single drop of chloroform to prevent bacterial growth. The possibility that residual chloroform in the phage preparations might affect release was investigated by dilution of the stock phage up to 4-fold before attempting release. Neither this, nor the use of freshly-cored, rather than stored plaques, affected success in obtaining release.

3.4.4.4 Use of a new batch of *E. coli* NP66 cells

Because of the above problems a fresh batch of NP66 cells was supplied by the manufacturer, and two fresh plaques, one white (no. 31) and one blue (no. 32) were cored from plate 37 and released with the fresh stock of NP66 cells. Both samples grew in LB broth +Ap and phagemid DNA was prepared and digested with *EcoRI* for agarose gel electrophoresis. Even with enzyme digestion times extended to 4 h sample 31 remained uncut. Sample 32 was digested after 1 h to reveal no insert, as expected from a blue plaque.

3.4.5 Release of phagemids from phages B41.1 to B41.25

Three problems had been found during attempts to analyse the mean insert size of the pExCell library. These were, firstly, difficulties in obtaining release of phagemids, secondly, difficulties in release of inserts from the phagemids, and thirdly degradation of the phagemid DNA preparation during restriction enzyme digestion. Although all phagemids could not be released, a number were successfully released. From plaques B41.1-25 release was obtained consistently on four different occasions with plaques B41.2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 18, 21, 22 and 24, but other plaques (B41. 15, 16, 17, 19, 20 and 23) gave inconsistent results. For example, B41.15 was analysed on four separate occasions with the same protocol but grew in LB broth +Ap on one occasion.

In total, 16 phage were released successfully and analysed by Wizard minipreps, incorporating an additional phenol:chloroform and ethanol precipitation step. On digestion with*BamHI,* agarose gel analysis revealed that samples 2, 7 and 17 had low

concentrations of DNA, therefore, no conclusions can be drawn. Samples 3, 8, 14, 18, and 19 all showed the same profile corresponding to undigested phagemid, and samples 5 and 6 had a major product at approximately 4 kb indicative of phagemid only, with no insert present (Figure 17).

3.4.6 Use of different restriction enzymes to release phagemid inserts

To determine if incomplete digestion was due to choice of a particular enzyme, sample B41.22 from the above batch, digested with *BamHI,* was further digested with *HaeII* for 3 h and 5 µl was analysed on an agarose gel. This enzyme cuts pExCell at four different sites, generating products of 2.9 kb, 876 bp, 370 bp and 8 bp, so in conjunction with *BamHI* the double digest should generate products of 3.3 kb, 588 bp, 288 bp and 8 bp. If an insert was present all bands would be visible except 588 bp, which would be greater in size. Each sample, 5 µl, was also digested for 3 h with *HaeII* alone for comparison. The double digest of sample 22 resulted in incomplete digestion, whereas digestion with *HaeII* alone resulted in products of 2.9 kb, 900 bp and 350 bp approx., the expected profile for phagemid alone, as the 8 bp product would not be detectable. It was concluded that B41.22 contained no insert.

Heat-treated (65°C) samples of Wizard phagemid preparations B41.2, 3, 5, 6, 7, 8, 14, 18, 21, 22, 24 and 25 were double digested with *HindIII / SfiI* and *XhoI /BamHI* for 3 h. Gel analysis showed that B41.8 contained an insert of approximately 500bp, although others contained no detectable insert (Figure 18), although samples 2 and 21 showed bands of 3kb, 4kb and 7kb and required further analysis

Digestion of B41.2 with *BamHI* and *SfiI* individually gave a product of 8 kb indicating the presence of an insert of 3.8 kb. Further digestion of B41.6, 7, 22, 24, and 25 phagemid preparations with the above two enzymes resulted in 4 kb bands only, indicating the absence of an insert in each sample. Sample B41.21 gave a major product of 7.5 kb, showing the presence of an insert of 3.3 kb. B41.2, 7, 8 and 21 were further analysed with double digests of *XhoI* and *BamHI* ,which releases inserts, if present from pExCell. The presence of inserts in each case was confirmed, Figure 19.

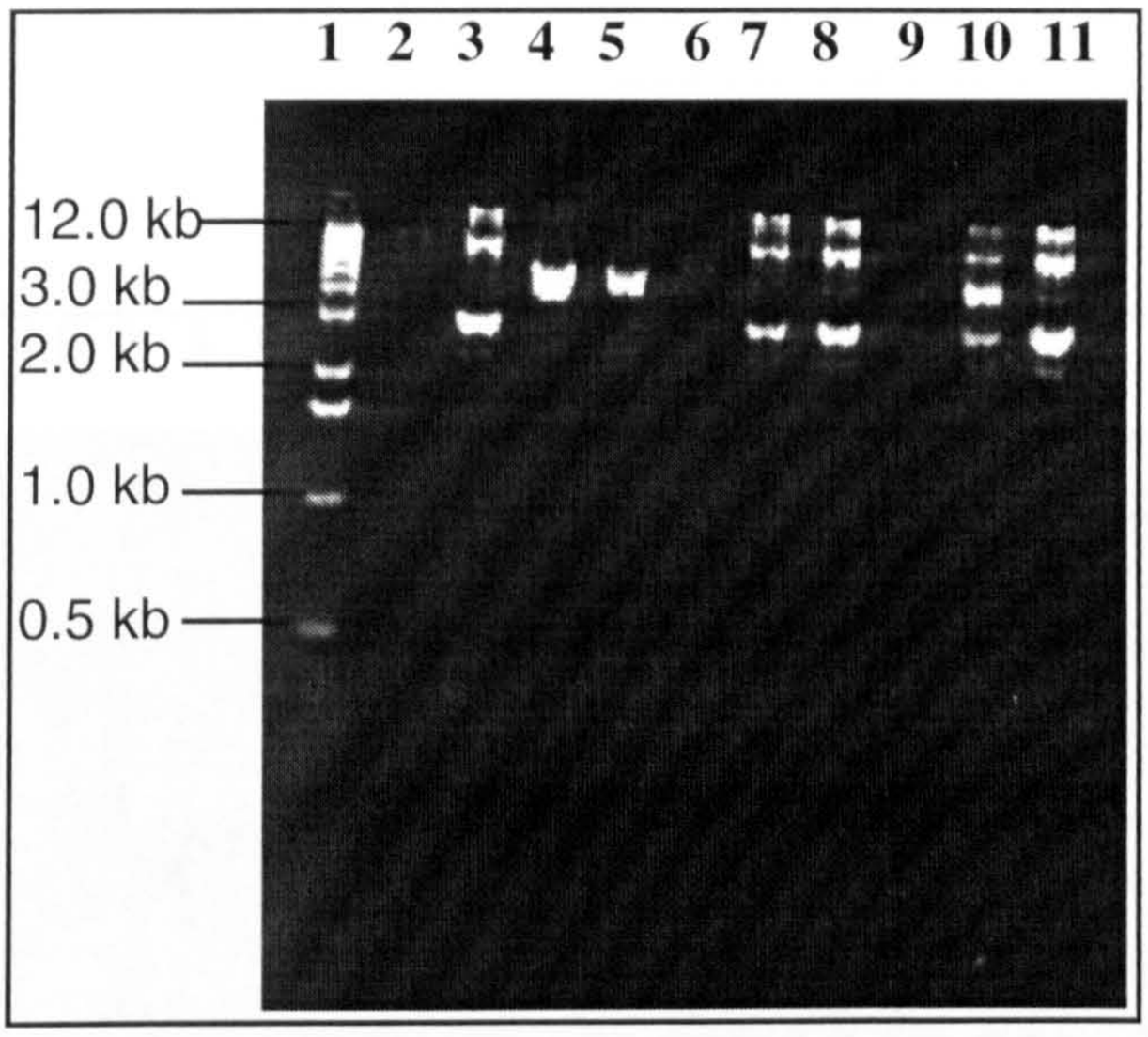


Figure 17 Analysis of phagemids B41.1-25

Release of phagemids B41.1-25 with an extra phenol:chloroform step incorporated into the Wizard prep. Each prep was digested with *BamHI* , 5 µl for 5 hr at 37°C. 5 µl was analysed, resulting in incomplete digestion in the majority of samples.

Lane 1 5 µl of KB ladder

Lane 2 10 µl B41.2 digest, very little to see

Lane 3 10 µl B41.3 digest, incomplete digestion

Lane 4 10 µl B41.5 digest, a major product at approx. 4 kb

Lane 5 10 µl B41.6 digest, a major product at approx. 4 kb

Lane 6 10 µl B41.7 digest, very little to see

Lane 7 10 µl B41.8 digest, incomplete digestion

Lane 8 10 µl B41.14 digest, incomplete digestion.

Lane 9 10 µl B41.17 digest, very little to see.

Lane 10 10 µl B41.18 digest, incomplete digestion with a major product at 4 kb

Lane 11 10 µl B41.19 digest, incomplete digestion.

Conclude that no inserts are present in samples B41.5, B41.6 and B41.18. The other samples have either very little DNA or digestion not complete.

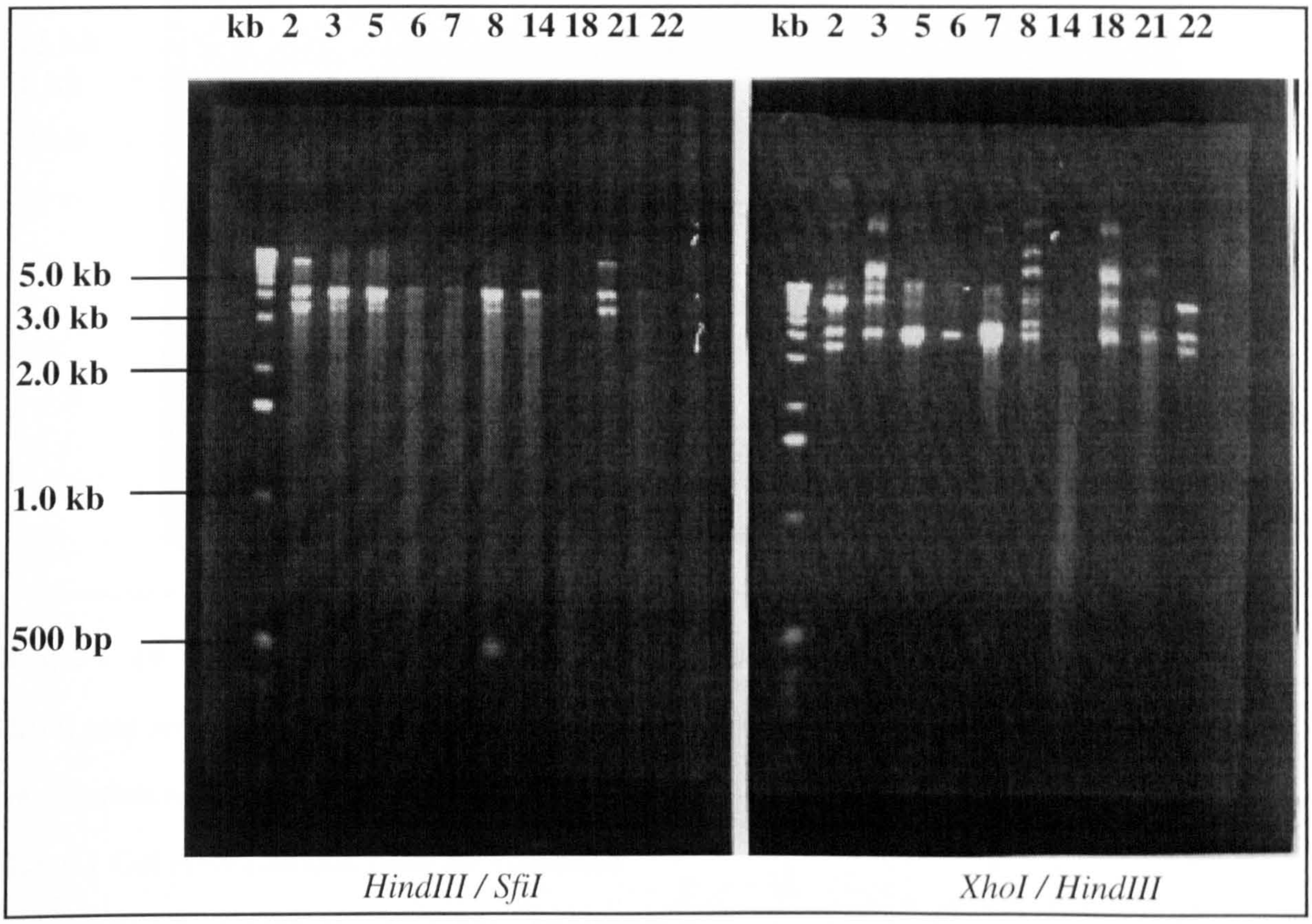


Figure 18 Double digests of selected B41 phagemids

B41.2, 3, 5, 6, 7, 8, 14 ,18, 21 and 22 with *HindIII/ SfiI* and *XhoI/HindIII*

Both gels contain the same order of samples digested with two sets of enzymes to generate different patterns.Conclusions; B41.8 has an insert of 500 bp

B41.2 and B41.21 inserts of approximately 3 kb? Requires further analysis

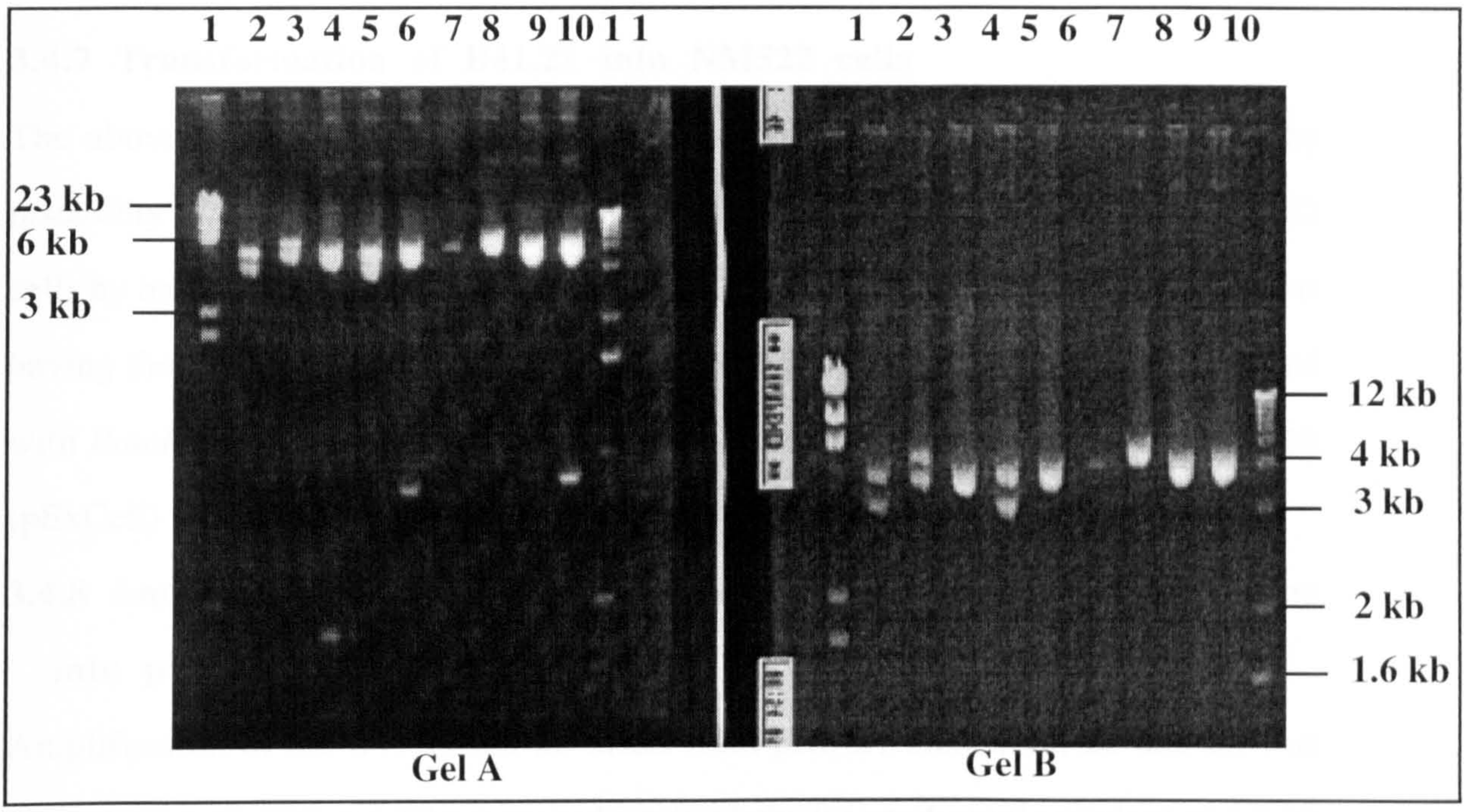


Figure 19 Analysis of pExCell inserts digested with *XhoI / BamHI*

Both gels are identical, Gel B was run for a longer period. Each insert from pExCell phagemids released in this study were digested on the same gel. The lane order follows;

Lane 1 Gel A+B Lambda Hind *III* standards

Lane 2 Gel A+B B41.21 digest, note 3 kb insert

Lane 3 Gel A+B B41.8 digest, note 3.5 kb insert

Lane 4 Gel A+B B41.7 digest, note 450 bp insert

Lane 5 Gel A+B B41.2 digest, note 3 kb insert

Lane 6 Gel A+B B11.15 digest, note 950 bp insert

Lane 7 Gel A+B B11.8 digest

Lane 8 Gel A+B S44 digest, no insert

Lane 9 Gel A+B S24 digest, no insert

Lane 10 Gel A+B S16 digest, 950 bp insert.

In summary, the presence of inserts of 3.5kb (B41.2), 950bp (B41.7) and 3.5kb (B41.8 and B41.21) was shown, and B41.1, 2, 3, 5, 8, 6, 7, 14, 18, 22, 24 and 25 had no inserts, refer to Table 9.

3.4.7 Transformation of B41.21 into NM522 cells

The above pExCell B41.21 was shown to contain a 3.5 kb insert, and due to the instability of *E. coli* NP66 cells, the phagemid was transformed into *E. coli* NM522 cells by heat shock. Transformed samples B41.21NM.12, 22 and 28 were identified as having the same profile as B41.21, and these subclones 12, 22 and 28 were digested with *BamHI* and *XhoI* ; agarose gel analysis showed the presence of bands of 4.2 kb (pExCell) and 3.5 kb (insert).

3.4.8 Amplification by PCR of the insert in pExCell B41.21 and cloning into pUC18

Amplification of the B41.21 3.5 kb insert from pNM21 clones 12, 22 and 28 was carried out with SP6 and T7 primers which flank the insert. A single product of 3.5 kb was generated, as expected. The PCR product was cloned into pUC18 using the Sureclone (Pharmacia) blunt-ended cloning system. Transformation produced only three colonies which were shown to contain inserts of 500 bp, 300 bp and no insert. A different approach was used in which blunt-ended (Sureclone) 3.5kb PCR product was ligated to pUC18/*SmaI* BAP with vector:insert ratios of 3:1 and 1:3. In this ligation GIBCO T4 DNA ligase and buffer were used with overnight incubation at 16°C. Although heat shock failed to yield any transformants, electroporation produced 13. The insert size was 500 bp in each case. Sequence analysis was performed on some of the above samples (see later). Repetition of the cloning with electroporation give 39 transformants, 12 of these were analysed to reveal, again, partial inserts. Due to the repeated failure in cloning with the Sureclone kit (used successfully in other experiments in the laboratory) a different strategy was adopted, by producing compatible ends on both insert and vector (pUC18) for ligation. pUC18 was digested with *SalI/ HindIII* and insert DNA (pNM28) was digested with *XhoI/ HindIII.* Of 20 transformants analysed one had an insert of 3.5 kb, and a maxiprep was made to

provide a large quantity of B41.21 3.5 kb insert in pUC18 DNA for further manipulations.

3.4.9 Analysis of other pExCell inserts

Following the above example of detecting inserts in pExCell other plaques were analysed in the same manner, identifying 900 bp insert in plaques B11.8 and B11.15, as shown above in Figure 19. S16 was also shown to contain an insert of 950 bp, this positive plaque resulting from anti-sera screening. Each of these inserts are analysed by sequencing in the next section.

NAME	ORIGIN	INSERT
S16	sera screen	950 bp
B11.8	Bijoux 11	450 bp
B11.15	Bijoux 11	900 bp
B41.2	Bijoux 41	3 kb
B41.7	Bijoux 41	450 bp
B41.8	Bijoux 41	3.5 kb
B41.21	Bijoux 41	3 kb

Table 9 Summary of inserts sizes in λExCell/*P. salmonis* library.

3.5 Nucleotide sequence analysis of clones from the *P. salmonis* Lambda ExCell library

3.5.1 Nucleotide sequence analysis of clone B41.21

As it had not been possible to release inserts from the pExCell *P. salmonis* clones by digestion with *EcoRI* the sequence of the pExCell multicloning site (MCS) was determined for clone B41.21 by automated sequencing. Several different DNA preparations and primers were used, as summarised in Table 10, together with the FASTA and BLAST results for these samples.

3.5.1.1 Sequence ag6

The FASTA alignment of sequence ag6 with that of pExCell is shown in Figure 20. Up to nt 39 of ag6, which is equivalent to the *EcoRI* site of pExCell at position 2110, there is 87.2% identity, and residues from nt 40 onwards are presumed to be of insert origin. The *EcoRI* site present in pExCell, GAATTC is not present in ag6 but is replaced by GCGTTC, explaining why the insert could not be released from this clone by digestion with *EcoRI*.

BLASTN 2.0.3 analysis of the insert sequence revealed close similarity between ag6 and *E. coli* sequences (accession numbers D90784, AE000241 and D90783). The identity, 89.2% over 426 nt (Figure 21), would be considerably higher if unidentified bases in ag6 were discounted. The region of identity within *E.coli* encodes the potH gene involved in putrescine transport. A BLASTX 2.0.3 analysis was also performed to determine the similarity at the protein level, and this also showed similarity to potH.

3.5.1.2 Sequence ag19

As with sequence ag6, this sample originated from pExCell clone B41.21 and was sequenced with the T7 reverse primer, which anneals to pExCell at nt 2175-2155 resulting in readthrough of the pExCell MCS from nt 2154 to 2110, from the *HindIII* restriction site to the *EcoRI* site. The sequence data was poor in the region of the MCS and did not allow definite identification of the *EcoRI* site. However, BLAST and FASTA searches comparing the insert sequence with databases again revealed high similarity with *E. coli* genes. FASTA analysis resulted in similarity to *E. coli* X73026

Table 10. Summary of nucleotide sequence analysis of *P. salmonis* clone B41.21 in lambda pExCell

Sequence number	Origin	Sequencing primer	sequence at putative *EcoRI* site	Sequence similarity identified in searches with: FASTA	BLAST
ag6	pExCEll B41.21	-40	GCGTTC	M93239	D90784 93%, 336nt
					AE000241
					D90783
ag19	pExCEll B41.21	T7	-	X73026	X73026 85%, 314nt
					X66836
					AE000374
ag27	pUC18. B41.21	-40	GCGTTC	AE000241;90.7%, 845nt)	AE000241 100%, 480nt
				D90783	D90783
				D90784	D90784
ag36	pUC18. B41.21	-40	GCGTTC	X66836; 96.6%, 546nt)	X66836 97%, 371nt
				AE000374	AE000374
				M28377	M28377
ag33	pUC18. B41.21	-40	GCGTTC	AE000241; 98.3%, 358nt)	AE000241 97%, 406nt
				D90783	D90783
				D90784	D90784

and M64630, encoding iciA and rpi genes, and the SerA promoter region, respectively. BLAST analysis revealed a similar picture with 85% similarity over 314 nt with *E. coli* X73026, X66836 and AE000374, all of which encode iciA, rpiA and SerA genes.

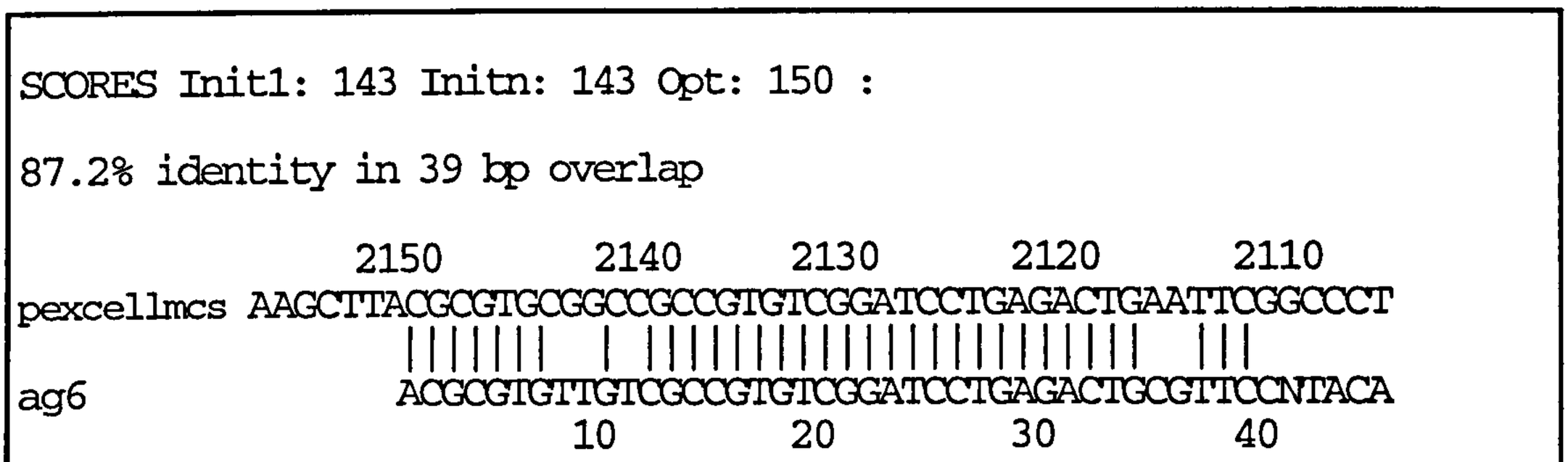


Figure 20 Comparison of ag6 and pExCell MCS

Identification of the multicloning site in sequence ag6. The sequences of ag6 and pExcell (Accession Number u13848) were compared using FASTA, from which a 39 nucleotide section was identified with 87.2% identity between the sequences.

3.5.1.3 Sequence ag27

The sequence ag27 was obtained by automated sequencing of a pUC18 clone containing the B41.21, 3.5 kb insert and the -40 primer, which anneals to pUC18 at nt 307-325. The sequence through the MCS revealed again that the *EcoRI* cloning site had been altered as in sequence ag6 (above), with bases AA replaced with CG. This is shown clearly on the original electrophoretogram (Appendix 2). The pExCell MCS sequence is evident through to position 2151 of pExCell, and after the putative *EcoRI* site lies the insert sequence (Figure 22).

```
Ae000241 Escherichia coli from bases 150678 (10160 nt): initn: 1060
init1: 1060 opt: 1704 Z-score: 1851.4: 89.202% identity in 426 nt overlap

       50         60         70         80         90        100
AG      AACGCGTGTTGTCGCCGTGTCGGATCCTGAGACTGCGTTCCNTACATTACATGCGGTGGG
                                          :::::::::: :::::::: :::::::::
ECAE24 TTTACAACATCTTGGGCTGGAACCACTGCTGACTGCGTTCCTTACATTACCTGCGGTGGG
     5540       5550       5560       5570       5580       5590

      110        120        130        140        150        160
AG      CGGAAATACNCTGTCAACTTCCGGGCTGGGGCGCTTTCTGGTG-TTCTCTATATCTGGTT
        ::::::::: :::::::::::::::::::::::::::::::: ::::::::::::::::
ECAE24 CGGAAATACGCTGTCAACTTCCGGGCTGGGGCGCTTTCTGGTGTTTCTCTATATCTGGTT
     5600       5610       5620       5630       5640       5650

       170        180        190        200        210        220
AG      GCCGTTCNTGATCCTGCCCGTTCAGGCGGTGCTTGAGCGTTTGCCGNCGTCATTGTTGCA
        ::::::: :::::::::::::::::::::: :::::::::::::::: ::::::::::::
ECAE24 GCCGTTCATGATCCTGCCCGTTCAGGCGGCGCTTGAGCGTTTGCCGCCGTCATTGTTGCA
     5660       5670       5680       5690       5700       5710

       230        240        250        260        270        280
AG      GGCGTCNGCTGATCTCGGCGCANGTCCNCTACAAACCTTTCGCTATGTGGTGCTGCCGCT
        :::::: ::::::::::::::: :::: : ::::::::::::::::::::::::::::
ECAE24 GGCGTCGGCTGATCTCGGCGCACGTCCACGACAAACCTTTCGCTATGTGGTGCTGCCGCT
     5720       5730       5740       5750       5760       5770

       290        300        310        320        330        340
AG      NGNTATCCCGGGTNTTGCCGCTGGCTCTATCTTTACCTTCTCACTCACACTGGGCGATTT
         : :::::::: ::::::::::::::::::::::::::::::::::::::::::::::::
ECAE24 GGCAATCCCGGGTATTGCCGCTGGCTCTATCTTTACCTTCTCACTCACACTGGGCGATTT
     5780       5790       5800       5810       5820       5830

       350        360        370        380        390        400
AG      TATCNTCCCNCATCTGGTTGGTCCTCCACGATATTTTTATCNGCAATATGGTTTATTCCC
        :::: :::: :: :::::::::::::: :::: ::::::: ::::::::::::::::::
ECAE24 TATCGTCCCGCAGCTGGTTGGTCCTCCAGGATA-TTTTATCGGCAATATGGTTTATTCCC
     5840       5850       5860       5870       5880       5890

       410        420        430        440        450        460
AG      ACCANGGGGCGATTGGCAATATGCCNATGGGNGTNGNNTTCANCCCTTNGTTGNCGATTA
        : :: :::::::::::::::::::: :::: :  :  :::: ::    : :: ::::::
ECAE24 AGCAGGGGGCGATTGGCAATATGCCGATGGCGGCGGCATTCACCC---TGGTGCCGATTA
     5900       5910       5920       5930       5940       5950

       470        480        490        500        510
AG      TTCCTCATCCNCACTGTTCCTGTCCGTTNCGTTNAAAACNTT
        :: ::::: : :::::: :::: :::: :::: ::: ::
ECAE24 TT-CTCAT-CGCACTGTACCTG-GCGTT-CGTGAAACGTCTG
         5960       5970       5980       5990
```

Figure 21 Comparison of sequences ag6 and *E. coli* A000241

The unedited sequence of ag6, determined by automated sequencing was submitted to a FASTA search of the EMBL database. The greatest similarity was with 3 *E. coli* sequences, of which one, A000241, is shown here.

```
SCORES      Init1:    75 Initn:    75 Opt:   138
  85.7% identity in 42 bp overlap
            2150       2140      2130       2120       2110       2100
pexcellmcs      AAGCTTACGCGTGCGGCCGCCGTGTCGGATCCTGAGACTGAATTCGGCCC
                     ||||| ||||||| || |||||||||||||||| ||||  |||
ag27  ANGGGCCTGCTNACTTAC-CGTGCGGNCG-CGTGTCGGATCCTGANACTGCGTTCCTTAC
                10         20         30         40         50
```

Figure 22 Identification of the multicloning site in sequence ag27

The sequences of ag27 and pExcell (Accession Number u13848) were compared using FASTA, from which a 42 nucleotide section was identified with 85.7% identity between the sequences.

FASTA and BLAST analysis provided identical results in each case, with three identical *E. coli* sequences AE000241, D90783 and D90784 resulted in high similarity, 90.7% over 845 nt for FASTA and 100% over 480 nt for BLAST (Figure 25 & 26). These sequences code for the potH gene and other genes involved in putrescine transport. The sequence was analysed at the protein level to confirm the results.

Sequences producing significant alignments:	Score (bits)	E Value
dbj|D90783|D90783 E.coli genomic DNA, Kohara clone #272(32.4-32...	1094	0.0
gb|AE000241|ECAE000241 Escherichia coli K-12 MG1655 section 131..	1094	0.0
dbj|D90784|D90784 E.coli genomic DNA, Kohara clone #273(32.5-32...	1094	0.0
gb|U79490|LLU79490 Lactococcus lactis cremoris putative O-acety...	40	0.45
emb|X66412|CRENOLM C.reinhardtii mRNA for enolase	38	1.8

Figure 23.BLASTN analysis of ag27
Database sequences showing significant alignment with sequence ag27 by BLASTN analysis.

Figure 24. Comparison of sequences ag27 and *E. coli* D90783.

The unedited sequence of ag6, determined by automated sequencing was submitted to a BLASTN search of the EMBL database. The greatest similarity was with 3 *E. coli* sequences, of which one, D90783, is shown here.

```
dbj|D90783|D90783 E.coli genomic DNA, Kohara clone #272(32.4-32.7 min.)
Length = 15399
Score = 1094 bits (552), Expect = 0.0
Identities = 665/692 (96%), Positives = 665/692 (96%), Gaps = 10/692 (1%)


Query: 45    actgcgttccttacattacctgcggtgggcggaaatacgctgtcaacttccgggctgggg 104
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 12709 actgcgttccttacattacctgcggtgggcggaaatacgctgtcaacttccgggctgggg 12768


Query: 105   cgctttctggtgtttctctatatctggttgccgttcatgatcctgcccgttcaggcggcg 164
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 12769 cgctttctggtgtttctctatatctggttgccgttcatgatcctgcccgttcaggcggcg 12828

Query: 165   cttgagcgtttgccgccgtcattgttgcaggcgtcggctgatctcggcgcacgtccacga 224
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 12829 cttgagcgtttgccgccgtcattgttgcaggcgtcggctgatctcggcgcacgtccacga 12888


Query: 225   caaacctttcgctatgtggtgctgccgctggcaatcccgggtattgccgctggctctatc 284
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 12889 caaacctttcgctatgtggtgctgccgctggcaatcccgggtattgccgctggctctatc 12948


Query: 285   tttaccttctcactcacactgggcgattttatcgtcccgcagctggttggtcctccagga 344
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 12949 tttaccttctcactcacactgggcgattttatcgtcccgcagctggttggtcctccagga 13008


Query: 345   tattttatcggcaatatggtttattcccagcaggggcgattggcaatatgccgatggcg 404
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 13009 tattttatcggcaatatggtttattcccagcaggggcgattggcaatatgccgatggcg 13068


Query: 405   gcggcattcaccctggtgccgattattctcatcgcactgtacctggcgttcgtgaaacgt 464
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 13069 gcggcattcaccctggtgccgattattctcatcgcactgtacctggcgttcgtgaaacgt 13128


Query: 465   ctgggagcgttcgatgcactctgaacgcgcaccgtttttcctcaaactggcggcctgggg 524
              ||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct: 13129 ctgggagcgttcgatgcactctgaacgcgcaccgtttttcctcaaactggcggcct-ggg 13187


Query: 525   gcggcgttgttttcctacatttttcccatcctgataatcccccgcctatgcgtttaacac 584
              |||||||||||||||||| |||||||||||||||| | ||||||||||||||||||||
Sbjct: 13188 gcggcgttgttttcctaca-ttttcccatcctgataat-cgccgcctatgcgtttaacac 13245


Query: 585   tgaaaat-ccgcgtttaatttttccaccgcaagggnctgacgctgcgctggtttaacctt 643
              |||| || | |||||| | |||||||||| |||| |||||||||||||||| | | |
Sbjct: 13246 tgaagatgcggcgttt-agttttccaccgc-agggcctgacgctgcgctggttt-agcgt 13302


Query: 644   ggcancacagcgtaantgatatccttgangccgtgacactgttcacttaaaattggcggn 703
              |||| |||||||| | |||||| ||||| |||||||||||| |||||| ||| ||||||
Sbjct: 13303 ggcagcacagcgt-agtgatattcttgatgccgtgacactg-tcactt-aaagtggcggc 13359


Query: 704   gctggcaanattaattgcnctggtgttangga 735
              |||||| | |||||||||| |||||||| |||
Sbjct: 13360 gctggcgacattaattgcgctggtgttaggga 13391
```

3.5.1.4 Sequence ag36

The sequence ag36 resulted from automated sequencing, with the T7 primer, of a clone of pUC18 containing the 3.5 kb insert of B41.21. The pExCell MCS lay too close to the primer to give determination of a readable sequence with certainty.

FASTA and BLAST analysis of the insert sequence again revealed almost identical results. BLAST showed high homology with *E. coli* X73026, X66836 and AE000374, at 97% over 371 nt. FASTA analysis revealed high homology with *E. coli* X66836, AE000374 and U28377 over 564 nt at 96.6%. Each of these sequences encode the genes iciA, rpiA and SerA.

This is the same pattern as was found with the sample ag19. No protein analysis was performed.

3.5.1.5 Sequence ag33

The sequence of this sample was generated from a PCR product amplified from pExCell B41.21 using primers designed in this study to amplify specifically the region flanking the pExCell MCS. The primers should result in a 484 bp product if no insert is present and a product greater than 484 bp if an insert is present. In this case the PCR product was 3.9 kb, as expected. For sequencing, the primer -40, which anneals to pExCell within the flanking region at residues 2217-2201 was used. The position of the putative *EcoRI* site at nt 101 of ag33 is shown in Figure 25; the MCS is clearly identified through to nt 2154 of pExCell. The equivalent of the *EcoRI* site again has AA replaced by CG. Analysis of the following insert sequence by FASTA and BLAST again revealed homology with *E. coli* ; BLAST analysis found 97% identity over 406 nt with AE00241, D90783 and D90784, whilst FASTA showed 98.3% identity with the same sequences over 358 nt. As expected from earlier sequence analysis the region of high homology encodes the potH gene.

Thus, samples ag6, 27 and 33 have similar sequences and all contain nt CG instead of AA at the *EcoRI* cloning site.

```
SCORES      Init1:   160 Initn:   160 Opt:   186
88.9% identity in 45 bp overlap

                2100        2110      2120       2130       2140       2150
pexcellmcs                   AAGCTTACGCGTGCGGCCGCCGTGTCGGATCCTGAGACTGAATTCG
                             |||||||||||||||||||||||||||||||||||  | ||||  |||
ag33       CCCNCCCAANANAAGGAAGCTTACGCGTGCGGCCGCCGTGTCGGATCCNNACACTGCGTTCC
                    10        20         30         40         50         60
```

Figure 25 Identification of the multicloning site in sequence ag33.

The sequences of ag33 and pExCell (Accession Number u13848) were compared using FASTA, from which a 45 nt section was identified with 88.9% identity between the sequences.

3.5.2 Nucleotide Sequence Analysis of B41.7

The partial nucleotide sequence of *P. salmonis* lambda clone B41.7 was also determined by automated sequencing with a range of DNA samples and primers. B41.7 contains an insert of 450 bp, this was sequenced to determine the insert origin and analysis of pExCell MCS.

3.5.2.1 Sequence ag26

Sample ag26 was sequenced directly from a pExCell Wizard SV plus miniprep using the -40 primer. The sequence was analysed to locate the pExCell MCS and *EcoRI* cloning site by FASTA alignment with the pExCell DNA Sequence. Sample ag26 matches the MCS from nt 39 to approximately approximately nt 90, corresponding approximately to nt 2154-2100 in pExCell. The ag26 sequence also matched pExCell at positions 530-740 corresponding to positions 2011-1891 of pExCell (Figure 26). Therefore, one can conclude that the sequence from nt 80 to 530 of sample ag26 is of insert origin.

BLAST analysis revealed 98% identity with 463 nt of *E. coli* sequences (EMBL Accession Numbers, AE000169, D90705 and D90704), which do not encode any known functional genes.

Table 11. Summary of nucleotide sequence analysis of *P. salmonis* clone B41.7 in lambda pExCell

Sequence	Origin	Sequencing primer	sequence at putative *EcoRI* site	Sequence similarity identified in BLAST search
ag26	B41.7 SV Plus prep.	-40	GAATGG	AE000169 98%, 463nt X65315 X65304
ag31	B41.7 PCR 1841f-2325r	-40	GAATNG	AE000169 91%, 151nt D90705 D90704
ag34	B41.7 PCR 1841f- 2325r	2325	GAATGG	AE000169 70%, 220nt D90705 D90704

3.5.2.2 Sequence ag31

The data from this sequence analysis was of a poorer quality than for sample ag26, this sample was a PCR product, amplified directly from pExCell B41.7 using primers flanking the pExCell MCS, nevertheless, the pExCell MCS site could be aligned to approximately positions 83-9, equivalent to positions 2114-2194 of pExCell (Figure 27).

BLAST analysis gave a similar result to the above with 91% identity over 151 nt for *E. coli* sequences AE000169, D90705 and D90704.

3.5.2.3 Sequence ag34

The sample for ag34, as for ag31 was a 450 bp PCR product of B41.7 and the sequencing primer was 2325r, as used in the PCR amplification. The resultant sequence was of poor quality but could still be analysed, with the pExCell MCS aligned to ag34 at nt 220-63 approximately, corresponding to positions 2114-2270 of pExCell, as seen below (Figure 28). Again, the sequence GAATGG (reverse of sequence CCATTC) was found at the position of the *EcoRI* site in the pExCell MCS.

BLAST results gave again a similar pattern to the previous two samples. 70% homology over 220 nt for the *E. coli* sequence

Other samples were sequenced including pExCell B41.2, which contains a 3.5 kb insert amplified from PCR. The MCS of pExCell was analysed and was GCGTTC. FASTA and BLAST analysis on this sample concluded 95.6 % similarity over 371 nt with AE000241, D90783 AND D90784. Similar results were obtained with BLAST analysis, 95% over 418 nt with the above accession numbers. These results are remarkably similar to BLAST and FASTA analysis of ag6, ag27 and ag33, these sample are analysis of B41.21 which also contains a 3.5 kb nsert.

The unsucessful transformation of B41.21 into pUC18, which resulted in partial inserts in more than one sample was also sequenced to determine the origin of the insert. BLAST analysis of this sample showed the highest degree of similarity to Lambda cloning vectors, this sample was not analysed further.

```
SCORES      Init1:   495 Initn:   575 Opt:   814
  89.9% identity in 218 bp overlap

                2139      2129      2119      2109      2099      2089
u13848.gb_sy GTGCGGCCGCCGTGTCGGATCCTGAGACTGAATTCGGCCCTCGAGGCCTGTATTCTATAG
                                          | ||||||||||||  ||||||||||||||||
ag26           CGGANAATGTCTATTTGCGTCGTGCTTTCAATTTCGGCCCTCGAAGCCTGTATTCTATAG
             500       510       520       530       540       550

                2079      2069      2059      2049      2039      2029
u13848.gb_sy TGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTAT
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ag26           TGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTAT
             560       570       580       590       600       610

                2019      2009      1999      1989      1979      1969
u13848.gb_sy CCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCC
             |||||||| ||||| ||| |||||||| || ||| |||||| ||| |||||||||| |
ag26           CCGCTCACNATTCC-CACNACATACGAACCNGAAACATAAA-TGTTTAGCCTGGGGTG-C
             620       630       640       650       660       670

                1959      1949      1939      1929      1919      1909
u13848.gb_sy TAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGA
             |||||||||| || |||| |||||||||| |||||||||||| || ||||||  || |||
ag26           TAATGAGTGA-CT-ACTCNCATTAATTGC-TTGCGCTCACTGNCC-CTTTCCCATCNGGA
              680       690       700       710       720       730

                1899      1889      1879      1869      1859      1849
u13848.gb_sy AACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGT
             || |||||
ag26           AA-CTGTCTNCCACTGCATTATGAAACCGGCCACCCCCGGGAAAAGNGGTTGCNTTTTNG
                   740       750       760       770       780       790
```

Figure 26 Identification of the multicloning site in sequence ag26.

The sequences of ag26 and pExCell (Accession Number u13848) were compared using FASTA, from which a 218 nucleotide section was identified with 89.9 % identity between the sequences.

```
SCORES      Init1:   173 Initn:   224 Opt:   208
  73.8% identity in 80 bp overlap

                  109       99        89        79        69        59
ag31           GACGAAGAAAGNGAGGCTTTCCNGAGACNATTCAGTCTCAGGATCCGACNCGNCNCCCC
                                                 ||||||||||||||||||| || |  ||
u13848         ACACTATAGAATACAGGCCTCGAGGCCGAATTCAGTCTCAGGATCCGACACGGCGGCCG
                    2090      2100      2110      2120      2130      2140

                   49        39        29        19         9
ag31           CANNCGTTAGCTTCCCCATNCTGACTCGTCTCA-TCAA---ACNGGCCCTANAAANAT
               ||  ||| |||||||| ||  ||| |||| | |  |||   || |||| |
u13848         CACGCGTAAGCTTCCCTATAGTGAGTCGTATTACGCAATTCACTGGCCGTCGTTTTACAA
                    2150      2160      2170      2180      2190      2200
```

Figure 27 Identification of the multicloning site in sequence ag31

The sequences of ag31 and pExCell (Accession Number u13848) were compared using FASTA, from which a 80 nucleotide section was identified with 73.8 % identity between the sequences.

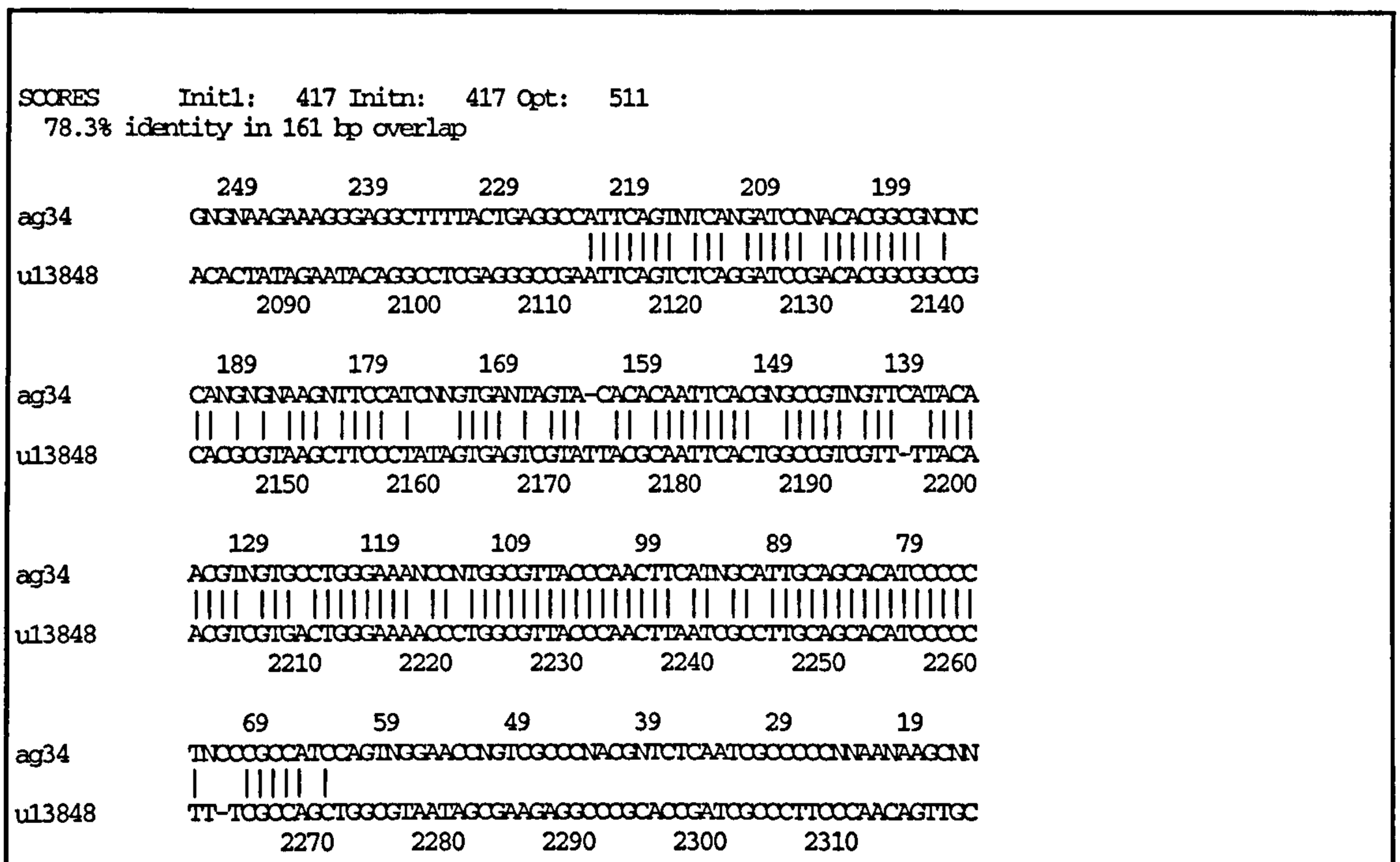


Figure 28 Identification of the multicloning site in sequence ag34.

The sequences of ag34 and pExCell (Accession Number u13848) were compared using FASTA, from which a 161 nucleotide section was identified with 78.3 % identity between the sequences.

CHAPTER FOUR: *PISCIRICKETTSIA SALMONIS* ARDINTOUL

4. 1. Growth of *P. salmonis* Ardintoul in tissue culture

4.1.1 Introduction

In the course of this study an outbreak of a disease with symptoms similar to those of piscirickettsiosis was detected by Marine Harvest MacConnell (Grant *et al.*, 1996). Two sea loch sites stocked with smolts in April 1995 experienced an outbreak of disease in November 1995 when water temperatures had fallen to 11°C. Abnormal swimming, skin damage and increased mortalities were noted. Histopathology showed few obvious changes except for splenomegaly. However, histopathological changes were evident in the brain with basophilic intracellular coccoid bodies associated with areas of encephalitis and vasculitis. From explants of brain tissue cytopathic effects were caused in CHSE cells within 9 days. The RLO harvested after passage in CHSE-214 cells reproduced the disease in salmon smolts, from which the organism could again be recovered in tissue culture. This new RLO isolate was called Ardintoul after the site of origin. To confirm the relatedness of this isolate to LF89 further work was undertaken in this laboratory.

4.1.2 Growth in tissue culture

The Ardintoul isolate was supplied by Marine Harvest MacConnell growing in CHSE-214 cells. Growth in tissue culture was monitored by light microscopy on a regular basis. The same cytopathic effects were seen in CHSE-214 cells with the new isolate, i.e. the cells rounded up, vacuoles were apparent in the cytoplasm after 3-5 days, and bacteria were visible within these vacuoles (Figure 29 and 30). Eventually the monolayer was completely destroyed by the damage caused by the bacteria. The new isolate was maintained in CHSE-214 cells for several passages to build up a large stock of infected material for analysis.

4.1.3 Electron microscopy

To confirm the nature of the organism electron microscopy was performed on infected cell culture supernatant harvested from a flask of cells showing confluent lysis, (Figures 31 and 32). These showed clusters of organisms within vacuoles in degenerated cells. The organism was coccoid, approximately 1µm in diameter, and had

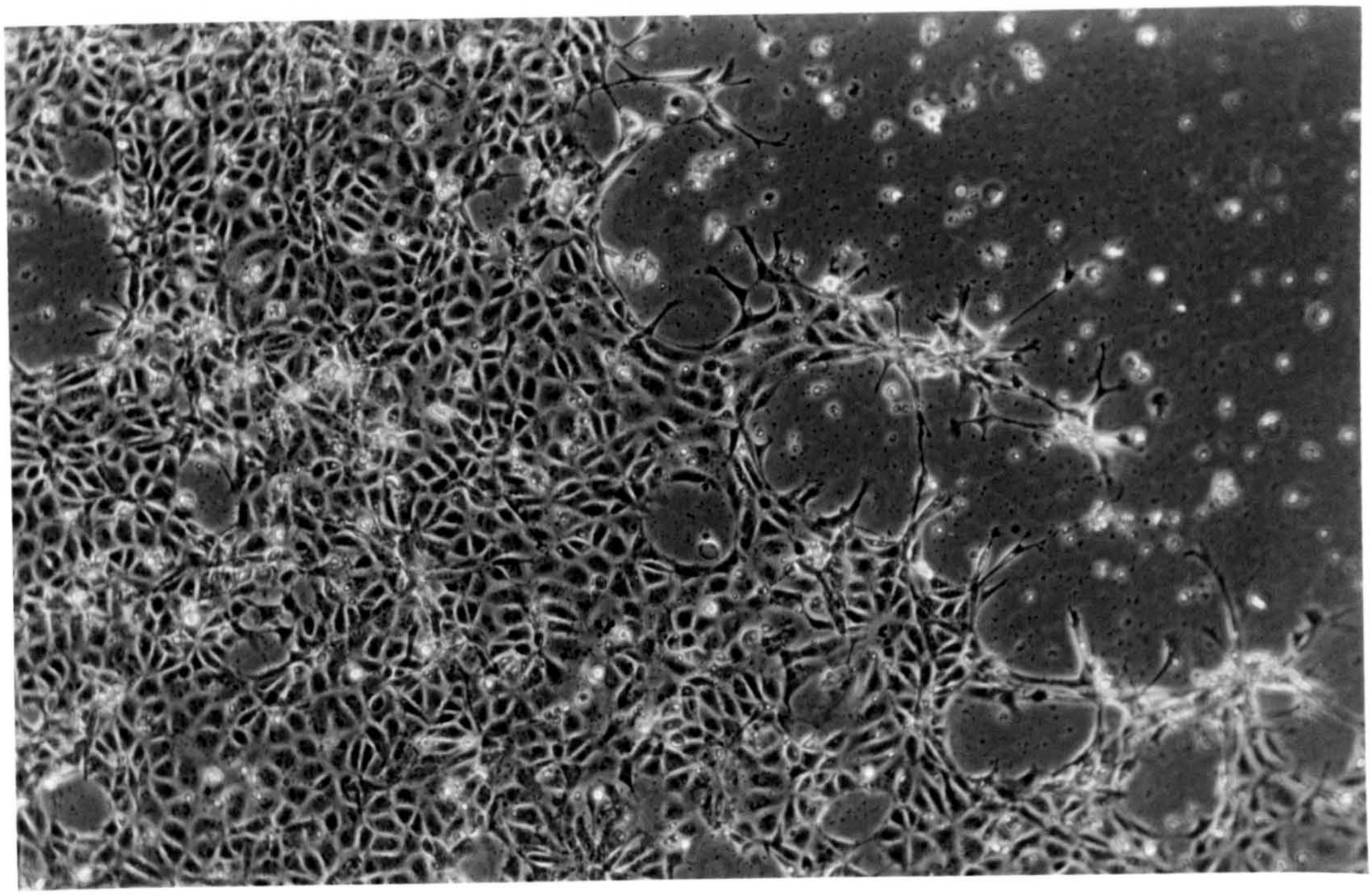


Figure 29 CHSE cells infected with *P. salmonis* Ardintoul viewed by light microscopy
Cytopathic effect produced by *P. salmonis* Ardintoul isolated from the infected brain of a salmon displaying typical diseases signs, in CHSE cells. Day 6 post-infection. Phase contrast, x40 magnification.

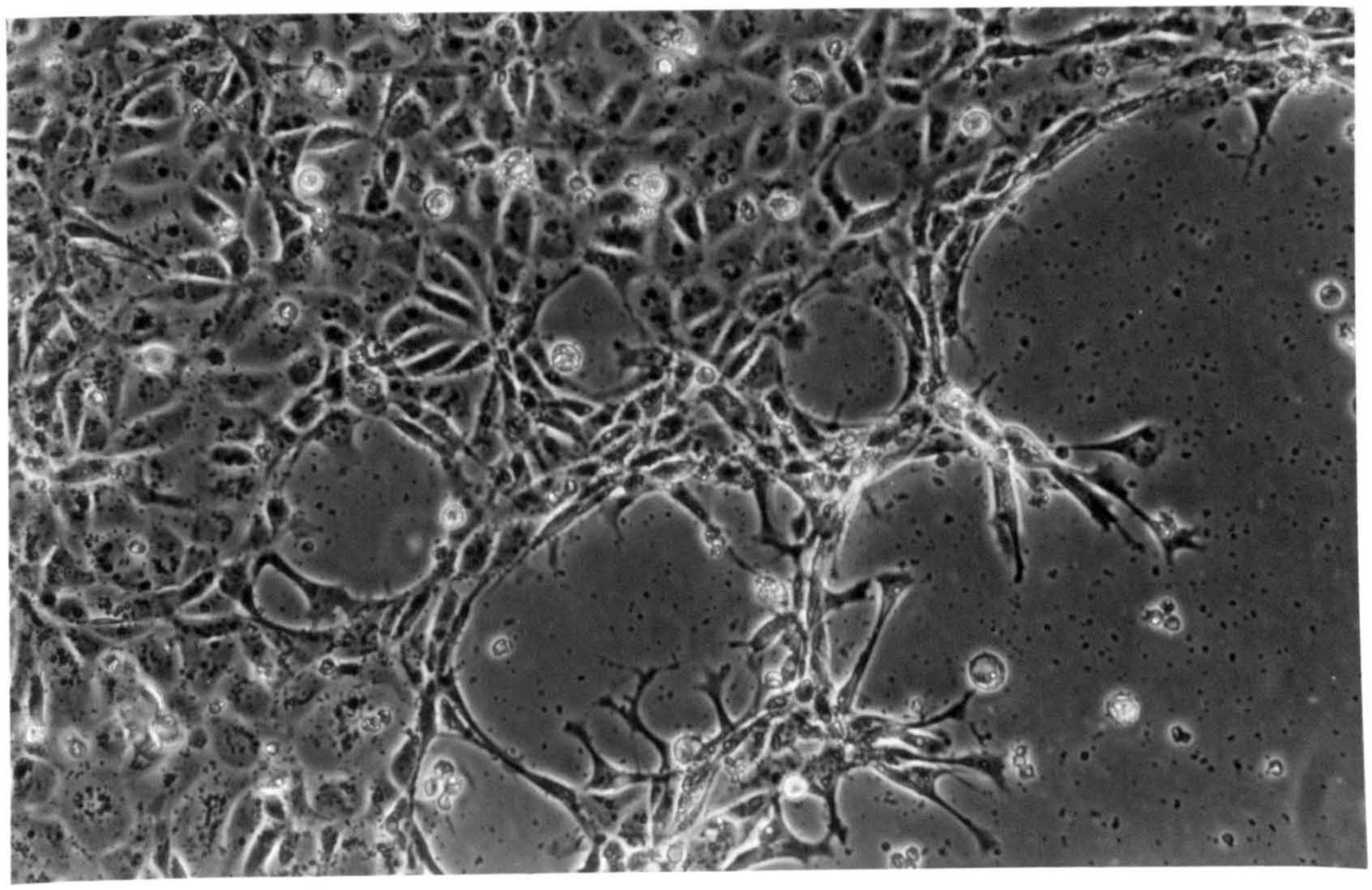


Figure 30 CHSE cells infected with *P. salmonis* Ardintoul viewed by light microscopy
Cytopathic effect produced by *P. salmonis* Ardintoul isolated from the infected brain of a salmon displaying typical diseases signs, in CHSE cells. Day 8 post-infection. Phase contrast, x100 magnification.

a characteristically rippled outer membrane. In Figure 31 a cell undergoing division can be seen.

4.1.4 Optimal conditions for growth in tissue culture

To determine the optimal temperature for growth the experiment described above (3.1.3) for *P. salmonis* LF89 was repeated with infected and uninfected CHSE cells in 25 cm^2 sterile, tissue culture flasks incubated at 15, 17 and 20°C. Growth was monitored by light microscopy on a daily basis; as before, 17°C was considered the optimum incubation temperature to support productive infection of the cells.

4.1.5 Growth of Ardintoul RLO in XTC cells

The Ardintoul RLO was passaged routinely in CHSE cells but it was desirable to be able to culture the agent in other cells for future work, e.g. immunoblotting, and an aliquot of infected supernatant was filtered (4.5 µm) and transferred to monolayers of XTC cells at 17°C. XTC cells are derived from frogs (Rokaw, 1996). The supernatant after 21 days was transferred to a fresh monolayer of XTC cells and the procedure repeated. Growth was monitored by light microscopy and the organism caused cytopathic effects in these cells, although fewer organisms were visible in the supernatant of infected cultures.

4.2 16S rRNA gene sequence of the Ardintoul agent

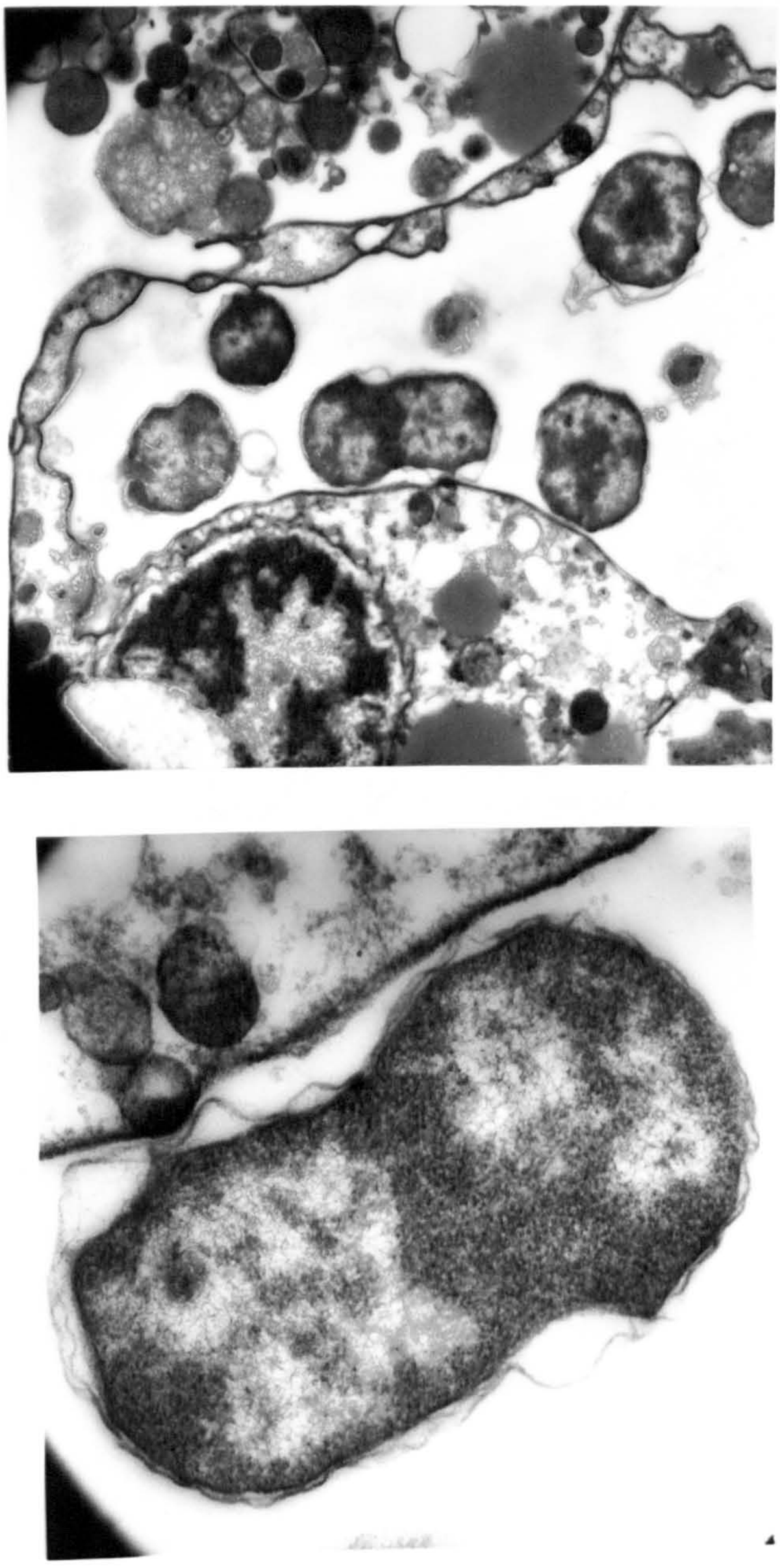
4.2.1 Determination of 16S rRNA gene sequence

To determine the degree of relatedness of the Ardintoul agent to other recognised piscirickettsia the sequence of the 16S rRNA gene was determined. DNA was extracted from infected CHSE cells and used, either directly, or after cloning into pUC18, for automated sequence analysis.

Sequence data was assembled manually using the program SEQMAN to generate contigs and the final sequence is shown in Appendix 3.

4.2.2 Comparison of 16S rRNA sequence with that of other rickettsia

The above sequence was subjected to a FASTA search for comparison with sequences in the EMBL database. As expected, this showed a high degree of identity with other piscirickettsial sequences (Table 12), the closest similarity being with *P. salmonis*



A

B

Figure 31. Electron microscopy of CHSE cells infected with *P. salmonis* Ardintoul isolate.

A number of organisms can be seen within membrane bound vacoules, with one organsim appearing to divide by binary fission.

Magnification = x 13,200. Scale 12 mm = 0.6 µm. Photo A

Magnification = x 150, 000. Scale 12 mm = 0.15 µm. Photo B

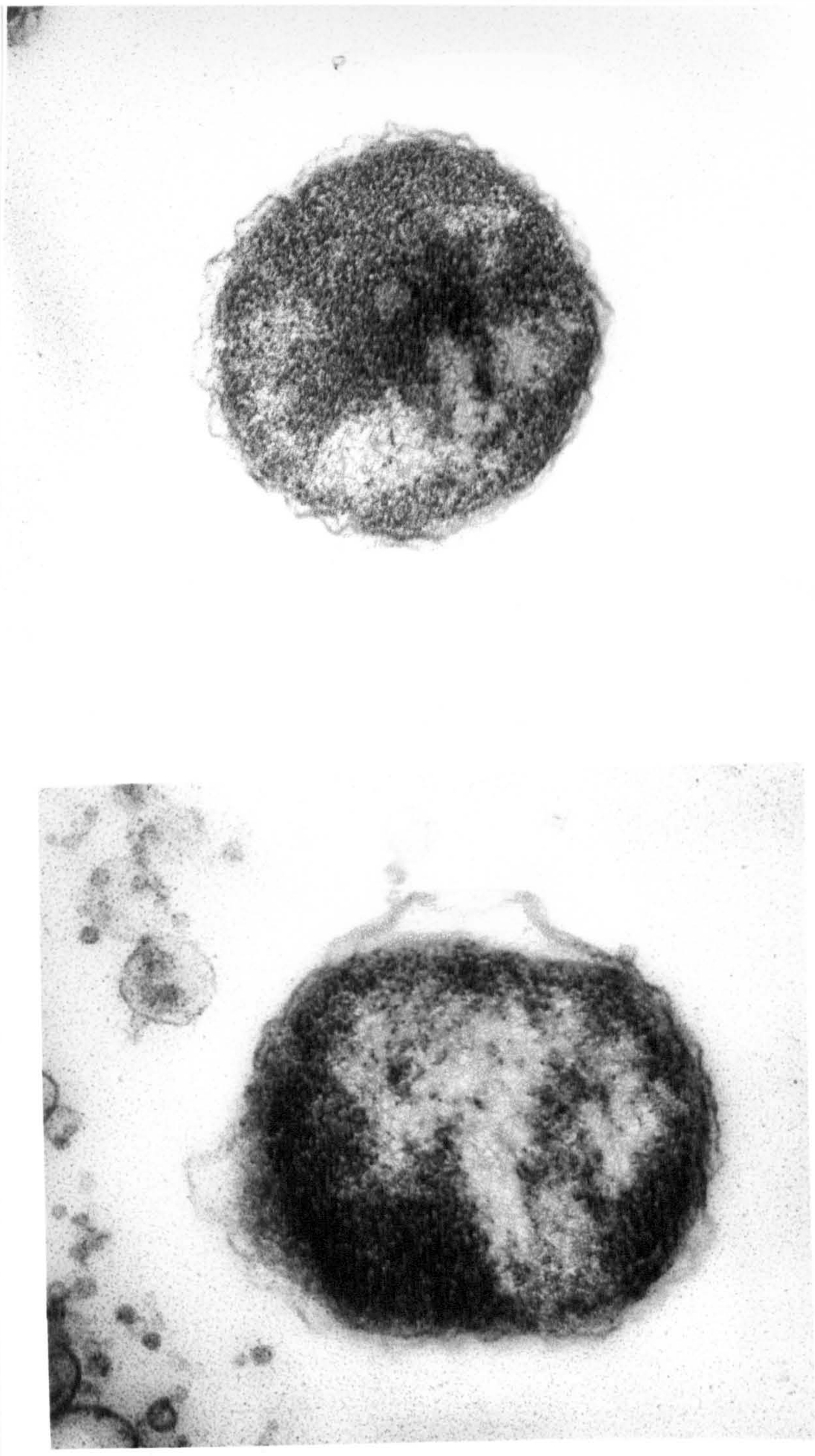


Figure 32.Transmission electron micrograph of a cell of *P. salmonis* Ardintoul isolate.

An undulating outer cell membrane and electron dense area connected by fibrillar strands are visible, in each case.

Magnification = x 150,000 14 mm = 0.15 μm.

Database: Non-redundant GenBank+EMBL+DDBJ+PDB sequences
360,863 sequences; 796,746,568 total letters.
Searching.....:.....done

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
gb|U55015|PSU55015	Piscirickettsia salmonis 16S rRNA ...	7580	0.0	1
dbj|AB013828|AB013828	Pseudomonas sp. 16S rRNA gene, clo...	5018	0.0	3
gb|U77480|REU77480	Ridgeia piscesae endosymbiont 16S ...	4901	0.0	2
gb|U77478|REU77478	Riftia pachyptila endosymbiont 16S...	4810	0.0	2
gb|U77481|LEU77481	*Lamellibrachia columna* endosymbion...	4797	0.0	2
gb|U36941|PSU36941	Piscirickettsia salmonis LF-89 16S...	4590	0.0	4
gb|U36942|PSU36942	Piscirickettsia salmonis NOR-92 16...	4338	0.0	3
gb|M99451|RIT16SRRNA	Riftia pachyptila trophosome symbi...	4309	0.0	2
gb|U77479|LEU77479	Lamellibrachia sp. endosymbiont 16...	4044	0.0	4
gb|AF068008|AF068008	Stenotrophomonas maltophilia strai...	4008	0.0	3
emb|X84979|COS16SR	C.orbicularis symbiont 16S rRNA gene	3550	0.0	3
emb|X95229|LNS16SRRN	Lucina nassula gill symbiont 16S r...	3514	0.0	3
gb|U77482|EEU77482	Escarpia spicata endosymbiont 16S ...	3121	0.0	5
gb|AF026979|	Unidentified beta proteobacterium ...	2959	0.0	4
gb|AF035724|AF035724	Calyptogena phaseoliformis gill sy...	2929	0.0	2
gb|AF035722|AF035722	Calyptogena sp. Florida gill symbi...	2920	0.0	2
emb|Y11150|PGY11150	Pseudomonas graminis sp. nov. 16S ...	2903	0.0	4
gb|AF035721|AF035721	Calyptogena magnifica gill symbion...	2892	0.0	2
dbj|D84020|PSEIAM19	Pseudomonas putida 16S rRNA gene, ...	2891	0.0	4
dbj|D84004|FLSIAM03	Flavimonas oryzihabitans 16S rRNA ...	2891	0.0	4
dbj|AB005655|AB005655	Unidentified gamma proteobacterium...	2888	0.0	3
gb|AF064458|AF064458	Pseudomonas monteilii 16S ribosoma...	2885	0.0	5
gb|U85843|CSU85843	Colwellia sp. (strain ACAM 605) 16...	2882	0.0	2
gb|U85842|CSU85842	Colwellia psychroerythrus IC064 16...	2879	0.0	3
gb|U85841|CSU85841	Colwellia psychroerythrus ACAM 604...	2879	0.0	2
gb|AF064459|AF064459	Pseudomonas rhodesiae 16S ribosoma...	2871	0.0	4
gb|AF074383|AF074383	Pseudomonas migulae 16S ribosomal ...	2868	0.0	4
gb|AF064461|AF064461	Pseudomonas cedrella 16S ribosomal...	2868	0.0	4
gb|AF058286|AF058286	Pseudomonas mandelii 16S ribosomal...	2868	0.0	4
dbj|D84025|PSEIAM24	Pseudomonas *syrxantha* 16S rRNA gen...	2868	0.0	4
dbj|AB015254|AB015254	Unidentified gamma proteobacterium...	2867	0.0	3
gb|AF068259|AF068259	Pseudomonas jessenii 16S ribosomal...	2863	0.0	4
gb|U61848|MCU61848	Marinobacter sp. CAB 16S ribosomal...	2862	0.0	3
dbj|D84013|PSEIAM12	Pseudomonas fluorescens 16S rRNA g...	2862	0.0	4
gb|AF057645|AF057645	Pseudomonas libaniensis 16S riboso...	2859	0.0	4
emb|Z76659|PCZ76659	P.citronellolis 16S rRNA gene	2859	0.0	4
dbj|D84009|PSEIAM08	Pseudomonas azotoformans 16S rRNA ...	2858	0.0	5
emb|X87271|AH16S3018	A.hydrophila 16S rRNA gene	2854	0.0	4
gb|AF074384|AF074384	Pseudomonas gessardii 16S ribosoma...	2853	0.0	4
gb|AF064460|AF064460	Pseudomonas veronii 16S ribosomal ...	2853	0.0	4
gb|AF064457|AF064457	Pseudomonas orientalis 16S ribosom...	2850	0.0	4
emb|AJ000215|SBAJ215	Shewanella baltica OS155 16S rRNA ...	2850	0.0	4
emb|X81623|SPRRNA16S	S.putrefaciens 16S rRNA gene	2848	0.0	4
gb|AF072688|AF072688	Pseudomonas mosselii 16S ribosomal...	2847	0.0	5
dbj|D84011|PSEIAM10	Pseudomonas chlororaphis 16S rRNA ...	2846	0.0	4
emb|Z76671|PVZ76671	P.viridiflava 16S rRNA gene	2842	0.0	4
emb|AJ000216|SBAJ216	Shewanella baltica 16S rRNA gene	2838	0.0	4
gb|M35016|DIHRRDA	Dichelobacter nodosus 16S ribosoma...	2838	0.0	5
dbj|D84002|CH3IAM01	Chryseomonas luteola 16S rRNA gene...	2831	0.0	4
emb|AJ000214|SBAJ214	Shewanella baltica NCTC10735 16S r...	2828	0.0	4
emb|Z76662|PFZ76662	P.fluorescens 16S rRNA gene	2828	0.0	4
dbj|D84027|PSEIAM26	Pseudomonas taetrolens 16S rRNA ge...	2826	0.0	4
emb|X81621|SH16RRNAB	S.alga 16S rRNA gene (Bry)	2821	0.0	4
dbj|AB013829|AB013829	Pseudomonas sp. 16S rRNA gene, clo...	2816	0.0	4
gb|AF063219|AF063219	Pseudomonas stutzeri 16S ribosomal...	2814	0.0	4
emb|Z76657|PCZ76657	P.chlororaphis (strain LMG 5004T) ...	2813	0.0	4
emb|Z76669|PSZ76669	P.syringae 16S rRNA gene	2813	0.0	4
gb|AF005249|	Shewanella alga 16S ribosomal RNA ...	2812	0.0	4
emb|X81622|SH16RRNAF	S.alga 16S rRNA gene (FeRed)	2812	0.0	4
emb|AJ002805|PSPAJ2805	Pseudomonas sp. 16S rRNA gene, iso...	2810	0.0	4

Table 12 FASTA search Ardintoul and EMBL sequences

FASTA analysis was performed using the GCG Software Package, as described elsewhere. The greatest similarities occur with other *P. salmonis* isolates.

SLGO-94. In addition to the piscirickettsiae there was close similarity with a number of endosymbiotic bacteria, such as those of *Ridgeia piscesae* and *Riftia pachyptila* (Table 12).

PILEUP analysis of the 16S rRNA gene sequences of the Ardintoul agent with those of recognised piscirickettsia is also shown in Appendix 3 and indicates that, compared to the Ardintoul sequence, there were differences in 9 bases with SLGO-94 and LF-89 strains, 16 differences with NOR-92 and ATL-4.91 strains, and 48 differences with strain EM-90. The position at which differences occur is summarised in Table 13 The comparative sequence data allowed the generation of a PAUP tree showing the relatedness between these bacteria (Figure 33), and it is clear that the Ardintoul agent is distinct from previously identified strains of *P. salmonis*.

Base number	Ardintoul	SLGO-94	LF-89	NOR-92	ATL 4.91	EM-90
12	C		A	A		
201	A	T	T			
258	G					
269	G				•	
374	G					A
385	C					A
386	A					•
387	G					•
390	A					•
391	T					C
392	G					A
426	G					T
445	•				G	•
503	A				•	
519	C					G
537	T					C
612	C			A		
676	G					
702	T	C	C	C	C	•
715	G					C
724	G					C
733	T	C	C	C	• C	• C
873	A					
874	C			•	•	
876	G	C		C	• C	C
890	G					
891	G			•		
894	G			C	C	C
910	A			C	C	C
911	A					
912	G					•
914	A					•
916	T					G
918	G					A
919	A	T	T	T	G	T
929	G	C	•	C	C	• C
930	C					
931	A					A
932	C					C
934	A					A
957	T					G
958	C					•
959	G					•
960	A					C
961	T					G
962	G					A
1002	T		•	•		T
1004	C					G
1005	T					T
1006	G		•			G
1007	C				•	A
1014	G					T
1015	C					A
1016	G					T
1019	A					T
1095	G					T
1135	T			•		C
1403	T	C				A

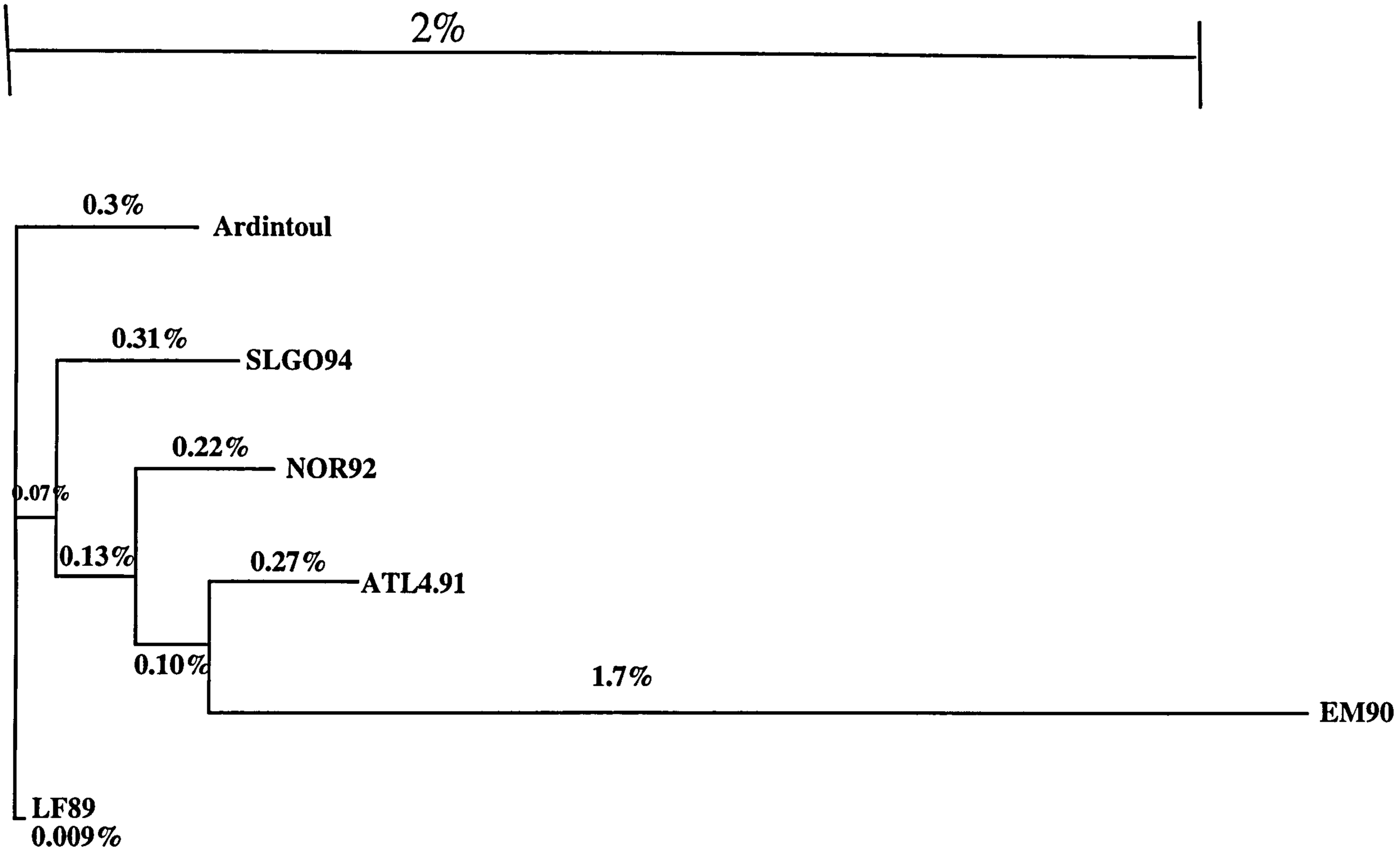


Figure 33 PAUP figure showing phylogenetic relationship between 16S rRNA genes of *P. salmonis* isolates

Phylogenetic tree assembled using PAUP analysis, software obtained from GCG Software Package, Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wis. The tree is heuristic based on distance with minimum evolution. The scale bar indicates the distance equivalent to % differences.The accession numbers of each isolates are listed below and were obtained from the EMBL database, with the exception of Ardintoul. ARD = Ardintoul isolate, not publihed. SLGO94 = U55015. NOR92 = U36942. ATL4.91 = U36915. EM90 = U36940. LF89 = U36941. Raw data used to draw figure is found in Appendix 6b.

4.3 Preparation and analysis of a *P. salmonis* genomic library in λFixII

4.3.1 Preparation of a *P. salmonis* library in λFixII

Purified *P. salmonis* Ardintoul bacteria were prepared (Section 2.5), the DNA extracted by the CTAB method (Section 2.6) and 2µg DNA was partially digested with *Sau3AI* to generate compatible ends for cloning into the vector λFixII (Figure 35), the overhanging ends were then partially filled-in to generate 3' AG 5' overhangs, Figure 36.

Ligation, with 2 µg insert DNA and 1 µg λFixII vector, and packaging to form infective lambda recombinants were performed, along with control ligations with vector alone (1 µg) and vector (1 µg) plus control insert (pMF, 0.3 µg). The ligations were packaged in *E. coli* strains XL1-Blue MRA and XLI-Blue MRA P2. λFixII is a replacement vector which is sensitive to P2 inhibition. Lambda phages containing active *red* and *gam* genes are able to grow on lysogenic strains, XLI-Blue MRA P2 is a lysogenic strain. The *red* and *gam* genes in λFixII are located on the stuffer fragment, therefore wild type λFixII cannot grow on the lysogenic strain. When the stuffer is replaced with an insert the recombinant λFixII becomes $red^{-}gam^{-}$, and the phage grows on the lysogenic strain. By plating the library on XL1-Blue MRA (P2) only recombinant phages grow, the XL1-Blue MRA strain is provided as a control. A control packaging reaction was also set up to test the efficiency using an extract of the *E. coli* strain VCS257 with wild-type λcI857 Sam7. Part of each packaging mixture was immediately titrated and the remaining material stored at 4°C. The results of the phage titrations are shown in Table 14. The packaging efficiency of wild-type lambda with *E. coli* strain VCS257 was 1 x 10^8 recombinants per µg DNA, comparable to that expected from the manufacturer (>10^8 recombinants per µg DNA). Similarly, the number of plaques generated with the ligated test insert was 1.75 x 10^7 recombinants per µg DNA, compared to that expected by the manufacturer (5 x 10^6 recombinants per µg DNA). The transfection efficiency, calculated from the number of plaques at the 10^{-}

^{1} dilution was 2.75 x 10^{5} recombinants per µg DNA; this compares well with other published results for recombinant libraries from rickettsia.

4.3.2 Screening the *P. salmonis* λFixII library

A portion of the packaged *P. salmonis* genomic library in λFixII was plaque assayed (20 µl), the resultant plaques from 5 x 150 mm plates were transferred to Hybond N+ membranes, according to manufacturer's instructions.

These were numbered 1-5, and hybridisations were performed at 68°C overnight with a variety of probes and washed with a medium stringency of 0.5 x. Results of screening are shown in Table 15.

21 positive plaques resulted from screening plate 2 with the 16S DIG-labelled probe, and membranes 2 and 3, previously probed with DIG-16S rRNA probe were stripped of DIG-labelled 16S probe and reprobed with DIG-MIP, resulting in 3 positive plaques, named MIP 1-3. Each positive plaque was individually cored and numbered accordingly.

4.3.3 Secondary screening

A selection of plaques resulting from the primary screening were assayed and hybridised with their respective probes to eliminate any false positives. The results of secondary screening (Table 16) indicate that all samples were false positives except MIP3 which generated a further 6 positive plaques, numbered 1-9, subsequently analysed by tertiary screening.

4.3.4 Tertiary screening

Phage were isolated from MIP-positive plaques 4, 5 and 6 from secondary screening, assayed and probed with DIG-MIP at a constant hybridisation temperature of 68°C; the stringency was varied from low (1x, MIP4), to medium (0.5x, MIP5) and high (0.1x, MIP6). No hybridisation was found at any stringency. Positive controls with 1 or 10 µl volumes of *L. pneumophila* DNA at dilutions of 1/25 and neat MIP-PCR product were tested and all spots gave strong positive reactions after 1 h, results not shown. Thus, the sensitivity of the reaction was adequate to detect MIP-DNA in the form of the PCR

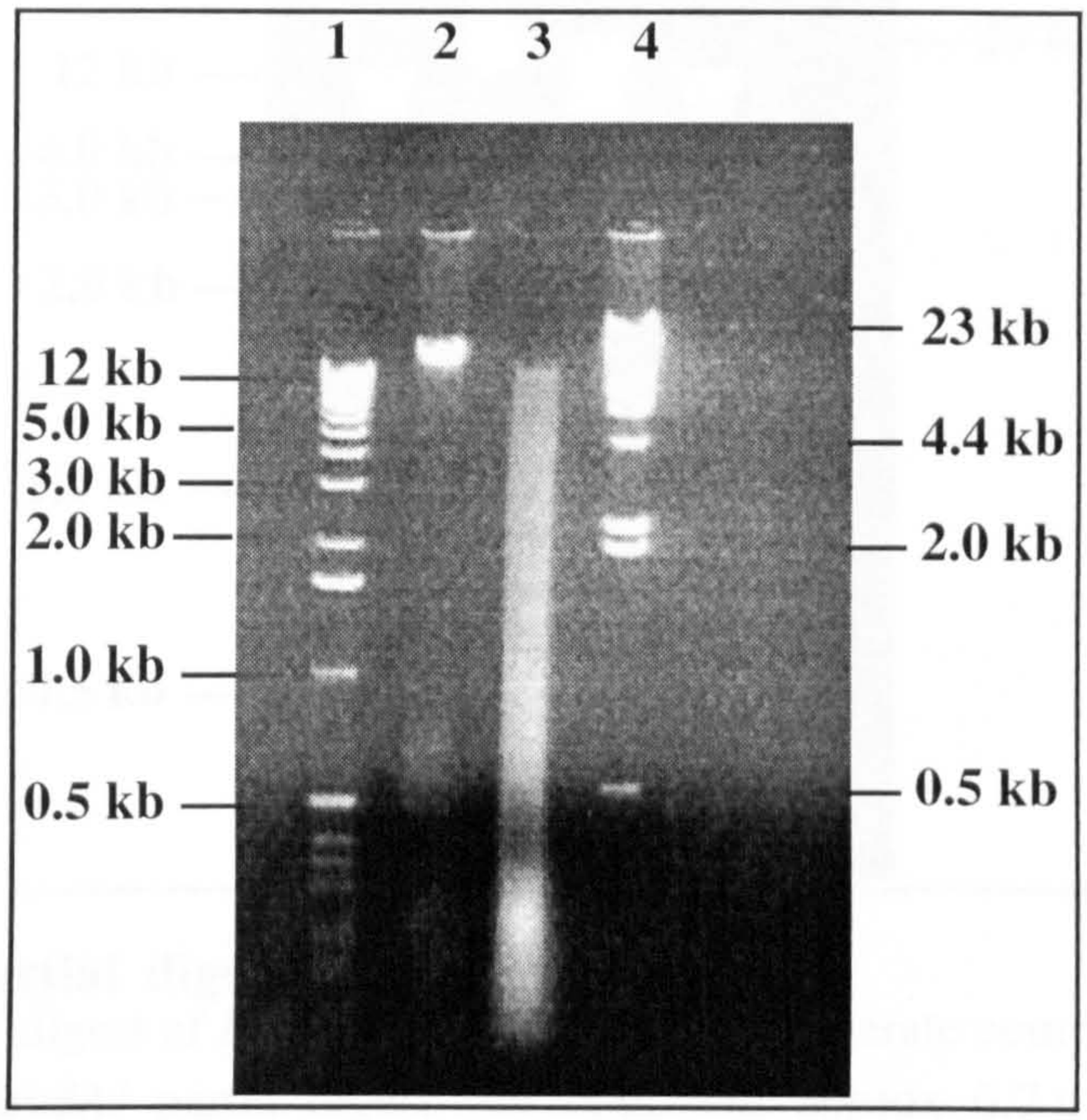


Figure 34 Ardintoul extracted DNA

P.salmonis isolated from tissue culture material by differential centrifugation and isolation on a Percoll gradient, resulted in two bands an upper and lower. Upper composed of tissue culture debris and lower composed of bacteria. Both bands underwent CTAB genomic prep. Concentration of *P.salmonis* equivalent to 80 ng/µl therefore got 10 µl = 800 ng, together with previous prep which generated 1600 ng, adding both together gives a total of 2.4 µg, conclude approx. 2 µg to allow for errors in estimation.

Lane 1	KB ladder
Lane 2	*P.salmonis* Ard isolate CTAB-genomic prep
Lane 3	Upper band CTAB genomic prep (tissue culture material degraded)
Lane 4	λHindIII (to determine concentration of prep)

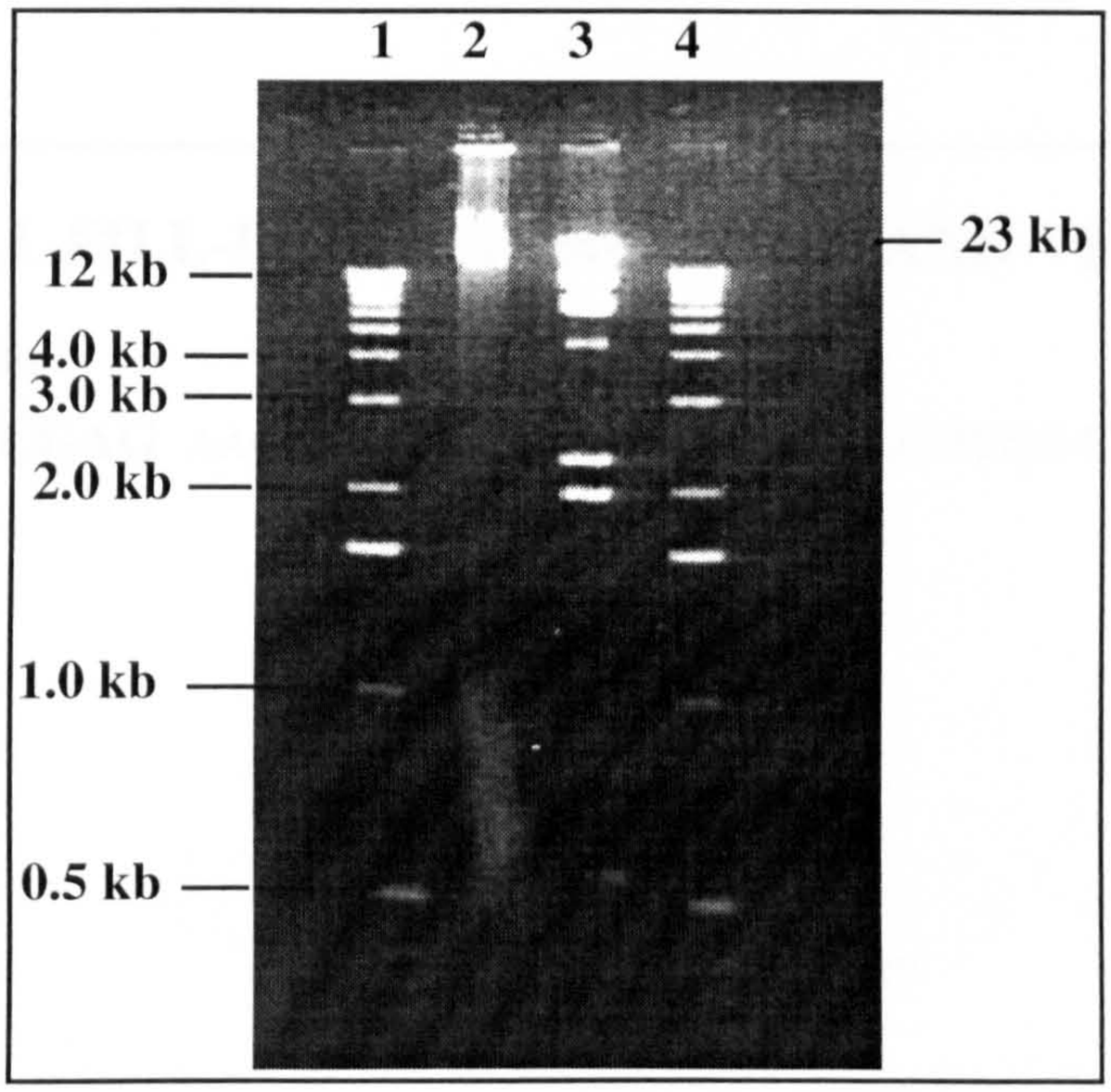


Figure 35 Partial digest of Ardintoul DNA

Partial *Sau3AI* digest of *P.salmonis* Ard isolate to generate compatible ends for cloning. Ard *Sau3AI* partial digest was set up with approx. 0.7 µg of Ard CTAB-genomic prep and digested for 5, 10 and 20 min at 37°C. The three digests were pooled to give 30 µl, 1 µl was analysed to check digestion and the remaining 29 µl was phenol extracted and EtOH precipitated and resuspended in 10 µl of dH_2O.

Lane 1 KB ladder
Lane 2 Ard *Sau3AI* partial digest.
Lane 3 λHindIII
Lane 4 KB ladder

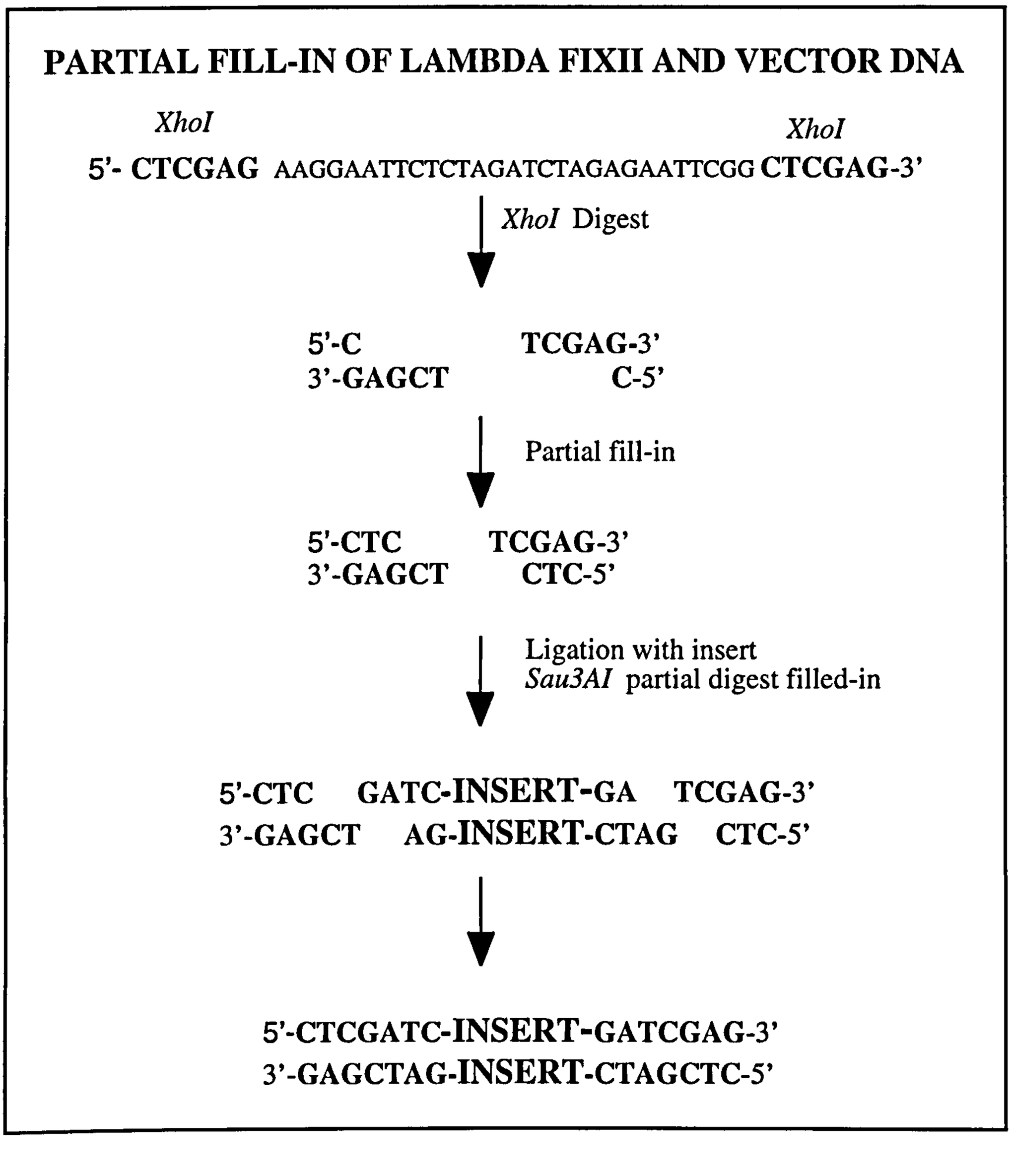


Figure 36 Schematic to show digestion and partial fill-in of λFixII and insert DNA.
The final ligated insert plus vector is shown at the bottom, resulting in the removal of two *XhoI*, *EcoRI* and *XbaI* restriction sites from the MCS of lambda FixII.

Dilution of packaging mixture	Number of colonies	
λFixII	XL1-Blue MRA P2	XL1-Blue MRA
neat	0	60
10^{-1}	0	18
10^{-2}	0	1
10^{-3}	0	0
10^{-4}	0	0
λFixII + pMF		
neat	TMTC	TMTC
10^{-1}	TMTC	TMTC
10^{-2}	105	90
10^{-3}	12	8
10^{-4}	1	0
λFixII + Ard		
neat	86	74
10^{-1}	11	12
10^{-2}	0	1
10^{-3}	0	0
λcI857	VCS257	
neat	TMTC	
10^{-1}	TMTC	
10^{-2}	TMTC	
10^{-3}	0	
10^{-4}	0	

Table 14. Preparation of a *P. salmonis* recombinant library in λFixII
The number of plaques is shown for each dilution of the packaged plaque assayed.
Control packaging reactions are expected to yield greater than 1×10^8 plaques per μg; value found = 1×10^8 plaques per μg.
The control vector plus test insert is expected to yield 5×10^6 recombinants per μg; value found = 1.75×10^6 recombinants per μg.
TMTC = too many to count

MEMBRANE	PROBE	POSITIVES	NAME
1	MIP	-	-
2	16S	21	2.1-21
2	MIP	3	MIP1-3
3	16S	-	-
3	MIP	-	-
4	16S	-	-
5	3.5 KB	8	3.5.1-8

Table 15 Summary of Primary Screening

The membranes were numbered 1-5 and probed with different probes, the resulting positives are recorded.

PLAQUE	PROBE	NUMBER OF POSITIVE PLAQUES
MIP	MIP	0
MIP2	MIP	0
MIP3	MIP	6
2.1	16S	0
2.10	16S	0
2.21	16S	0
3.5.1	B41.21 3.5 kB	0
3.5.5	B41.21 3.5 kb	0
3.5.8	B41.21 3.5 kb	0

Table 16. Secondary screening of *P. salmonis* recombinant library in λFixII

The origin of each plaque and the number of positives plaques generated by hybridisation at 68°C overnight with a medium stringency are shown.

PROBE	NUMBER OF POSITIVE
MIP	
PRIMARY SCREEN	3
SECONDARY SCREEN	6
TERTIARY SCREEN	0 (only 3 analysed)
16S	
PRIMARY SCREEN	21
SECONDARY SCREEN	0 (only 3 analysed)
3.5 KB	
PRIMARY SCREEN	8
SECONDARY SCREEN	0 (only 3 analysed)

Table 17. Summary of Screening
Each screening step is summarised above, showing the positives.

product or *L. pneumophila* DNA, although this cannot be an absolute control as the DNA has been extracted and is not within a cell or phage where proteinases and cell debris are present to contaminate and interfere with hybridisation reactions. However, it was considered that the putative MIP-positive phages were all false positives.

4.4 Investigation of recombinant inserts in λFixII library by PCR differential screening

Differential screening was performed to isolate insert DNA from recombinant λFixII plaques by PCR (Luo *et al.*, 1994). The procedure used primers complementary to left and right lambda arms, the section between this contains the cloned DNA. Positive plaques from the primary screen were cored, freeze/thawed to release DNA and used as template in the PCR reaction with primers recognising sections of the lambda L and R arms (T7/T3 primers). Plaques reacting positively with the DIG-16S rRNA probe did not yield PCR products, nor did DNA extracted from phage preparations. Lambda minipreps were, therefore, made for each of the 16S positive plaques (2.1-2.6) to

provide clean template DNA for PCR amplification with eubacterial 16S primers 926f and 1525r. A 600 bp product was successfully amplified from each sample (Figure 37). To determine the origin of the above 600 bp PCR product, restriction enzyme analysis was performed to distinguish between *P. salmonis* and *E. coli* sequences. The restriction enzyme *PstI* should digest the *P. salmonis* 16S rRNA gene but not that of *E. coli*. None of the recombinant lambda positive isolates were digested after overnight incubation at 37°C. Subsequently, phages from 10 plaques from five different plates, selected at random (2 per plate) were used in PCR with the 16S primers 926f and 1525r and all produced PCR products of approximately 600 bp, from which it was concluded that positive results had arisen from the *E. coli* DNA inevitably present in all samples from its use as the host cell for growth of phage lambda (Figure 38).

4.5 Analysis of average insert size of recombinant *P. salmonis* genomic library in λFixII

To determine the average insert size, DNA was prepared from phage from 10 well-separated plaques from five different plates, as above and digested with a variety of restriction enzymes (*EcoRI, NotI* and *BglII*) and analysed by agarose gel electrophoresis. Sample 1, digested with *EcoRI* , clearly showed an insert of 7 kb (Figure 39), and the *NotI* digest of samples 6 and 7 indicated the presence of 9 kb and 10 kb inserts. Other samples gave bands which were too faint for reliable analysis and further analysis with larger quantities of freshly prepared lambda DNA is required.

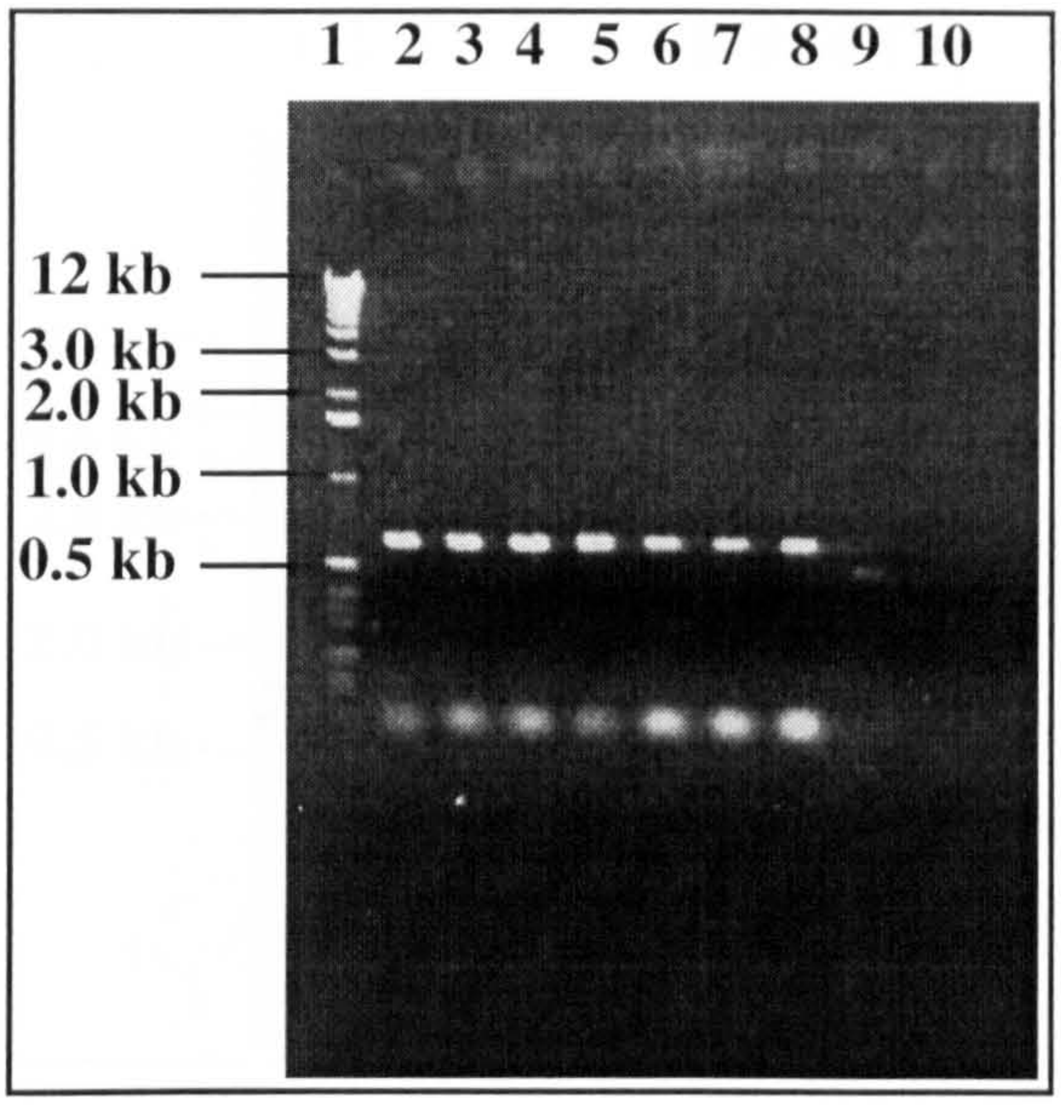


Figure 37. PCR amplification of 16SrRNA from recombinant λ

Using 16S primers, 926f and 1525r PCR was performed on recombinant λFixII/ *P. salmonis* clones. Template DNA was prepared by λ minipreps and 2 µl amplified, 7 µl of each reaction was analysed by agarose gel electrophoresis.

Lane 1	KB ladder
Lane 2	λ 2.1, product approx. 600 bp
Lane 3	λ 2.2, product approx. 600 bp
Lane 4	λ 2.3, product approx. 600 bp
Lane 5	λ 2.4, product approx. 600 bp
Lane 6	λ 2.5, product approx. 600 bp
Lane 7	λ 2.6, product approx. 600 bp
Lane 8	λ 2.21, product approx. 600 bp
Lane 9	PCR reaction with no primers
Lane 10	PCR reaction with no DNA or primers

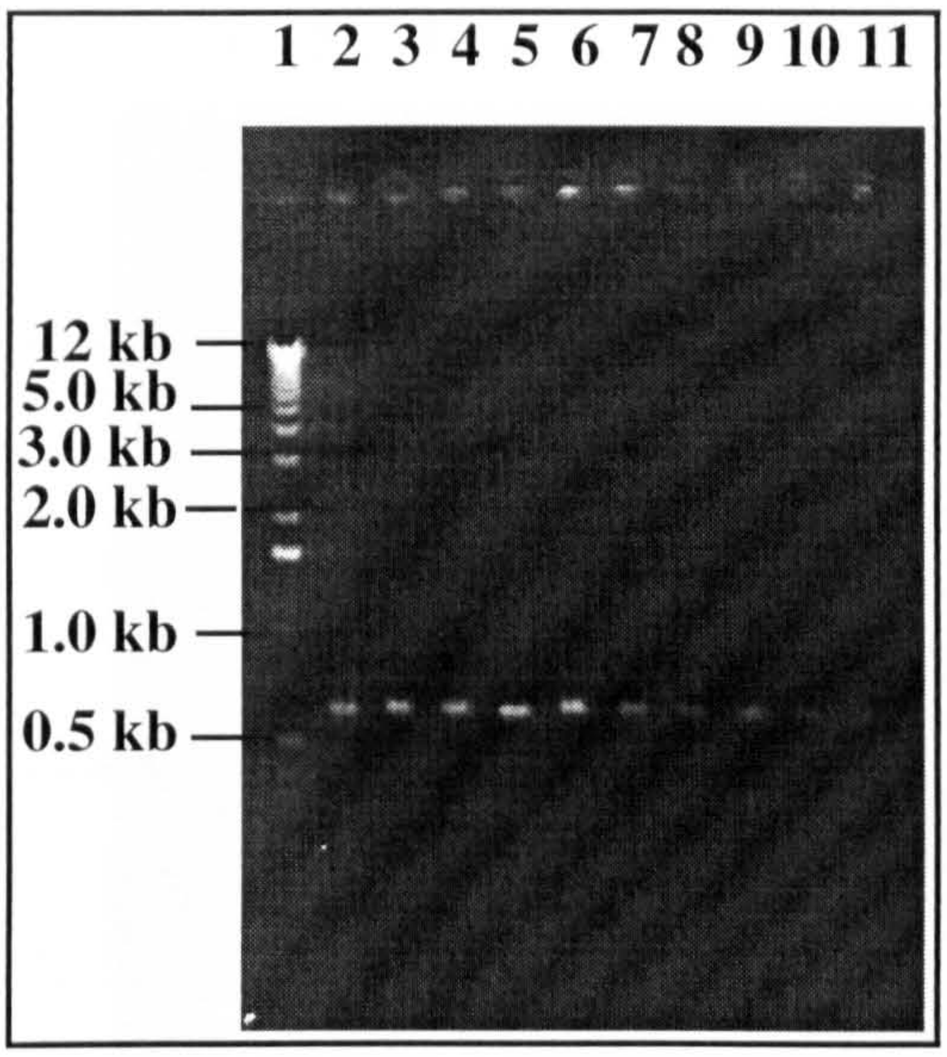


Figure 38. *PstI* digestion of λ 16S rRNA positives

16S products amplified from λ plaques by PCR were digested (5 µl) with the restriction enzyme *PstI* to determine origin of 16S if *E. coli* or *P. salmonis* ? *PstI* cuts *P. salmonis* at position 1007 of 16S gene, region amplified is betwen 926 and 1525, there are no *PstI* sites within the *E. coli* 16S gene in the region above. 10 µl analysed on gel, .

Lane 1	KB ladder
Lane 2	λ2.1 uncut
Lane 3	λ2.1 *PstI* digest
Lane 4	λ2.2 *PstI* digest
Lane 5	λ2.3 *PstI* digest
Lane 6	λ2.4 *PstI* digest
Lane 7	λ2.5 *PstI* digest
Lane 8	λ2.6 *PstI* digest
Lane 9	PCR controls
Lane 10	PCR controls

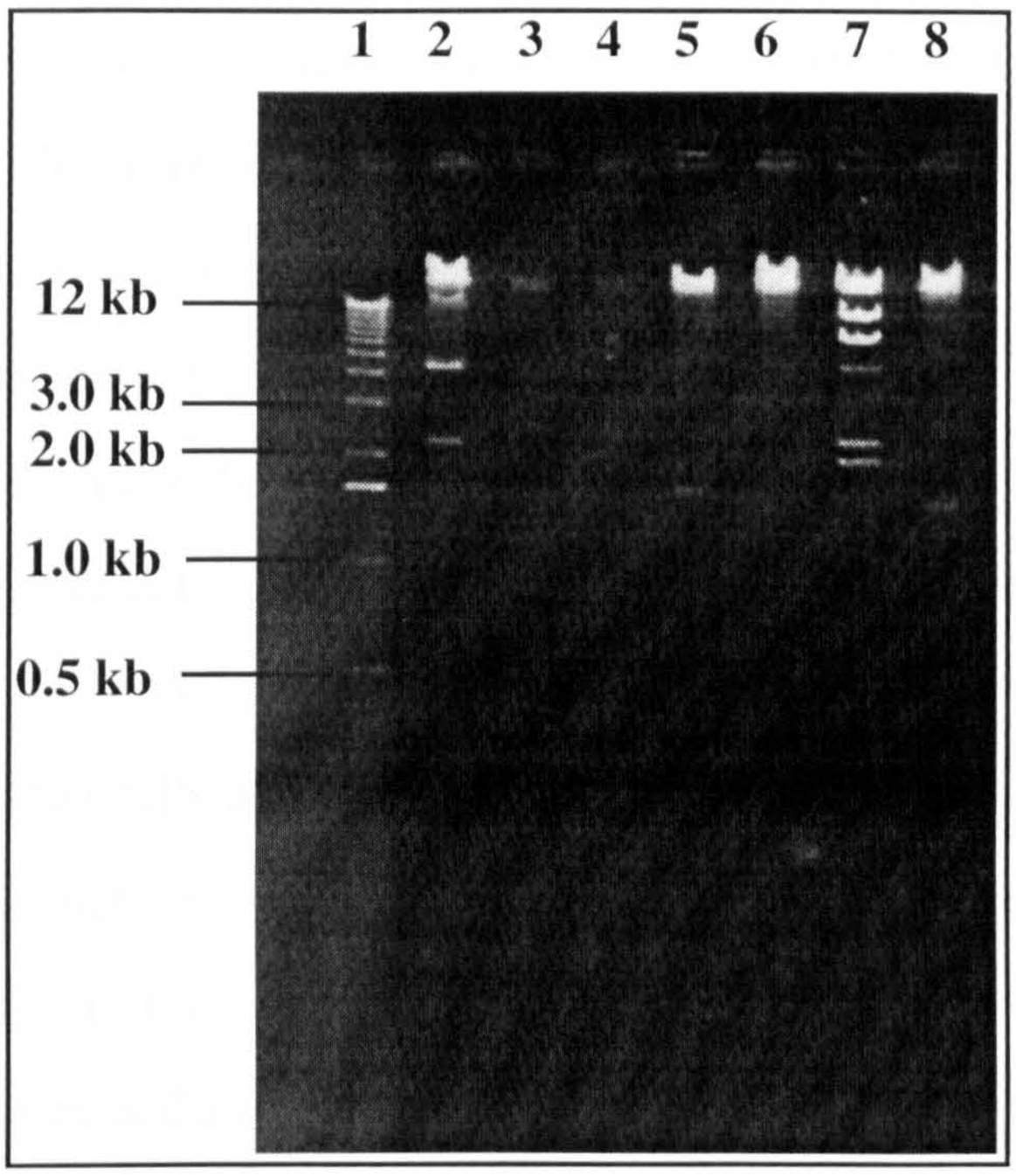


Figure 39*EcoRI* digest of λ DNA samples 1, 2, 4, 5, 6, and 7.

λ DNA was prepared by modifed Wizard Clean-up protocol of 10 well separated λ FixII/Ard and 7 µl digested with *EcoRI* for 1.5 hr at 37°C.Samples 3, 8, 9 and 10 were not analysed.

Lane 1 KB ladder

Lane 2 λ1 *EcoRI* digest

Lane 3 λ2 *EcoRI* digest

Lane 4 λ4 *EcoRI* digest

Lane 5 λ5 *EcoRI* digest

Lane 6 λ6 *EcoRI* digest

Lane 7 λ HindIII standard

Lane 8 λ7 *EcoRI* digest

4.6 Preparation and analysis of antiserum to *P. salmonis* Ardintoul

Purified Ardintoul bacteria were used for production of antiserum in a sheep at the Scottish Antibody Production Unit, and the sera obtained after immunisation for 1, 2, 3 and 8 months were compared with pre-immunisation serum.

4.6.1 Comparison of sera by ELISA

ELISA was performed to determine the reactivity of the different sera with rickettsiae and the host cells (CHSE) from which they had been purified. For this purpose extracts of uninfected cells (both CHSE-214 and XTC), and *P. salmonis* (Ardintoul)-infected CHSE and XTC cells were used. The results are shown in Figures 40 and 41, as graphs of serum concentration against A595 nm. Some cross-reactivity of antisera to uninfected cells occurred, although the reactivity with both CHSE and XTC antigens suggest that this is mainly a non-specific cross-reactivity which could be removed by absorption of the sera. The reactivity against extracts of Ardintoul-infected cells was much greater than with uninfected cells and increased progressively with immunisation time.

4.6.2 Indirect fluorescent antibody test (IFAT)

To determine whether the anti-*P. salmonis* antisera could detect Ardintoul and LF89 bacteria directly in infected tissue cultures, an IFAT was performed (Lannan *et al.*, 1991). The antiserum did not react with uninfected cells, and pre-immune serum (1/50) gave slight background fluorescence with extracts of either Ardintoul or LF-89-infected CHSE cells, but the anti-*P. salmonis* immune sera (1/50) clearly detected both LF89 and Ardintoul bacteria as brightly fluorescing cocci in infected cell cultures (Figure 42).

4.6.3 SDS-PAGE and immunoblotting analysis of rickettsia-infected cells

Extracts of uninfected and infected CHSE and XTC cells were adjusted to a standard protein concentration and compared by SDS-PAGE. Few differences could be seen between the Coomassie Blue-stained gel profiles for infected and uninfected cells (Figure 43). Immunoblotting with anti-*P. salmonis* antisera (pre-immune, and 8 months post-immunisation) showed very little reaction with extracts of CHSE cells,

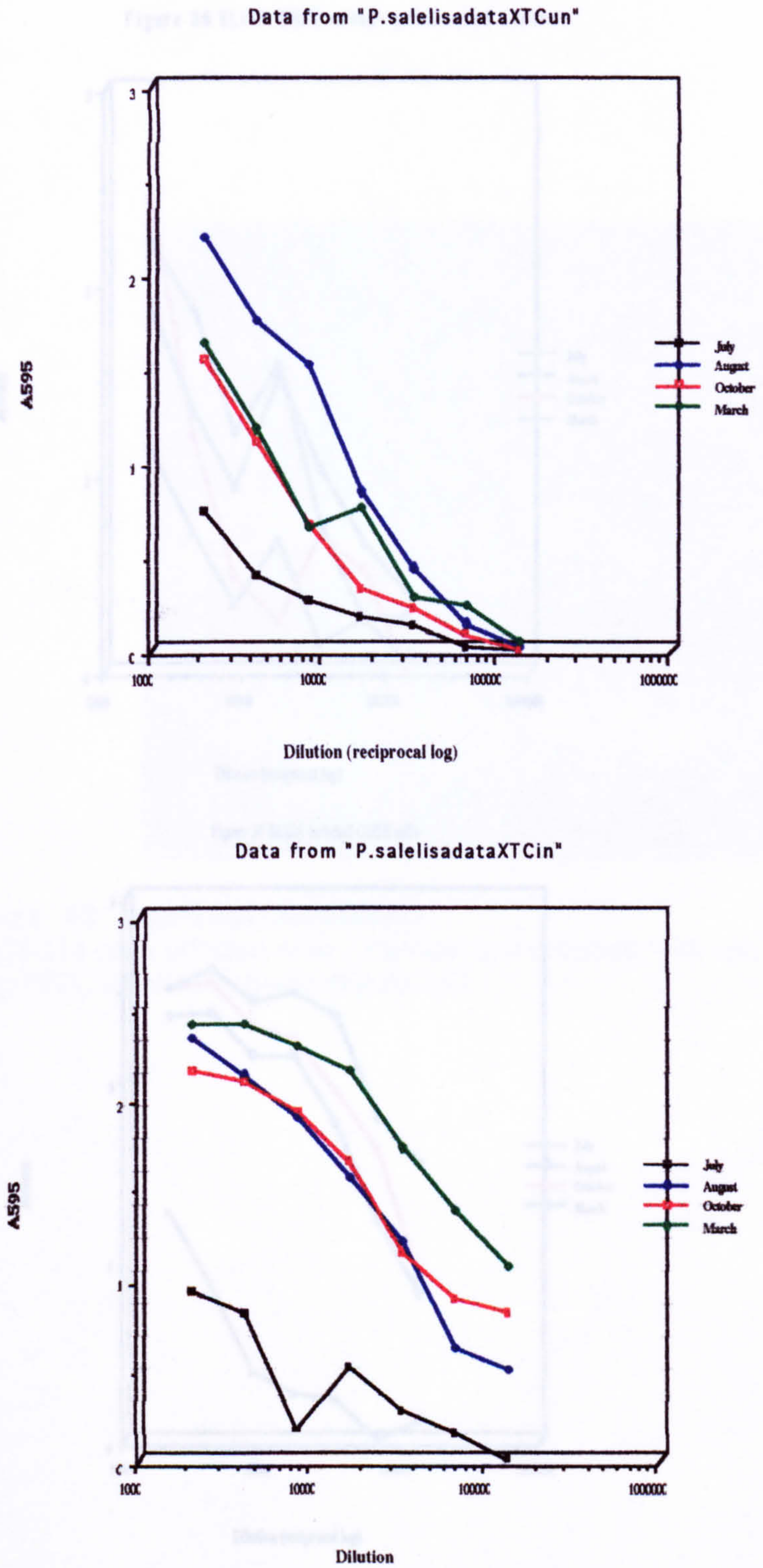


Figure 40 Graph of ELISA data
The data from ELISA are plotted Dilution against A595. The top graph represents uninfected XTC cells and the bottom, Ardintoul infected XTC cells.

Figure 36 ELISA CHSE cells- uninfected control

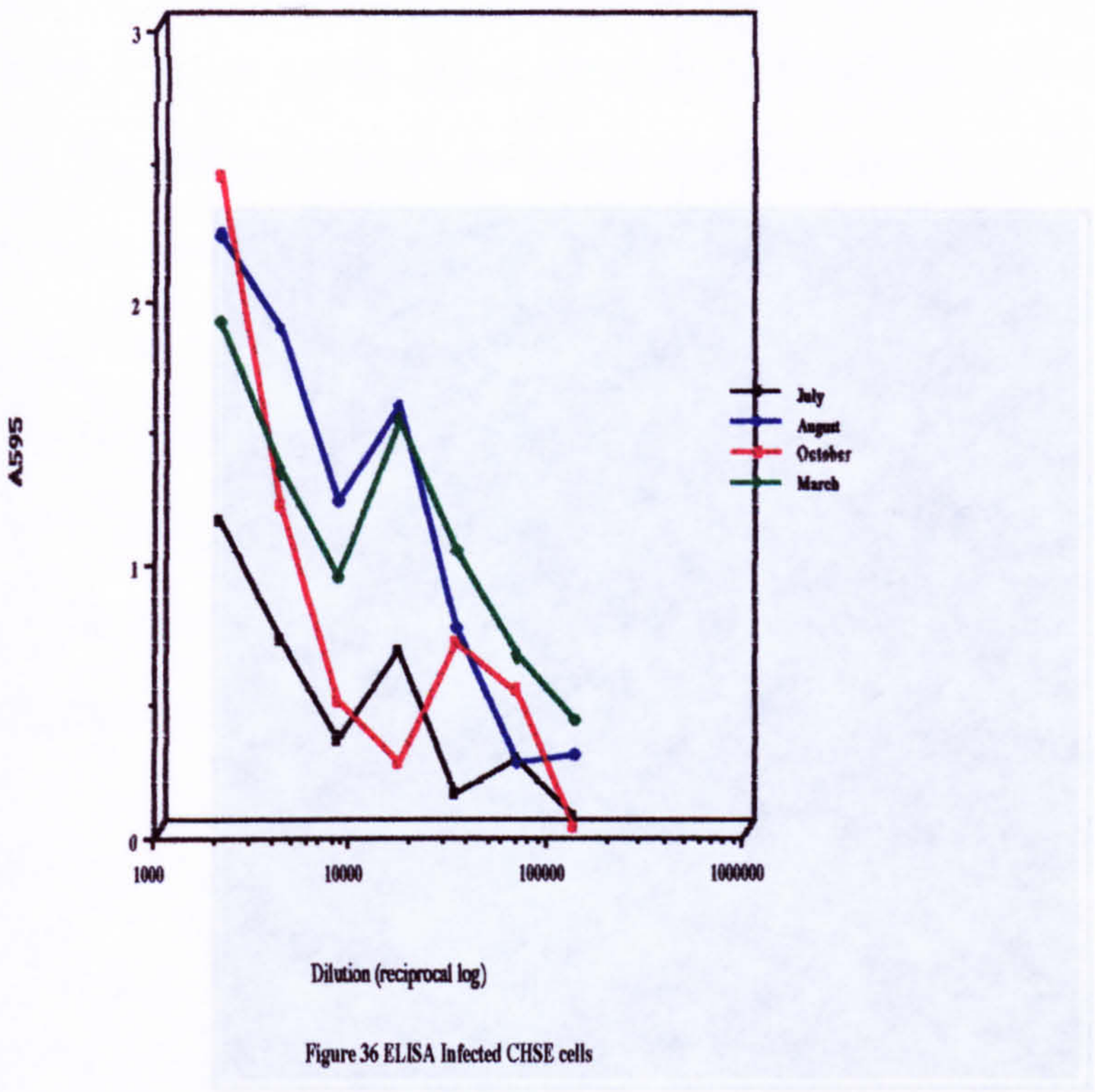


Figure 36 ELISA Infected CHSE cells

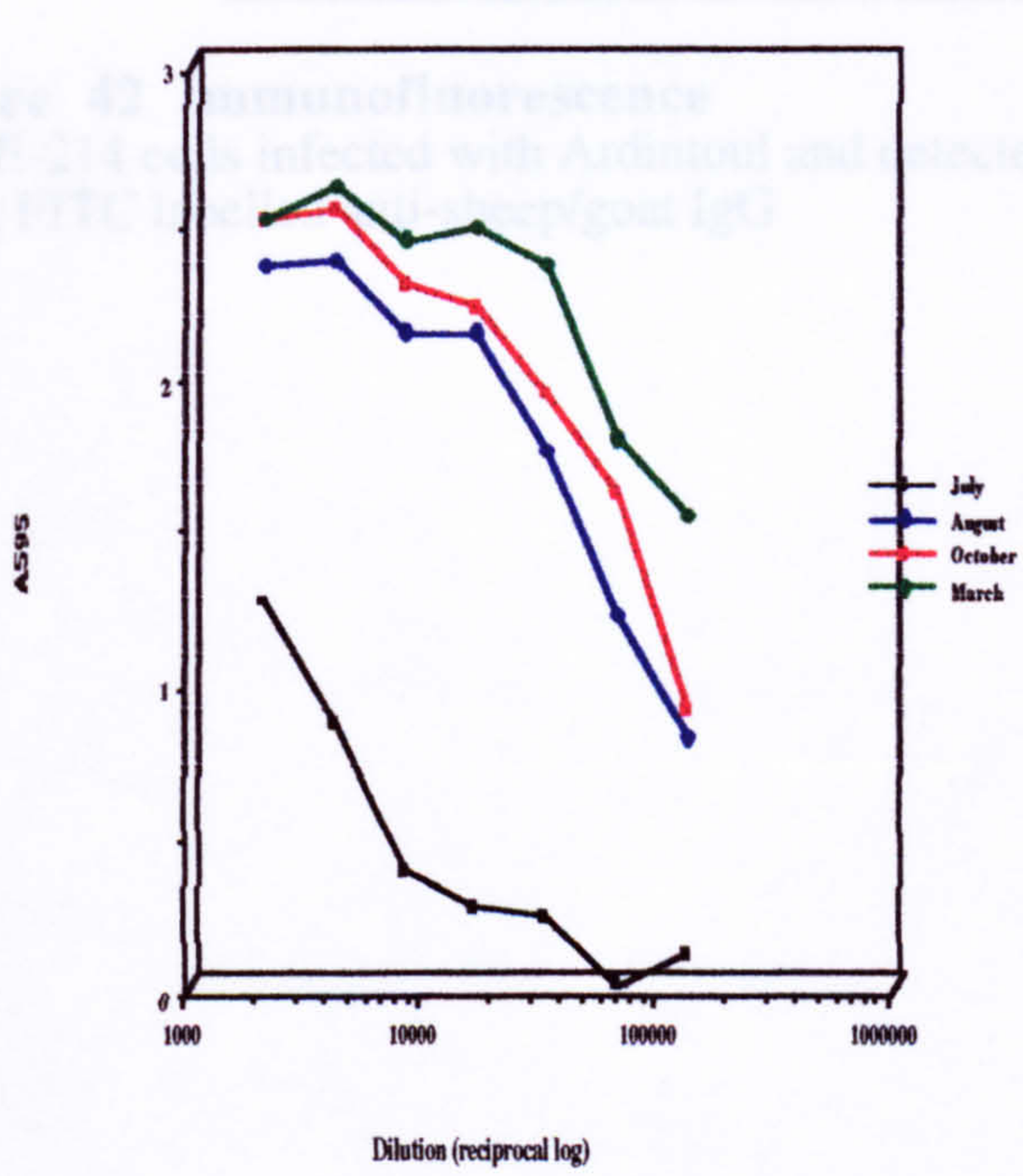


Figure 41 ELISA of infected and uninfected CHSE cells with anti-*P. salmonis*

The ELISA data is plotted Dilution against A595. The top graph represents the uninfected control CHSE cells and the lower graph the infected CHSE Ardintoul cells.

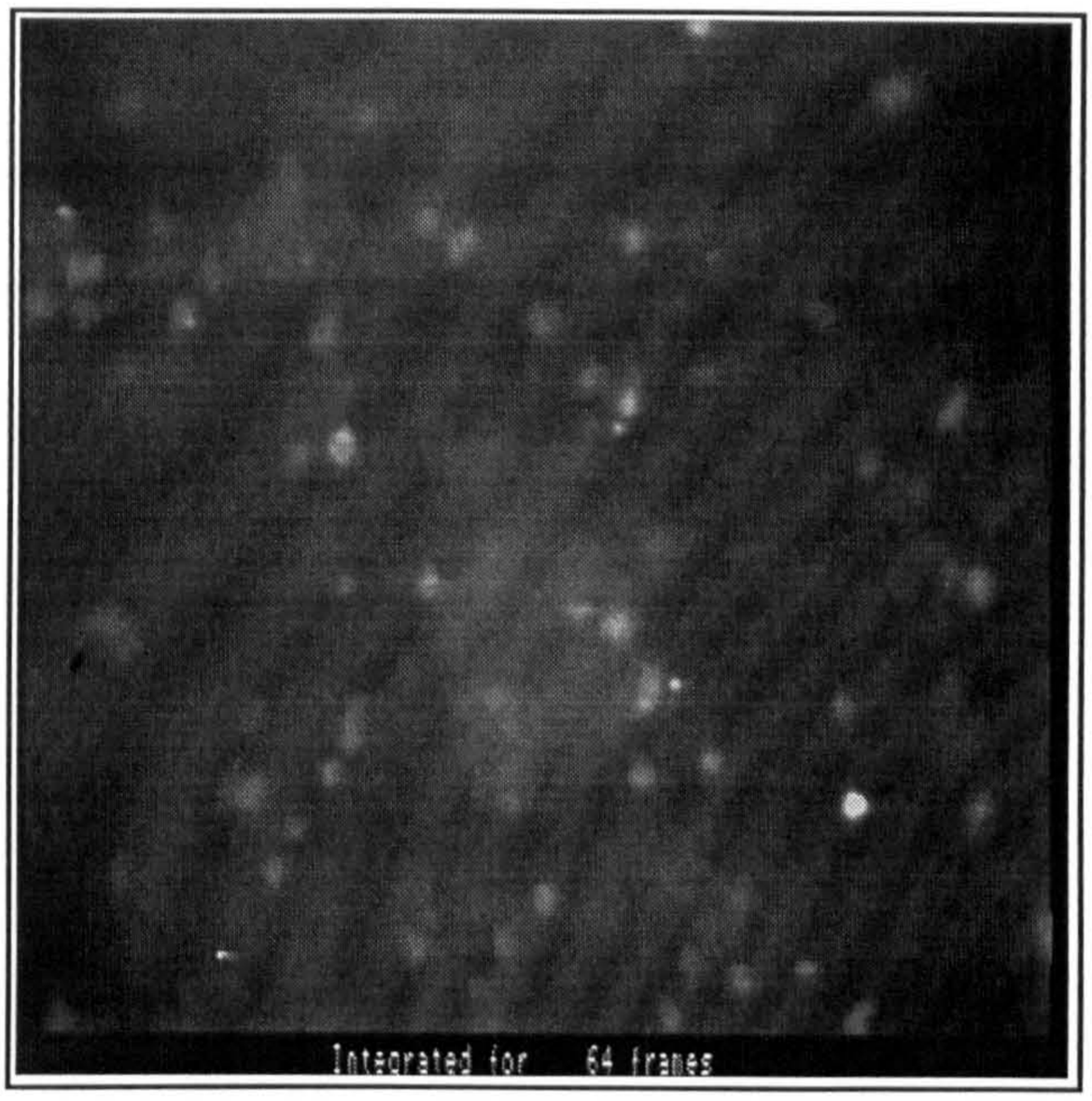


Figure 42 Immunofluorescence
CHSE-214 cells infected with Ardintoul and detected with anti-*P. salmonis* antisera using FITC labelled anti-sheep/goat IgG

suggesting that there was little contamination of the purified rickettsiae with cellular material. With extracts of uninfected XTC cells 4 polypeptides of MW > 66k reacted with the immune serum and at least two of these reacted with control, pre-immune serum. The antigens recognised by the antiserum in extracts of both cell types infected with Ardintoul rickettsiae were very similar, probably identical, the dominant polypeptides being of MW approximately 80k, 70k, 55k, 50k and 20k, with at least a further 10 minor bands evident (Figure 43).

SDS-PAGE and immunoblotting were repeated with purified Ardintoul agent (Figure 44). Although not as clear as the previous gel the dominant bands recognised by antiserum were of MW approximately 70kd, 50kd and 20kd.

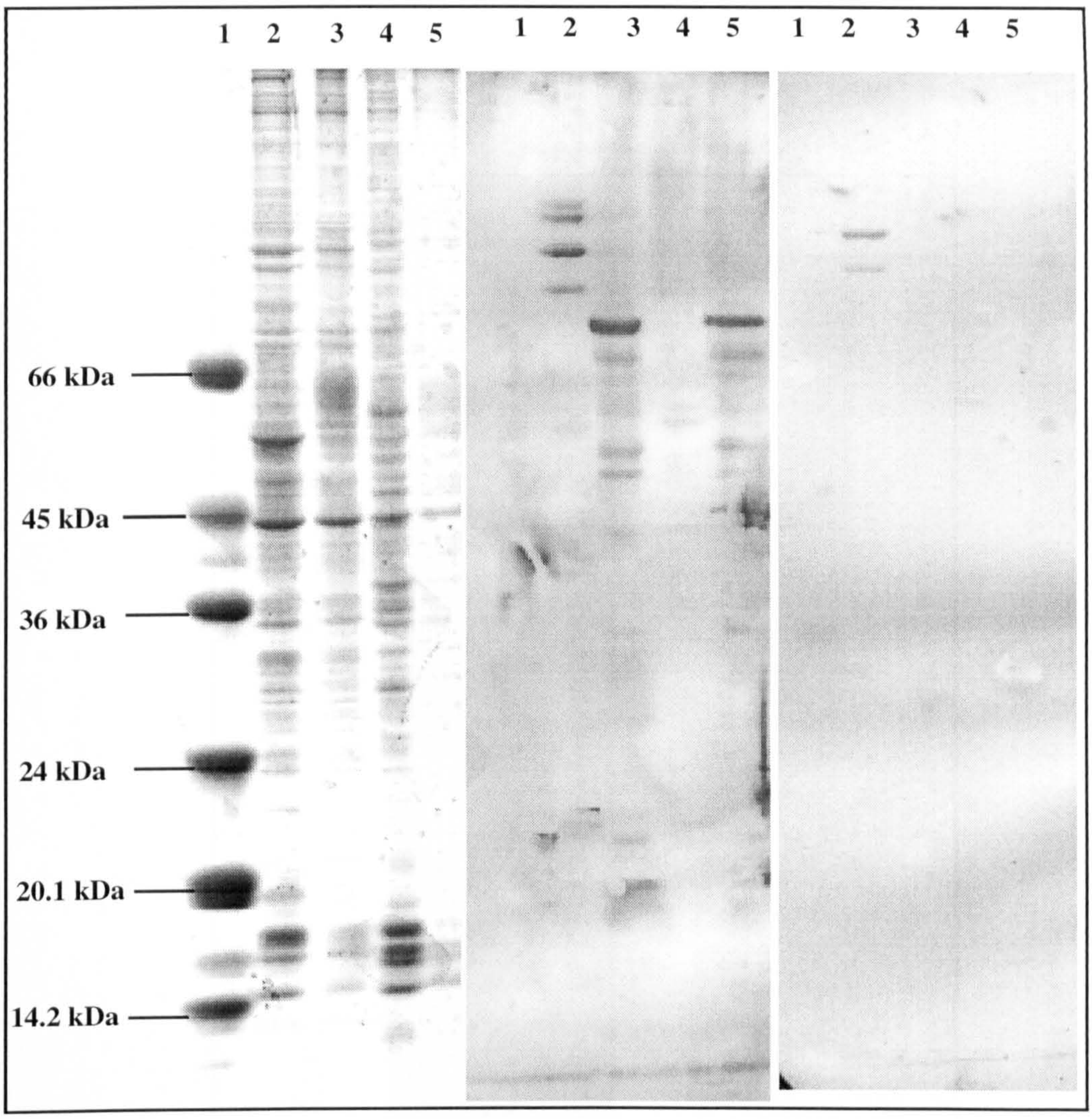


Figure 43 SDS-PAGE and Immunoblotting
On the left; Coomassie stained SDS-PAGE gel of uninfected and infected XTC and CHSE cells with the Ardintoul agent.
Lane 1 SDS-PAGE Molecular weight markers
Lane 2 Uninfected control XTC cells
Lane 3 Infected XTC cells with Ardintoul
Lane 4 Uninfected control CHSE cells
Lane 5 Infected CHSE cells with Ardintoul
In the centre and right are the same samples transferred and blotted with anti-*P. salmonis* antisera, 8 months post-infection (centre) and pre-bleed sample (right). Immunodominant bands of approx. 80 k, 70k, 55k, 50k and 20k are present in the infected samples with 8 months post-infection sera.

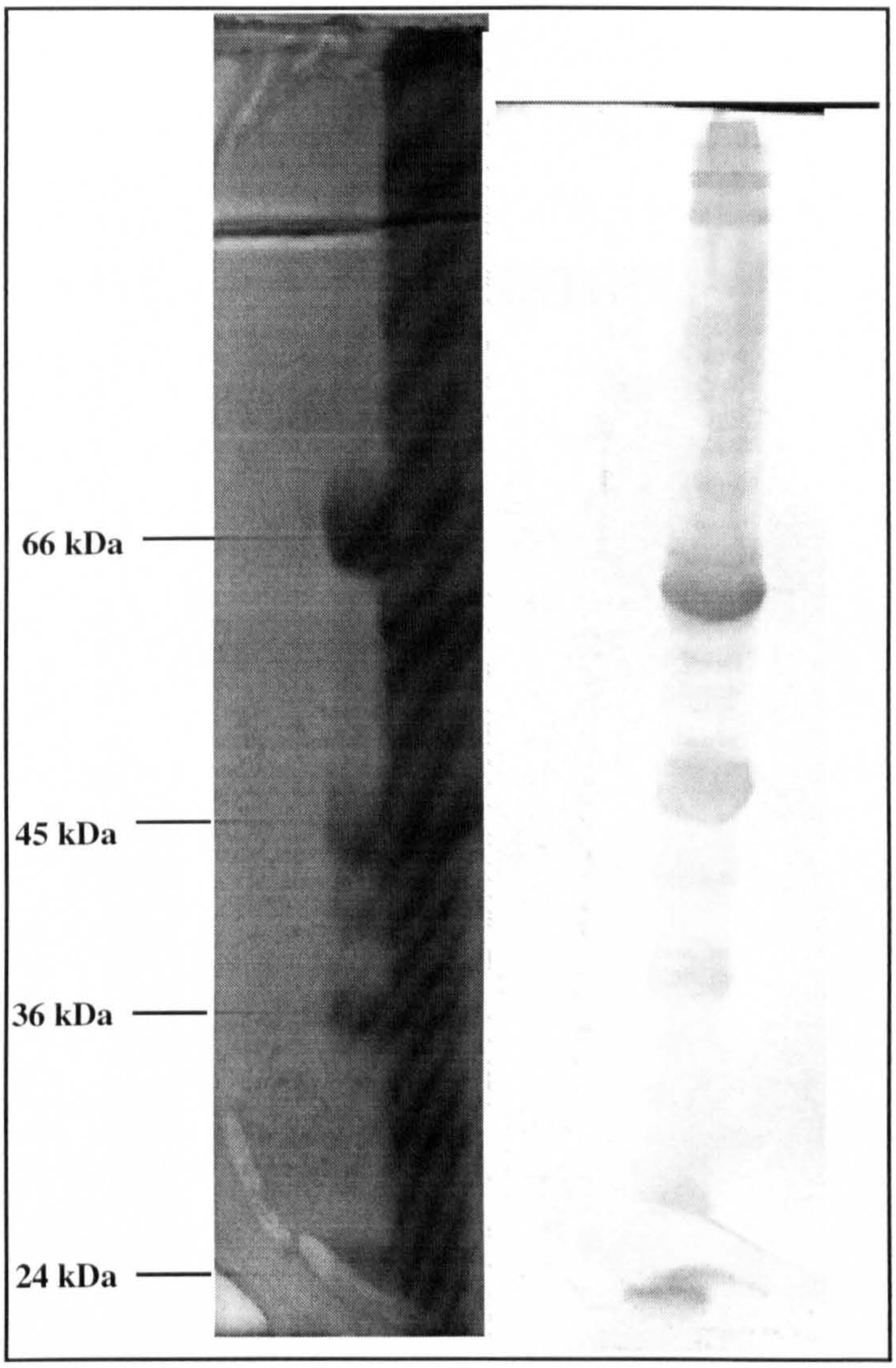


Figure 44 SDS-PAGE and Immunoblotting
Comparison of Coomassie stained SDS-PAGE gel of purified Ardintoul bacteria and the corresponding profile when probed with anti-*P. salmonis* sera. Major protein bands of approximately 70 kDa, 50 kDa and 20 kDa are recognised by the anti-sera.

DISCUSSION

The aims of this thesis were to purify *P. salmonis* for preparation of DNA, to prepare a recombinant library in a phage lambda vector and to characterise the library.

5.1 Growth of *P. salmonis* in cell cultures

To fulfil these aims it was necessary to grow the organism on a large enough scale to obtain sufficient purified bacteria for further manipulations. As rickettsiae had not been grown previously in this laboratory it was also necessary to confirm the culture characteristics of the organism and to determine the optimal conditions for growth.

Growth of rickettsial isolates from Chile was obtained in CHSE-214 and RTG-2 cells once these had been cultured in antibiotic free media. Residual antibiotics in the tissue culture cells affected growth of *P. salmonis* and it took 3 cell passages to remove antibiotics and allow successful cultivation of *P. salmonis* LF89. The CHSE cells were not fastidious in their culture requirements and growth was supported by a range of media (results not shown). The optimum temperature for growth of *P. salmonis* in cell culture was reported by Fryer *et al.*, (1990) to be within the range 15-18^{o}C, and here, good replication was found at 17^{o}C, but not at 20^{o}C, thus confirming the observations of Fryer.

Initially, infection was monitored by light microscopy and *P. salmonis* LF89 was readily visible within intracytoplasmic vacuoles in CHSE cells. At lower magnification, discrete foci of infection were observed in both CHSE-214 and RTG-2 cells, in which cells became rounded and then detached from the monolayer as seen in viral plaque assays. Again, this was consistent with the published literature (Fryer *et al*, 1990). Rounded cells and vacuoles were apparent within 5 days when a high inoculum was used and the cytoplasmic vacuoles increased in size until the nucleus was pushed to the side of the cell. This was clearly seen by Giemsa staining (Figure 6), and the organisms displayed the characteristics of *P. salmonis,* in size, morphology and growth within cytoplasmic vacuoles, e.g. Gram negative coccoid organism.

P. salmonis has been isolated from a range of salmon species, including Coho salmon, *Oncorhynchus kisutch* (Walbaum), (Garces *et al.*, 1991), Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), Atlantic salmon, *Salmo salar* L. and

Rainbow trout, *Oncorhynchus mykiss* (Walbaum)(Fryer *et al.*, 1992). Subsequent to the isolations from salmonids in sea water, reports soon followed of the isolation of *P. salmonis* from diseased rainbow trout and Coho salmon in freshwater (Bravo *et al.*, 1994; Gaggero *et al.*, 1995). The isolation of this organism at freshwater and sea water sites, and in a wide range of salmon species highlights the importance of this organism as an emerging pathogen of salmon (Fryer & Mauel, 1997). The organism is not geographically restricted, as isolates have subsequently been reported from a range of distant locations including Canada (Brocklebank *et al.*, 1992), Ireland (Rodger & Drinan 1993; Palmer, 1997), Norway (Olsen *et al* 1993) and Scotland (Grant *et al.*, 1996).

P. salmonis has been successfully cultivated in several fish cell lines, namely : CHSE-214 (ATCC CRL 1681); RTG-2 (ATCC CCL 55); EPC, originating from Common Carp; CSE-119, Coho salmon embryo; CHH-1 from Chum salmon and FHM, Fathead Minnow. Initial attempts to culture the agent in BB cells, originating from Brown Bullhead, *Ictalurus nebubsus* (Lesuesur) (ATCC CCL 91) and BF-2 Bluegill *Lepomis macrochirus* Rafinesque were unsuccessful (Cvitanich *et al* ., 1991), but growth in these cell lines was recently reported by Almendras *et al.*, (1997), highlighting the apparently widespread infectivity of *P. salmonis* . Isolation of the organism from natural infections in an ever increasing number of fish species, its ready growth in many fish cell lines and the diversity of strains isolated suggests either that the organism has unknown species of fish as its natural host, or that it readily adapts to infect a range of species of fish. These are questions which should be addressed in the near future.

There is no evidence to date for maintenance of the organism in mammalian cell lines and this was not achieved in this study. The inability to grow in mammalian cell cultures is probably due to temperature requirements, as most such cell lines grow optimally at 37^{o}C and not at all below 30^{o}C. *P. salmonis* cannot be grown at temperatures greater than 20^{o}C. However, this study has shown for the first time the growth of *P. salmonis* LF89 in a cell line not derived from fish. XTC cells, which is a

frog-derived cell line, supported growth of the organism within intracytoplasmic vacuoles and to high concentration. This indicates a possibly wider range of hosts which might be susceptible, and it is worth testing whether *P. salmonis* is able to replicate in insect cells, and that invertebrates could act as vectors, as with other rickettsiae. Published evidence on the vector theory indicates the role of sea lice as the alternative to the terrestrial arthropod in rickettsial infections, as (Cvitanich *et al.*, 1991) found sea lice on a number of *P. salmonis* infected salmon, although this was only a small number of the total salmon infected with the disease. Other reports suggest that the bacteria are ingested in the natural diet. However, the recent demonstration by P. Smith (Santiago, Chile; AFS Meeting, Baltimore 1998) that salmon could be infected by placing on the skin (gills) for only a few minutes a small piece of filter paper soaked in rickettsia suggests that direct transmission via the water, with possible entry though an abrasion or superficial lesion is highly probable. As yet, there are no reports of the disease occurring naturally in wild salmon.

P. salmonis infected CHSE-214 cells employed in this study seemed to display long extensions between infected cells; these were only apparent at the end of infection, before complete lysis of the monolayer. It is not known whether they were due to cell degradation or a phenomenon caused by the infection process. Evidence of movement within cells and between cells of infected rickettsia has been published by Teysseire *et al.*, (1992) who showed evidence of actin polymerisation in *R. conorii* and *R. typhi*. To evaluate whether these cellular extensions were composed of actin the bacteria could be stained by immunofluorescence, and actin labelled and detected by phallacidin (Heinzen *et al.*, 1993).

During infection of CHSE cells *P. salmonis* tended to burst from the cells due to the increase in size of the vacuoles which burst leading to death of the cells. When viewed by electron microscopy burst cells show signs of apoptosis, as shown by the presence of cellular debris surrounding bacteria. Phospholipase A2 has been postulated to be involved in the entry and exit of *R. prowazekii* and *R. rickettsii* into and from cells (Winkler & Daugherty, 1989). Walker *et al.*, (1984) added the phospholipase inhibitor

phentermine, to agarose overlays of *R. rickettsii*-infected Vero cells and chick embryo fibroblasts, and this reduced the number of plaques formed in the cell monolayer. To determine whether the phospholipase activity was rickettsial or cellular in origin, Silverman *et al.*, (1992) used an alternative phospholipase inhibitor, p-bromophenacylbromide, on both cells and rickettsia and concluded that the phospholipase was rickettsial in origin. Similar experiments would be of interest to determine whether phospholipase has any role in *P. salmonis* infections.

5.2 Macrophage Infectivity Potentiator MIP

Early in the course of this study Cianciotto *et al.*, (1995) produced evidence for *mip* -like sequences and Mip-related proteins in the family Rickettsiaceae. Mip, a virulence factor of *Legionella sp.*, is a 24 kDa surface protein necessary for the intracellular infection of *Legionella pneumophila* (Cianciotto *et al.*, 1990; Cianciotto & Fields, 1992). There is a high degree of conservation of the *mip* gene among the 14 serogroups of *L. pneumophila* (Cianciotto *et al.*, 1990) and other Legionella species. For example, Bangsborg *et al.*, (1991) showed that the *mip* gene of *L. micdadei* has 71% homology with the equivalent gene of *L. pneumophila,* and that the predicted secondary structures for each were very similar. Analysis of *mip* sequences from *L. pneumophila* and *L. micdadei* revealed homology to FK506 binding proteins at their C-terminus. FK506 binding proteins are found in a number of eukaryotic organisms; they are immunophilins and possess peptidyl-prolyl cis/trans isomerase activity, which catalyses the isomerisation of prolyl bonds in proteins *in vitro* , reviewed by Hacker & Fischer, 1993; in which the study showed peptidyl-prolyl cis/trans isomerase activity was coded for by the *mip* gene, suggesting that a role for the Mip protein in modifying bacterial cell surfaces, thus enhancing entry and, therefore, intracellular survival. Further support for this theory was obtained by preparation of a *mip* mutant of *L. pneumophila* (Cianciotto *et al.*, 1990) which had reduced virulence for U937 cells, explanted human macrophages and guinea pigs. Therefore, a role for Mip in the internalisation of bacteria into host cells has been postulated. Hacker and Fischer (1993) showed that mip-like sequences were present in the genomes of *Chlamydia*

trachomatis, Neisseria meningitidis, Coxiella burnetii and *Pseudomnas aeruginosa.* The mip-like sequences detected by Cianciotto *et al.*, in 1995 were from *C. burnetii* and *Rochalimea quintana* and were detected under low stringency conditions using mip-probes prepared from *L. pneumophila* and *L. micdadei.* Mip-related proteins were detected in the following Rickettsiaceae using both monoclonal and polyclonal antisera to Mip; *C. burnetii, Rochalimea quintana, Rochalimaea vinsonii, R. australis, R. canada, R. conorii, R. montana, R. parkerii, R. prowazekii, R. rickettsii, R. typhi* and *Ehrlichia sennetsu.* The *C. burnetii* Mip protein was cloned by Mo & Cianciotto (1995) and exhibited 46% similarity to Mip of *L. pneumophila* and 30% with *C. trachomatis* Mip, which had been identified by Lundemose *et al.* (1992). The C-terminus of *C. burnetii* Mip showed 35% similarity to eukaryotic FKBPs, similar to the Mip of *L. pneumophila*. The above evidence for the existence of Mip in the Rickettsiaceae stimulated attempts in this work to investigate the possible existence of Mip in *P. salmonis* for a number of reasons; firstly, due to the relatedness of *P. salmonis* to *C. burnetii* and *L. pneumophila* as demonstrated by 16SrRNA analysis (Figure 1), secondly, because all three organisms are intracellular pathogens and can infect macrophages, and thirdly, because it appears to be an important virulence factor. The *L. pneumophila mip* gene sequence published by Engleberg *et al.* (1989) was used to construct a probe for the *L. pneumophila mip* gene. Although the DNA sequence was not determined, from the size of the PCR-amplified gene product, its restriction enzyme digest pattern, and the strong reactivity of a DIG-labelled *mip* probe with *L. pneumophila* DNA it was considered that it did represent the *mip* gene.

5.3 Purification of rickettisae from cell cultures

Published procedures for separation of human-pathogenic rickettsiae from host cell components is usually based on the method of Wisseman (1951) (cited by Weiss *et al.* 1975), which allowed the preservation of the biological properties of the bacteria. Typically, this involves differential centrifugation followed by density gradient centrifugation, some groups used Renografin for density gradients (Weiss *et al.*, 1975), and others preferring a sucrose cushion (Aniskovich *et al.*, 1989; Eremeeva *et*

al., 1993). Differential centrifugation is used to remove the larger host cell debris from the bacteria, which are then separated from smaller cell fragments and molecules on a density gradient. As Percoll was used routinely in this laboratory for density gradient separation of cells the above methods were successfully modified to separate *P. salmonis* from fish cells. A slightly different version was also used by (Kuzyk *et al.*, 1996) for the same purpose, with similar results.

5.4 Construction of a λExCell/ *P. salmonis* LF-89 library

The purpose of preparing a genomic library of *P. salmonis* was as a first step in using molecular techniques to identify putative protective antigens of *P. salmonis*, through screening with antisera or oligonucleotide probes. This approach has been used in the past to identify antigens of numerous pathogens, including rickettsiae (Emelyanov *et al.*, 1993). There is little published work on genetic manipulation in rickettsiae, although a small number of genomic libraries have been created. The most commonly used vector appears to be λgt11, used by Uchiyama *et al.*, (1996) to create a genomic library in *R. japonica.* Other libraries constructed in λgt11 include *R. prowazekii* (Emelyanov *et al.*, 1993), *R. conorii* (Schuenke & Walker 1994), *R. tsutsugamushi* (Hickman, 1991) and *R. typhi* (Hahn *et al.*, 1993). The vector λZapII was used by Brayton *et al.*, (1997) and Gilmore *et al.*, (1991) who constructed genomic expression libraries of *Cowdria ruminantium* and *R. rickettsii*, respectively. The λ vector ExCell was chosen as it appeared to have the additional advantage of containing a phagemid containing the insert genomic DNA that could be released and manipulated as a plasmid. This would simplify the task of identifying insert sizes in the recombinant library, releasing them via the *EcoRI* cloning site, and facilitating subcloning. From the literature the vector did not appear to have been used for rickettsial libraries but was similar in design to λZapII.

5.5 Analysis of the λExCell *P. salmonis* library

An estimated 20 ng of rickettsial DNA was used in construction of the library, compared with the 70 ng used by Emelyanov *et al.*, (1993) in construction of a rickettsial library in λgt11. Although this is low in comparison with libraries prepared

for most bacteria the number of recombinants/ μg DNA was consistent with the data published for the rickettsial libraries described above (see Table 8), and the library initially appeared to be satisfactory. However, problems were encountered in release of phagemids but once this had been overcome it did not prove possible to release insert DNA by digestion with *EcoRI*. Various procedures were tested to eliminate possible contamination by nucleases and other enzymes but to no avail. Eventually, inserts were released from a small number of phagemids using enzymes which flanked the *EcoRI* cloning site.

Although λExCell will accept inserts of up to 6kb the inserts detected were mainly small in size, and many 'white' plaques appeared to have no insert or one which was extremely small. More importantly, sequence analysis of inserts showed the absence of an intact *EcoRI* site, explaining the inability to release inserts with *EcoRI*. In the sequences determined here, the expected *EcoRI* recognition sequence, GAATTC, was replaced with GCGTTC or CCATTC. Correct incorporation of an insert should lead to two *EcoRI* sites flanking the insert, yet no sites were found in the recombinants analysed. This suggests that these phages were either part of the 'background' present in the library or resulted from rearrangement events at the cloning site. It is possible that the use of such a low amount of rickettsial DNA in preparation of the library, and thus production of a relatively small number of recombinants, rendered the background phage in λExCell much more prominent than would normally occur.

Sequence analysis also revealed that all the inserts investigated seemed to be of *E. coli* origin as extremely high identity was recorded with *E. coli* database sequences. The entire *E.coli* genome has been determined (Blattner *et al.*, 1997), but few rickettsial sequences have been recorded. It could be argued that most rickettsial sequences would share features with those of other bacteria, and since the entire *E. coli* genome is known one would expect similarities to *E. coli* sequences to be recorded.

However, the degree of identity was so high that it must be concluded that the inserts were *E. coli* DNA.

A recent publication by Jha *et al.*, (1997) reported the occurrence of *E. coli* sequences in their plasmid vector whilst subcloning troponinI cDNA. They postulated that some sort of rearrangement event had taken place. Investigation into these suspect clones by sequence analysis showed they mapped to various positions on the *E. coli* genome (Blattner *et al.*, 1997) namely 64-65 min and 92.8-00.1 min. It is interesting to observe that the *E. coli* sequence similarity that was obtained with the λExCell/*P. salmonis* clones were within or very close to the above regions. For example rpiA maps to 63 min and rpiB to 92.85 min, refer to sequencing section. Obviously when libraries are constructed investigators are interested in one gene or segment of the genome and the remainder of the library is not evaluated. Another interesting observation by Jha was the presence in the databases of *E. coli* sequence within a rat hepatic glutathione transporter cDNA sequence. The message from this paper seems to be interpret sequence with care.

5.6 Screening the λExCell *P. salmonis* library

The above discussion indicates why no success was obtained in detection of phages which reacted with the probes for 16S rRNA, MIP or immunological screening with antisera. Further discussion on screening is contained later for the Ardintoul agent.

5.7 Characterisation of the *P. salmonis* Ardintoul isolate

The occurrence of an outbreak of rickettsiosis in Scotland during this study was in many ways fortuitous as it allowed characterisation of the organism and its comparison with existing previously described strains.

The organism was isolated at Marine Harvest McConnell laboratories from the brain, kidney and spleen of moribund and dead fish which were used to infect cultured CHSE cells. The bacteria exhibited typical characteristics of *P. salmonis,* namely, cytopathic effects in infected cells within 7 days, bacteria visible within intracytoplasmic vacuoles, susceptibity to antibiotics, Gram-negative coccoid morphology, and an inability to grow on conventional bacteriological media. To compare the organism with the LF89 strain the isolate was cultured in our laboratory. The Ardintoul isolate was cultured readily in CHSE and RTG-2 cells and appeared to replicate more efficiently than LF89,

giving more rapid lysis of the cell monolayer. Electron microscopy showed an outer rippled cell membrane similar to that reported by Fryer *et al.*, (1992) for LF89. The Ardintoul isolate appeared possibly slightly larger than LF89, from the scale bars from electron micrographs, and viewing by light microscopy.

Confirmation that LF89 and Ardintoul were related was obtained from the 16S rRNA sequence, mainly determined in a separate project but analysed here, with the complete sequence shown in Appendix 3. Comparison of this sequence with those in the databases shows high identity with recognised *P. salmonis* isolates, except for strain EM-90, for which the sequence reported by Fryer and co-workers is quite distinct from other piscirickettsia studied thus far. The base differences are shown in Table 13. Mauel *et al.*, 1996 and Fryer and Mauel, 1997 analysed the 16S rRNA sequences of isolates from Chile (LF-89, EM-90), Norway (NOR-92) and Canada (ATL 4.91 and SLGO-94) and found that strain EM-90 was quite different from other strains which were more closely related but still easily distinguishable on the basis of 16S rRNA sequence. The Chilean isolates could be distinguished by use of different primers to amplify the 16S rRNA sequence in a PCR detection method. The Ardintoul isolate would fall into the LF-89, NOR-92, ATL 4.91, SLGO-94 group in PCR with the primers of Mauel *et al.*, (1996) and differed by 8 bases from the SLGO-94 and LF-89 strains.

Another interesting observation from sequence comparisons is the relatedness of the *P. salmonis* isolates to endosymbiotic bacteria, some of which - *Ridgeia piscesae*, *Riftia pachyptila* and *Lamellibranchia columna* endosymbionts -recorded higher similarity scores than some *P. salmonis* isolates (Table 12). These bacteria are associated with animals residing near hydrothermal vents, and a closer comparison of these micro-organisms with rickettsiae, including culture studies with poikilothermic tissue culture cell lines would be of interest in confirming possible evolutionary links between these organisms, and suggesting a possible origin for the fish-pathogenic rickettsia which should be explored. The stability of piscirickettsiae in sea water (Lannan & Fryer 1994) for several weeks means that transmission could occur via water over considerable

distances. Rickettsia and RLO have been recorded in many fish and invertebrates (see Section 1) and they should also, where possible, be included in such comparisons.

5.8 Construction of λFixII/ *P. salmonis* library

Due to the failure of the first genomic library for *P. salmonis* LF-89 it was decided to attempt construction of a library for further molecular analysis of the Ardintoul agent as this appeared to be more readily grown in large quantities. Several litres of infected culture fluid were produced and bacteria purified by Percoll gradient centrifugation. Extraction of DNA proved difficult from this strain as it resulted in an insoluble product, considered to be possibly due to co-extraction of LPS with the DNA. The use of CTAB to extract LPS and oligosaccharides appeared to overcome this problem. The difference between strain LF-89 and Ardintoul in this respect may be due to production of greater quantities of LPS or oligosaccharides by the Ardintoul agent. It should be noted that human-pathogenic rickettsiae may produce slime layers which differ in thickness according to the state of nutrition of their host ticks, as mentioned in the Introduction and differences in metabolism may lead to differences between piscirickettsiae.

Lambda Fix®II (Stratagene) is a replacement vector suitable for cloning fragments of genomic DNA of between 9 and 23kb, and the resultant recombinant λ clones can be purified and the insert released from the vector DNA by *NotI* digestion. This represents a different approach to the use of λExCell and screening is effectively with nucleic acid probes only. The aim was to construct a good library from *P. salmonis* DNA, then to analyse purified rickettsia by SDS-PAGE such that N-terminal sequencing of relevant proteins could be carried out and probes constructed for use with the library.

The λFIXII library appeared to contain an adequate number of recombinants for comprehensive coverage of the *P. salmonis* genome, and random screening showed the presence of inserts of 4 to 9kb. More recent work (T.H. Birkbeck, data not shown) has detected inserts of up to 16kb, and as the inserts can be released with *NotI* it suggests that a library may have been produced which is suitable for further work.

5.9 Screening of the λFIXII library

The DIG-labelled 16S rRNA probe was based on the 16S rRNA gene sequence of strain LF89 but its use in screening the library led only to the production of false positive results. This can be explained since the 16S rRNA gene of *E. coli* will show a high degree of relatedness to that of *P. salmonis* and hybridisation almost certainly occurred to *E. coli* chromosomal DNA, present in high concentration on phage assay plates. Once the stringency was increased in hybridisation reactions the interaction was no longer seen. Further confirmation came from the perceived lack of an *EcoRI* site in the PCR product, characteristic of *E. coli*, and not *P. salmonis*. To overcome this problem specific *P. salmonis* 16S rRNA gene primers should be used, perhaps based on the sequences identified by Mauel *et al.*, (1996).

Use of the MIP gene probe also led to false positive results, and more detailed work is needed to determine whether *P. salmonis* does contain a homologue of the *mip* gene. This could use a PCR approach with primers based on the sequences of the different *mip* genes published more recently.

5.10 Preparation and analysis of antisera to *P. salmonis* Ardintoul

Ideally, the preparation of antisera would have taken place early in the project to provide large supplies of good quality product for immunoblotting and screening. However, early batches of purified bacteria were committed to production of DNA for library preparation and it was only later in the project that antiserum became available. The purity of the bacteria obtained from Percoll gradients is shown by the low reactivity of antisera in immunoblotting with extracts of the CHSE host cells in comparison with the high reactivity with *P. salmonis*-infected CHSE or XTC cells.

ELISA revealed a rising antibody titre to the CHSE- and XTC-infected cells with repeated immunisation but there was a residual, non-specific background reactivity with cellular antigens, both to XTC and CHSE cells.

Immunoblotting revealed at least 15 polypeptides which reacted with immune serum, the dominant bands being of MW c. 80k, 70k, 55k, 50k and 20k. Antigenic characterisation of *P. salmonis* was reported recently by Kuzyk *et al.*, (1996) who

noted the presence of polypeptides of MW 65k, 60k, 54k, 51k, and carbohydrate antigens of 16k and 11k, the latter being attributed to lipopolysaccharide. Given the differences between SDS-PAGE systems there is reasonable agreement between these studies. The main difference evident in immunoblotting is the dominance of the MW 70k antigen in eliciting an immune response to *P. salmonis* Ardintoul.

Preliminary data was obtained for analysis of purified Ardintoul agent (Figure 37) and, although not as clear as results with infected cell cultures, dominant polypeptide bands of MW approximately 70kd, 50kd and 20kd were recognised by antiserum, as in infected cells. The 70kDa antigen of *P. salmonis* appeared to be immunodominant in stimulating the sheep immune response, as the relevant polypeptide was not evident above the background cellular material on SDS-PAGE (Figures 43 & 44), and this antigen could provide a primary target for future library screening.

The other method of use of the antiserum was in indirect immunofluorescence, in which rickettsia in smears from infected tissue cultures were readily stained by anti-*P. salmonis* antiserum and FITC-labelled second antibody. Further development is required to test this antiserum in IFAT for routine diagnosis and in establishing the distribution of organisms in infected fish (Grant *et al.*, 1996).

5.11 General conclusions and future work

The continued prevalence of *P. salmonis* as a pathogen in Chilean waters and of related rickettisae from several geographically distinct regions in the Northern hemisphere suggest that these organisms are truly serious emerging pathogens (Fryer and Mauel, 1997) with the potential to cause severe losses in aquaculture. Many aspects of *P. salmonis* require more detailed study, particularly the origin and natural hosts of the organisms, their virulence determinants, and antigenic structure of the surface components. In particular, there is an important need for development of an effective vaccine.

The route chosen in this study (and others) towards the development of a vaccine recognises that it is likely to prove impossible to grow, in tissue culture cells, sufficient

quantities of bacteria for production of vaccines, and that a recombinant approach is required. The principal targets for such an approach are the outer membrane proteins in the expectation that all or part of such proteins, expressed from plasmids in *E. coli*, or other systems, will induce opsonising antibodies or cellular immunity. If effective opsonising antibodies are directed against polysaccharide antigens, e.g. lipopolysaccharide, then a different approach will be required. The antiserum raised in this work reacts strongly with several polypeptides of *P. salmonis* and should prove the basis for screening expression libraries prepared from *P. salmonis* DNA. Two approaches could be considered. Firstly, preparation of sufficient purified bacteria, solubilisation of outer membrane proteins and analysis by SDS-PAGE should yield sufficient 70kDa protein (assuming that the 70kDa polypeptide is an outer membrane protein) for N-terminal sequence determination and preparation of a suitable oligonucleotide probe for screening the current λFixII library. Secondly, the possibility of transferring the library to an expression vector, or preparing a further library in an expression vector should be considered.

REFERENCES

Aliabadi, Z., Winkler, H.H. and Wood, D.O.(1993) Isolation and Characterisation of the *Rickettsia prowazekii* gene encoding the flavoprotein subunit of succinate dehydrogenase. *Gene* **133** 135.

Almendras, F.E., Fuentealba, I.C., Jones, S.R.M., Markham, F. and Spangler, E. (1997) Experimental infection and horizontal transmission of *Piscirickettsia salmonis* in freshwater raised Atlantic salmon. *Journal of Fish Diseases* **20** 409-418.

Altschul, S. F. (1990) Basic alignment search tool. *Journal of Molecular Biology* **215** 403-410.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein datbase search programs. *Nucleic Acids Research* **25** 3389-3402 .

Amano, K.I., Fujita, M. and Suto, T. (1993) Chemical properties of lipopolysaccharides from Spotted Fever group rickettsiae and their common antigenicity with lipopolysaccharide form *Proteus* species. *Infection and Immunity* **61** 4350-4355.

Anderson, D.R. (1965) Comparison of the ultrastructure of several rickettsiae, ornithosis virus and mycoplsma in tissue culture. *Journal of Bacteriology* **90** 1387-1404 .

Anderson, D.R. and Tzianabos, T. (1989) Comparitive sequence analysis of a genus-common Rickettsial antigen gene. *Journal of Bacteriology*. **171** 5199-5201.

Anderson, B.E., McDonald, G.A., Jones, D.C. and Regenery, R.L. (1990) A protective protein antigen of *Rickettsia rickettsii* has tandemly repeated near-identical sequences. *Infection and Immunity* **58** 2760-2769.

Aniskovich, L.P., Eremeeva, M.E., Balaeva, N., Ignatovich, V.F., Artmiev, M.I., Emelyanov, V.V. and Smirnova, N. S. (1989) Methods for purification of *Rickettsia prowazekii* separated from the host tissue: a step by step comparison. *Acta Virologica* **33** 361-370.

Aniskovich, L.P., Motin, V.L., Balayeva, N. and Smirnov, G.B. (1991) Cloning and expression in *E. coli* of the 37, 14 or 16 kDa antigen genes from *Rickettsia prowazekii* strain E. *Acta Virologica* **35** 90-102.

Ausebel, F.M., Brent, R., Kingston, R.E. Moore D.D., Smith, J.A., Seidman, J.G. and Struhl (1987) Current Protocols in Molecular Biology. John Wiley and Sons.

Bangsborg, J.M., Cianciotto, N.P. and Hinderson, P. (1991) Nucleotide sequence analysis of the *Legionella micdadei mip* gene encoding a 30 kDa analogue of the *Legionella pneuphilia mip* protein. *Infection and Immunity*. **59** 3836-3840.

Bernoth, E.-M. (1997) Furunculosis: the history of the disease and of disease research. pp 1-20 in: Bernoth, E.-M., Ellis, A.E., Midtlyng, P.J, Olivier, G. and Smith, P. (eds) *Furunculosis Multidisciplinary Fish Disease Research* Academic Press London

Blattner, F.R., Plunkett III, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Colado-Vides, J., Glasner, J.D, Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B. and Shao, Y. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277** 1453-1462 .

Borck, K., Beggs, J.D., Brammer, W.J., Hopkins, A.S. and Murray, N.E. (1976) The construction *in vitro* of transducing derivatives of phage lambda. *Molecular General Genetics* **146** 199-201.

Branson, E. J. and Diaz-Munoz, D. N. (1991) Description of a new disease condition occurring in farmed coho salmon, *Oncorhynchus kisutch* (Walbaum) in South America. *Journal of Fish Diseases* **14** 147-156.

Brayton, K.A., Fehrsen, J., Devilliers, E.P., Vankleef, M. and Allsopp, B.A. (1997) Construction and initial analysis of a representative lambda ZAPII expression library of the intracellular rickettsia *Cowdria ruminantium:* cloning of map1 and three other cowdria genes. *Veterinary Parasitology* **72** 185-199

Brocklebank, J.R., Speare, D.J., Armstrong, R.D. and Evelyn, T. (1992) Septicemia suspected to be caused by a rickettsia-like agent in farmed Atlantic salmon. *Canadian Veterinary Journal* **33** 407-408.

Burgdorfer, M. (1981) Rickettsiae and Rickettsial Diseases. Academic Press London

Chen, S.C., Tung, M.C., Chen, S.P., Tsai, J.F., Wang, P.C., Chen, R.S., Lin, S.C., and Adams, A. (1994) Systemic granulomas caused by a rickettsia-like organism in Nile Tilapia, *Oreochronuis niloticus* (L.), from Southern Taiwan. *Journal of Fish Diseases* **17** 591-599.

Cianciotto, N.P. Bangsborg, J.M., Eisenstein, B.I. and Engleberg, N.C. (1990) Identification of *mip*-like genes in the genus *Legionella* . *Infection and Immunity* **58** 2912-2918.

Cianciotto, N.P. and Fields, B.S. (1992) *Legionella pneumophillia mip* gene potentiates intacellular infection of protozoa and human macrophages. *Proceedings of the National Academy of Science USA* **89** 5188-5191.

Cianciotto, N., O'Connell, W., Dasch, G. and Mallavia, L. (1995) Detection of *mip*-like sequences and *mip*-related proteins within the family Rickettsiaceae. *Current Microbiology* **30** 149-153.

Crocquet-Valdes, P., Weiss, K. and Walker, D. (1994) Sequence analysis of the 190 kDa antigen encoding gene of *Rickettsia conorii* (Malish 7 strain). *Gene* **140** 115-119.

Cvitanich, J., Garate, O. and Smith, D. (1991). The isolation of a rickettsia-like organism causing disease and mortality in Chilean salmonids and its confirmation by Koch's postulates. *Journal of Fish Diseases* **14** 121-145.

Dasch, G., Samms, J. and Williams, J. (1981). Partial purification and characterisation of the major species-specific protein antigens of *Rickettsia typhi* and *Rickettsia prowazekii* identified by rocket electrophoresis. *Infection and Immunity* **31** 276-288.

Dasch, G. (1981) Isolation of species-specific protein antigens of *Rickettsia typhi* and *Rickettsia prowazekii* immunodiagnosis and immunoprophylaxis. *Journal of Clinical Microbiology* **14** 333-341.

Davies, A.J. (1986) A rickettsia-like organism from dragonets, *Callionymus lyra* L. (teleostei: Callionymidae) in Wales. *Bulletin of the European Association of Fish Pathologists* **6** 103

Drancourt, M. (1994) Taxonomic position of the Rickettsiae: Current knowledge. *FEMS Microbiology Letters*. **13** 13-24 .

Dumler, J.S., Wisseman, C.L., Fiset, P. and Clements, M.L. (1992) Cell-mediated immune-responses of adults to vaccination, challenge with *Rickettsia-rickettsii* *American Journal of Tropical Medicine and Hygiene,* **46** 105-115

Dunkin, S. and Wood, D. (1994) Isolation and characterisation of *Rickettsia prowazekii reca* gene. *Journal of Bacteriology* **176** 1777-1781.

Egidius, E., Wiik, R., Anderson, K., Hoff, K.A. and Hjeltnes, B. (1986) *Vibrio salmonicida sp. nov.*, a new fish pathogen. *International Journal of Systemic Bacteriology* **36** 518-520.

Ellis, A. E. (1995) Recent development in oral vaccine delivery systems. *Fish Pathology* **30** 293-300.

Emelyanov, V.V. (1993) Molecular cloning and expression of *R. prowazekii* genes for three outer membrane proteins in *E. coli. Microbial Pathogenesis 15* 7-16.

Emmerich, R. and Weibel E. (1894) Über eine durch Bacterien erzeugte Seuche unter den Forellen. *Archiv. für Hygiene* **21** 1-21.

Engleberg, N., Carter, C., Weber, D., Cianciotto, N. and Eisenstein, B. (1989) DNA sequence of *mip*, a *Legionella pneumophila* gene assocaiated with macrophage infectivity. *Infection and Immunity* **57** 1263-1270 .

Eremeeva, M., Balayev, N., Ignatovich, V. and Raoult, D. (1993) Proteinic and genomic identification of Spotted Fever group rickettsiae isolated in the former USSR. *Journal of Clinical Microbioogy* **31** 2625-2633.

Evelyn, T.P.T. (1992) Salmonid rickettsial septicemia. pp. 19-19. in: Kent, M.L. (ed) Diseases of seawater netpen-reared salmonid fishes in the Pacific Northwest. Canadian Special Publication Fish Aquatic Science **116**. Nanaimo (BC): Department of Fisheries and Oceans.

FAO (1992) *FAO Fisheries Circular* (Provisional) (**815** Rev.3 Provisional) 136 pp, Rome.

Falk, K., Namork, E., Rimstad, E. Mjaaland, S. and Danevig, B.H. (1997) Characterization of infectious salmon anaemia virus, an orthomyxo-like virus isolated from atlantic salmon (*Salmo salar* L.) *Journal of Virology*, **71**, 9016-9023.

Fryer, J.L., Nelson, J.S. and Garrison, R.L. (1978) Immunization of salmonids for control of vibriosis. *Marine Fisheries Review* **40**, 20-23.

Fryer, J.L. and Bartholemew, J.L. (1996) Established and emerging infectious diseases of fish. *American Society for Microbiology News*, **62**, 592-594.

Fryer, J., Lannan, C., Garces, L., Larenas, J. and Smith, P. (1990) Isolation of a rickettsiales-like organism from diseased Coho salmon (*Oncorhynchus kisutch*) in Chile. *Fish Pathology* **25** 107-114.

Fryer, J., Lannan, C., Giovannoni, S. and Wood, N. (1992) *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes, *International Journal of Systemic Bacteriology* **42** 120-126.

Fryer, J. and Lannan, C. (1994) Rickettsial and Chlamydial infections in freshwater and marine fishes, bivalves, and crustaceans. *Zoological Studies* **33** 95-107.

Fryer, J.L. and Mauel, M.J. (1997) The Rickettsia: an emerging group of pathogens in fish. *Emerging Infectious Diseases* **3** 137-144.

Gaggero, A., Castro, H. and Sandino, A.M. (1995) First isolation of *Piscirickettsia salmonis* from coho salmon, *Oncorhynchus kisutch* (Walbaum), and rainbow trout, *Oncorhynchus mykiss* (Walbaum), during the freshwater stage of their life cycle. *Journal of Fish Diseases* **18** 277-279.

Garces, L., Larenas, J., Smith, P., Sandino, S., Lannan, C. and Fryer, J. (1991) Infectivity of a rickettsia isolated from Coho salmon, *Oncorhynchus kisutch* . *Diseases of Aquatic Organisms*. **11** 93-97.

Gibson, T.J. (1984) Studies on the Epstein-Barr virus genome. PhD Thesis, Cambridge University, England.

Gilmore, R., Ciepak, W., Poicastro, F. and Hackstadt, T. (1991) The 120 kDa outer membrane protein (rOMP B) of *Rickettsia rickettsii* is encoded by an unusually long open reading frame; evidence for protein processing from a arge precursor. *Molecular Microbiology* **5** 2361-2270.

Grant, A.N., Brown, A.G., Cox, D.I., Birkbeck, T.H. and Griffen,A.A. (1996) Rickettsia-like organism in farmed salmon. *Veterinary Record* **138** 423

Hacker, J. and Fischer, G. (1993) Immunophilins: structure-function relationship and possible role in microbial pathogenicity. *Molecular Microbiology* **10** 445-456.

Hahn, D., Amann, R. and Zeyer, J. (1993) Whole-cell hybridisation of *Frankia* strains with flourescence or digoxigenin labeled 16S rRNA targeted oligonucleotide probes. *Applied and Enviromental Microbiology* **59** 1709-1716.

Hanson, B. (1985) Identification and partial characterisation of *Rickettsia tsutsugamushi* major protein immunogens. *Infection and Immunity* **50** 603-609.

Hase, T. (1985) Developmental sequence and surface membrane assembly of Rickettsiae. *Annual Review of Microbiology* **39** 69-88.

Heinzen, R.A., Frazier, M.E. nad Mallavia, L.P. (1991) Sequence and linkage analysis of the *Coxiella burnetii* citrate synthase-encoding gene. *Gene* **109** 63-69

Heinzen, R., Hayes, S., Peacock, M. and Hackstadt, T. (1993) Directional actin polymerisation associated with Spotted Fever group Rickettsia infection of *Vero* cells. *Infection and Immunity* **61** 1926-1935.

Hendrix, L. (1993) Cloning and sequencing of *Coxiella burnetii* outer membrane protein gene *com1*. *Infection and Immunity* **161** 470-477.

Hickman, C. (1991) Molecular cloning and sequence analysis of a *Rickettsia tsutsugmushi* 22 kDa antigen containing B and T cells epitopes. *Microbial Pathogenesis* **11** 19-31.

Jha, P. and Sarkar, S. (1997) DNA sequencing and comparitive sequence analysis reveal that the *E. coli* genomic DNA may replace the target DNA during molecular cloning: Evidence for the erroneous assembly of *E. coli* into the database sequences. *Comparitive Biochemistry and Physiology* **118B** 333-339.

Kelly, D., Dasch, G., Chan, T. and Ho, T. (1994) Detection and characterisation of *Rickettsia tsutsugamushi* in infected chiggers with PCR. *Journal of Entomology* **31** 691-699.

Kuzyk, M.A., Thornton, J.C. and Kay, W.W. (1996) Antigenic characterisation of the salmonis pathogen *Piscickettsia salmonis* . *Infection and Immunity* **64,** p5205-5210

Lannan, C., Winton, J. and Fryer, J. (1984) Fish cell lines: establishment and characterisation of nine cell lines from salmonids. *In Vitro* **20** 671-676.

Lannan, C.N., Ewing, S.A. and Fryer, J.L. (1991). A fluorescent antibody test for detection of the rickettsia causing disease in Chilean salmonids. *Journal of Aquatic Animal Health* **3** 229-234.

Lannan, C. and Fryer, J. (1994) Extracellular survival of *Piscirickettsia salmonis*. *Journal of Fish Diseases* **17** 545-548.

LeGall, G., and Mialhe, E. (1992) Purification of rickettsial-like organisms associated with *Pecten maximus* (Mollusca: Bivalvia): serological and biochemical characterisation, *Diseases of Aquatic Organisms*, **12**, 215-220 .

Leong, J., (1993) Molecular and biotechnological approaches to fish vaccines, *Current Opinions in Biotechnology,* **4,** 286-293 .

Leong, J. (1995) Molecular basis of immunity and disease: Molecular biological tools to detect fish pathogens. *Journal of Fish Biology,*. **47** 61-75.

Lillehaug, A. A. (1990) Field trial of vaccination against cold-water vibriosis in atlantic salmon (*Salmo salar* L) *Aquaculture* **84** 1-12

Ludwig, B., Rahfeld, J., Schmidt, B., Mann, K., Wintermeyer, E., Fischer, G. and Hacker, J. (1994) Characterisation of *mip* proteins of *Legionella pneumophila FEMS Microbiology Letters* **118** 23-30.

Lundemose, A., Rouch, D., Birkelund, S., Christiasen, G., and Pearce, J. (1992) *Chlamydia trachomatis* MIP-like protein. *Molecular Microbiology*. **17** 2539-2548.

Luo, GZ, an, G and Wu, R. (1994) A PCR differential screening method for rapid isolation of clones from a cDNA library. *Biotechniques* **16** 670-675.

Mackie, T.J. and Menzies, W.J.M. (1938) Investigations in Great Britain of furunculosis of the Salmonidae. *Journal of Comparative Pathology and Therapeutics*. **51** 225-234

Mahan, S., McGuire, T., Srmu, S., Bowie, M., Jonejan, F., Rurangirwa, F. and Barbet, A. (1994) Molecular cloning of a gene encoding the immunogenic 21 kDa protein of *Cowdria ruminatum.*. *Microbiology* **140** 2135-2142.

Mallavia, L.(1991) Genetics of rickettsiae. *European Journal of Epidemiology* **7** 213-221.

Manor, E., Carbonetti, N. and Silverman, D. (1994) *Rickettsia rickettsii* has proteins with cross-reacting epitopes to eukaryotic phospholipase A2 and phospholipase C. *Microbial Pathogenesis* **17** 99-109.

Mauel, M. J., Giovannoni, S. J. and Fryer, J. L. (1996) Development of polymerse chain reaction assays for detection, identification, and differentiation of *Piscirickettsia salmonis*. *Diseases of Aquatic Organisms* **26** 189-195.

McDonald, G. (1987) Cloned gene of *Rickettsia rickettsii* surface antigen, candidate vaccine for Rocky Mountain Spotted Fever. *Science* **235** 83.

Mege, J.-L., Maurin, M., Capo, C. and Raoult, D. (1997) *Coxiella burnetii* : the 'query' fever bacterium. A model of immune subversion by a strictly intracellular microorganism. *FEMS Microbiological Reviews* **19** 209-217.

Middtlyng, P.J. (1997) Vaccination against furunculosis. pp.382-404 in: Bernoth, E.-M., Ellis, A.E., Midtlyng, P.J, Olivier, G. and Smith, P. (eds) *Furunculosis Multidisciplinary Fish Disease Research* Academic Press London

Mo, Y. and Cianciotto, N. (1995) Molecular cloning of a *Coxiella burnetii* gene encoding a macrophage infectivity potentiator (*mip*) analogue. *Microbiology* **141** 2861-2871 .

Moulder, J. (1985) Comparitive biology of intracellular parasitism. *Microbiological Reviews* **49** 298-337.

Munro, A.L.S. and Gauld, J.A. (1996) SOAEFD Marine Laboratory Aberdeen, Scottish Fish Farms Annual Production Survey 1995, 35 pp.

Murray, N.E., Brammer, W.J. and Murray, K. (1977). Lambiod phages that simplify the recovery of *in vitro* recombinants. *Molecular General Genetics* **150** 53-56.m

Oaks, E., Rice, R., Kelly, D. and Stoner, C. (1989) Antigenic and genetic relatedness of eight *Rickettsia tsutsugamushi* antigens. *Infection and Immunity* **57** 3116-3122.

Ohashi, N., Nashimoto, H., Ikeda, H. and Tamnra, A. (1990) Cloning, sequencing of the gene (*tsg56*) encoding a type-specific antigen from *Rickettsia tsutsugamushi* . *Gene* **91** 119-122.

Olsen, A.B., Evensen, Ø, Speilberg, L., Melby, H.P., Håstein, T. (1993) Laksesykdom forårsaket av rickettsie. *Norsk Fiskeoppdrett NR.* **12** 40-41.

Olsen, A.B., Melby, H.P., Speilberg, L., Evensen, Ø and Håstein, T. (1997) *Piscirickettsia salmonis* infection in Atlantic salmon *Salmo salar* in Norway- epidemiological, pathological and microbiological findings. *Diseases of Aquatic Organisms.* **31,** 35-48

Ormsbee, R. (1985) Rickettsiae as organisms. *Acta Virologica* **29** 432-447.

Osterman, J. (1985) Rickettsiae and hosts. *Acta Virologica* **29** 166-173.

Ozel, M. and Schwanz-Pfitzner I. (1975) Vergleichende elektronenmikroskopische Untersuchungen an Rhabdoviren pflanzlicher und tierscher Herkunft: İII. Egtved-Virus (VHS) der Regenbogenforelle (*Salmo gairdneri*) und Rickettsienahliche Organismen. *Zentralblatt fuerr Bakteriologie Mikrobiologie und Hygiene, I Abt Originale A* **230** 1-14

Plamer, R., Ruttledge, M., Callanan, K. and Drinan, E. (1997) A Piscirickettsiosis-like disease in farmed Atlantic salmon in Ireland- Isolation of the agent.

Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Proceedings of the National Academy of Science USA* **85** 2444-2448.

Plumb, J. and Wolf, K. (1971) Fish cell growth rates: quantitative comparison of RTG-2 cell growth at 5-25 °C. *In Vitro* **7** 42-45.

Raoult, D. and Roux, V. (1997) Rickettsioses as paradigms of new or emerging infectious diseases.*Clinical Microbiology Reviews* **10** 694-719.

Regnery, R. L., Spruill, C. L. and Plikaytis, B.D. (1991) Genotypic identification of Rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettisal genes. *Journal of Bacteriology* **173** 1576-1589.

Renault, T. (1994) Rickettsia-like organisms in the cytoplasm of gill epithelial cells of the Pacific oyster, *Crassostrea gigas*. *Journal of Invertebrate Pathology* **64** 160-162.

Rkihisa, Y., Ewing, S. and Fox, J. (1994) Western Immunoblot analysis of *Ehrlichia chaffee*, *Ehrlichia canis*, or *Ehrlichia ewingii* infections in dogs and humans. *Journal of Clinical Microbiology* **32** 2107-2112 .

Rodger, H. and Drinan, E. (1993) Observation of a rickettsia-like organism in Atlantic salmon, *Salmo salar* L, in Ireland. *Journal of Fish Diseases* **16** 361-369.

Rokaw, M.D. (1996) Regulation of a sodium channel- associated G-protein by aldosterone. *Journal of Biological Chemistry* **271** 4491-4496.

Sambrook,J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Schuenke, K. and Walker, D. (1994) Cloning, sequencing and expression of the gene coding for an antigenic 120 kDa protein of *Rickettsia conorii* . *Infection and Immunity* **62** 904-909.

Seong, S.Y, Park, S.G., Huh, M.S., Jang, W.J., Kim, H.R., Han, T.H., Choi, M.S., Chang, W.H. and Kim, I.S. (1997) Mapping of antigenic determinant regions of the *Bor56* protein of *Orientia tsutsugamushi* . *Infection and Immunity* **65** 5250-5256.

Shankarappa, B., Dutta, S. and Mattingly-Napier, B. (1992) Antigenic and genomic relatedness among *Ehrlichia risticii*, *Ehrlichia sennetsu* and *Ehrlichia canis*. *International Journal of Systematic Bacteriology* **42** 127-132.

Shankarappa, B., Dutta, S. and Mattingly-Napier, B.(1992) Identification of the protective 44 kDa recombinant antigen of *Erlichia risticii*. *Infection and Immunity* **60** 612-617.

Shaw, H. and Wood, D. (1994) Characterisation of *Rickettsia rickettsii* DNA fragment analogous to the *firA* ORF 17-lpxA region of *Escherichia coli* . *Gene* **140** 109-113.

Silhavy, T.J., Lopilato, J.E., Garwin, G.L., Emr, S.J., Beckwith, J.R. (1984) D-Ribose metabolism in *Escherichia-coli* K-12 - genetics, regulation, and transport *Journal of Bacteriology*, **158**, 665-673

Silverman, D., Santucci, L., Meyers, N. and Sekeyova, Z. (1992) Penetration of host cells by *Rickettsia rickettsii* appears to be mediated by a phopholipase of rickettsial origin. *Infection and Immunity* **60** 2733-2740.

Sindermann, C.J. (1989) Mechanisms of Internal Defence Chapter 10, pp. 217-255 *In: Principal diseases of marine fish and shellfish Second Edition Vol. 1 Diseases of Marine Fish* Academic Press London.

Vaughan, L.M. (1997) *Aeromonas salmonicida* subspecies *salmonicida* - a molecular genetic perspective. Chapter 11, pp. 297- 322. *In :* Bernoth, E.-M., Ellis, A.E., Midtlyng, P.J, Olivier, G. and Smith, P. (eds) *Furunculosis Multidisciplinary Fish Disease Research* Academic Press London

Smith, D. (1979) Separation of inner and outer membranes of *Rickettsia prowazekii* and characterisation of their polypeptide compositions. *Journal of Bacteriology* **137** 963-971.

Starnbach, M.N., Falkow, S. and Tompkins, L.S. (1989). Species-specific detection of *Legionella pneumophila* in water by DNA amplification and hybridisation. *Journal of Clinical Microbiology* **27** 1257-1261.

Stover, C., Marana, D., Carter, M., Roe, B., Mardis, E. and Oak, E. (1990) The 56 kDa major protein antigen of *Rickettsia tsutsugamushi* ; Molecular cloning and sequencing analysis of the *sta56* gene and precise identification of a strain-specific epitope. *Infection and Immunity* **58** 2076-2084 .

Sumner, J.W., Sims, K.G., Jones, D. C. and Anderson, B.E. (1995) Protection of guinea-pigs from experimental Rocky Mountain Spotted Fever by immunisation with baculovirus-expressed *Rickettsia rickettsii* rOmpA protein. *Vaccine* **13** 29-35.

Teysseire, N., Chiche-Poriche, C. and Raoult, D. (1992) Intracellualr movements of *Rickettsia conorii* and *Rickettsia typhi* based on actin polymerisation. *Research in Microbiology* **143** 821-829.

Thorud, K. and Djupvik, H.O. (1988) Infectious anaemia in Atlantic salmon (*Salmo salar* L.) *Bulletin of the European Association of Fish Pathologists*, **8**, 109-111.

Uchiyama, T., Uchida, T., Wen, J. and Walker, D. (1994) Demonstration of heat-labile and heat stable epitopes of *Rickettsia japonica* on ultrathin sections. *Laboratory Investigation* **71** 432-437.

Valkova, D. and Kazar, J. (1995) A new plasmid (QpDV) common to *Coxiella burnetii* isolates associated with acute and chronic Q fever. *FEMS Microbiology Letters*. **125** 275-280.

Vaughan, L.M. (1997) *Aeromonas salmonicida* subspecies *salmonicida* - a molecular genetic perspective. Chapter 11, pp. 297- 322. *In :* Bernoth, E.-M., Ellis, A.E., Midtlyng, P.J, Olivier, G. and Smith, P. (eds) *Furunculosis Multidisciplinary Fish Disease Research* Academic Press London

Vodkin, M.H. and Williams, J.C. (1988) A heat-shock operon in *Coxiella-burnetii* produces a major antigen homologous to a protein in both mycobacteria and *Escherichia-coli. Journal of Bacteriology*, **.170** 1227-1234.

Walker, T. (1984) Rickettsial interactions with human endothelial cells *in vitro*: Adherence and entry. *Infection and Immunity* **44** 205-210.

Weisburg, W., Dobson, M., Samuel, J., Dasch, G., Mallavia, L., Baca, O., Mandelco, L., Sechrest, J., Weiss, J. and Woese, C. (1989) Phylogenetic diversity of the Rickettsiae. *Journal of Bacteriology* **171** 4202-4206.

Weiss, E., Coolbaugh, J. and Williams, J. (1975) Separation of viable *Rickettsia typhi* from yolk-sac and L-cell host components by Renografin Density Gradient centrifugation. *Applied Microbiology* **30** 546-463.

Weiss, E. (1982) The biology of Rickettsiae. *Annual Review of Microbiology* **36,** 345-370.

Weiss, E. and Moulder, J.W.(1984) Order 1. Rickettsiales Gieszczkiewicz 1939, 25[AL]. pp 687-729 In: Krieg N.R., (ed) *Bergey's Manual of Systematic Bacteriology, Vol. 2* Williams and Wilkins London

Weiss, A. A. (1994) Unorthodox secretion by Gram-negative bacteria pp. 341-349 in: Miller, V., Kaper, J., Portnoy, D., and Isberg, R., (eds.) *Molecular Genetics of Bacterial Pathogenesis*

Winkler, H. and Daugherty, R. (1989) Phospholipase A activity associated with the growth of *Rickettsia prowazekii* in L929 cells. *Infection and Immunity* **57** 36-40.

Winkler, H.H. (1990) Rickettsia Species (as organisms). *Annual Review of Microbiology* **44** 131-153.

Wood, D.O., Atkinson, W.H., Sikorski, R.S. and Winkler, H.H. (1983). Expression of the *R. prowazekii* citrate synthase gene in *E. coli*. **155**, p412-416.

Wood, D.O, Williamson, L.R., Winkler, H.H. and Krause, D.C. (1987) Nucleotide sequence of the *Rickettsia prowazekii* citrate synthase gene. *Journal of Bacteriology* **169** 3564-3572.

Wood, D., Solomon, M., and Speed, R. (1993) Characterisation of the *Rickettsia prowazeki pepA* gene encoding leucine aminopeptidase. *Journal of Bacteriology* **175** 159-165.

Xuejie, Y., Walker, D. and Jerrells, T. (1990) Polypeptides constituting the antigenic basis for identification of *Rickettsia sibirica* species by the standard sero-typing method for spotted fever group rickettsiae. *Acta Virologica*. **34** 71-79.

APPENDICES

APPENDIX 1

Composition of media, buffers and solutions

The following recipes are for broth cultures. For agar plates 15g/L of technical agar (Oxoid L13 agar No. 3) was added. Sterilisation was by autoclaving at 121°C for 15 min unless stated otherwise.

LB Medium **g/L**

Tryptone (Bacto)	10
Yeast extract	5
NaCl	10

Adjust to pH 7

To prepare Top Agarose for use in phage plaque assays add 0.7g/100 ml of molecular biology grade agarose.

SOC Medium **g/L**

Tryptone		20
Yeast extract		5
NaCl	(10 mM)	0.585
KCl	(2.5 mM)	0.186
Glucose	(20 mM)	3.6
$MgCl_2$	(10 mM)	0.952
$MgSO_4$	(10 mM)	1.204

M9 Minimal Medium

5x M9 salts solution	200 ml
20% glucose	20 ml
1M $MgSO_4$	2 ml
1M $CaCl_2$	0.1 ml

Make up to 1 L with dH_2O.

10X M9 Salts Solution

$Na_2HPO_4.7H_2O$	64g
KH_2PO_4	15g
NaCl	2.5g
NH_4Cl	5g

The salts are dissolved in 1L of dH_2O, divided into 200 ml aliquots and sterilised by autoclaving.

The $MgSO_4$ and $CaCl_2$ solutions are prepared separately and autoclaved before addition to diluted 5 x M9 salts solution. Glucose should be sterilised by filtration before it is added to the diluted M9 salts solution.

NZCYM MEDIUM

NZ amine (casein hydrolysate : ICN pharmaceuticals)	10g
NaCl	5g
Yeast extract	5g
Casamino acids	1g
$MgSO_4.7H_20$	2g

Sterilise by autoclaving and store at room temperature.

2 x YT BROTH

tryptone	16g
yeast extract	10g
NaCl	5g

Make up to 1L with dH_20 and shake until dissolved. Autoclave to sterilise.

SOC medium

tryptone	20 g
yeast extract	5 g
NaCl	0.5 g
250 mM KCl	10 ml

Mix until dissolved and adjust to 1 L with dH_2O. Sterilise by autoclaving then add 5 ml 2M $MgCl_2$ and 20 ml 1 M Glucose.

TE (Tris.EDTA) pH7.4

10 mM Tris.HCl	(pH 7.4)
1 mM EDTA	(pH 8.0)

TE pH 7.6 and TE pH 8.0 were prepared similarly using 10mM Tris.HCl solutions of appropriate pH.

TBE (Tris Borate EDTA Buffer)

Prepare a 10x stock and dilute 1/10 with dH_2O to prepare a working solution.

10x stock TBE	g/L
Tris	108 g
Boric acid	55 g
0.5 M EDTA (pH 8.0)	40 ml

SM buffer

NaCl	5.8 g
$MgSO_4.7H_2O$	2 g

Dissolve in 900 mls of dH_2O

Add 50 ml 1 M Tris.HCl (pH 7.5) and 5 ml 2% gelatin

Sterilise by autoclaving and store at room temperature.

0.5 M EDTA pH 8.0

186.1 g of disodium ethylenediaminetetraacetate.$2H_2O$ added to 800 ml of dH_2O, stirred vigorously on a magnetic stirrer before adjusting the pH to 8.0 with NaOH (this requires approx. 20 g of pellets). Dispensed into aliquots and sterilised by autoclaving.

Isopropylthio-b-D-galactoside (IPTG)

2 g of IPTG was dissolved in 8 ml of dH_2O, the volume was adjusted to 10 ml and the solution sterilised by filtration through a 0.22 µm disposable filter. The solution was dispensed into 1 ml aliquots and stored at -20°C.

Phosphate Buffered Saline (PBS)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Dissolve in 800 ml of dH_2O, adjust the pH to 7.4 with HCl and make up to 1 L with dH_2O. The solution was dispensed into aliquots, sterilised by autoclaving and stored at room temperature.

Proteinase K

A stock solution of 20 mg/ml was prepared for use at a final concentration of 20 µg/ml.

Lysozyme

A stock solution of 50 mg/ml was prepared in dH_2O, dispensed into small aliquots and stored at -20°C. After thawing for use, any surplus is discarded.

RNase (DNase-free)

RNase A was dissolved in 0.01 M sodium acetate solution (pH 5.2) at a concentration of 10 mg/ml, heated to 100°C for 15 min and cooled slowly to room temperature. The

pH is adjusted by addition of 0.1 vol. of 1 M Tris.HCl (pH7.4), the solution dispensed into aliquots and stored at -20°C

Digestion Buffer for isolation of *Legionella pneumophila* DNA

100 mM Tris.HCl

1 mM EDTA pH 8.5

2% Laureth 10

400 mg Proteinase K/ ml

Sepahaglas reagents

N.B. complete formulae are not given by the manufacturer.

Sephaglas : Sephaglas BP suspended in a solution of NaI Tris.HCl

Gel Solubilizer : Solution of NaI buffered with Tris.HCl

Elution Buffer : 10 mM Tris.HCl (pH8.0), 0.1 mM EDTA

Wash Buffer : Buffered salt solution with EtOH

SOLUTIONS REQUIRED FOR IMMUNOBLOTTING

Tris Buffered Saline TBS pH7.5

NaCl	29.22 g
Tris	2.42 g

Add dH_2O to 1L and sterilise by autoclaving.

TTS (Tris Tween Solution)

To 1L of sterile TBS add 0.5 ml of Tween 20

Colour Development Solution

4-chloro-1-napthol	60 mg
Methanol	20 ml

H_2O_2 0.06 ml

Make up to 100 ml with sterile TBS.

SOLUTIONS REQUIRED FOR PLASMID PREPARATIONS

Kado and Liu alkaline lysis buffer

Tris	0.303g
SDS	1.5g
H_2O	70 ml

Adjust to pH 12.4 by addition of 1N NaOH and make up to 100 ml with dH_2O.

PROMEGA Wizard Miniprep Buffers

Cell Resuspension Solution

50 mM Tris.HCl pH7.5

10 mM EDTA

100 mg/ml RNase A

Cell Lysis Solution : 1 M NaOH, 1% SDS

Neutralization solution : 1.32M potassium acetate

Column Wash Solution (concentration prior to EtOH addition)

200 mM NaCl

20 mM Tris.HCl pH7.5

5 mM EDTA

Dilute with 95% ethanol. Final ethanol concentration should be approx. 55%

Wizard Plus SV Cell Resuspension Solution

50 mM Tris-HCl, pH 7.5

10 mM EDTA

100 mg/ml RNase A

Wizard Plus SV Cell Lysis Solution

1 M NaOH

1% SDS

Wizard Plus SV Neutralization Solution

1 M Guanidine hydrochloride

0.759 M Potassium acetate

1 M Glacial acetic acid

Final pH is approximately 4.2.

Wizard Plus SV Column Wash Solution

1 mM Potassium acetate

27.1 mM Tris-HCl, pH 7.5

SOLUTIONS REQUIRED FOR SOUTHERN BLOTTING

20 X SSC	**per L**
NaCl	175.3 g
Sodium citrate	88.2 g

Add 800 ml of dH_2O and adjust the pH to 7.0 by careful addition of a few drops of 10N NaOH. Adjust the volume to 1L with dH_2O, dispense into aliquots and sterilis by autoclaving.

10% Sodium dodecyl sulphate (SDS)

100g of electrophoresis grade SDS was dissolved in 900 ml dH_2O and heated to 68°C to aid dissolution. The pH was adjusted to 7.2 by addition of dilute HCl, the volume adjusted to 1L with dH_2O and the solution dispensed into aliquots.

Southern blotting denaturation solution

1 M NaCl

0.5 M NaOH

Southern blotting neutralisation solution

1 M Tris.HCl

1.5 M NaCl pH 8.0

SOLUTIONS REQUIRED FOR CONSTRUCTION AND MANIPULATION OF THE LAMBDA FIXII LIBRARY

10 x STE

1 M NaCl

200 mM Tris.HCl 9pH 7.5)

100 mM EDTA

10 x Fill-in Buffer

60 mM Tris.HCl 9pH8.0)

60 mM NaCl

60 mM $MgCl_2$

0.5% gelatin

10 mM DTT

STRATAGENE Denaturing Solution

1.5 M NaCl

0.5 M NaOH

STRATAGENE Neutralisation Solution

1.5 M NaCL

0.5 M Tris.HCl (pH 8.0)

STRATAGENE Rinse Solution

0.2 M Tris.HCl (pH 7.5)

2 x SSC

SOLUTIONS REQUIRED FOR DIG HYBRIDISATIONS

DIG Prehybridisation Solution

5 x SSC

1 % blocking solution

0.1% N-lauroylsarcosine

0.02% SDS

DIG Hybridisation solution

As in prehybridisation with DIG-labelled probe.

DIG Maleic Acid Buffer

0.1 M Maleic acid

0.15 M NaCl pH 7.5

DIG Wash Buffer

0.1 M Maleic acid

0.15 M NaCl pH 7.5

0.3% Tween-20

DIG Detection Buffer

0.1 M Tris.HCl

0.1 M NaCl

50 mM $MgCl_2$ pH 9.5

DIG Salt stringency wash solutions

2 x SSC, 0.1% SDS

0.1 x SSC, 0.1% SDS

SOLUTIONS REQUIRED FOR MANUAL SEQUENCING

10% Ammonium Persulphate

0.1 g of ammonium persulphate added to 1 ml of dH_2O. The solution must be prepared freshly when required.

SOLUTIONS FOR TISSUE CULTURE

MEM

Minimum Essential Media with Earles, w/o Glutamine. and the addition of 10% FCS and 1% L-Glutamine.

APPENDIX 2

ag27 electrophoetogram

The pExCell MCS lines up with ag27 at nt. 2153-2109. The *EcoRI* site is visibly absent.

ABI PRISM™

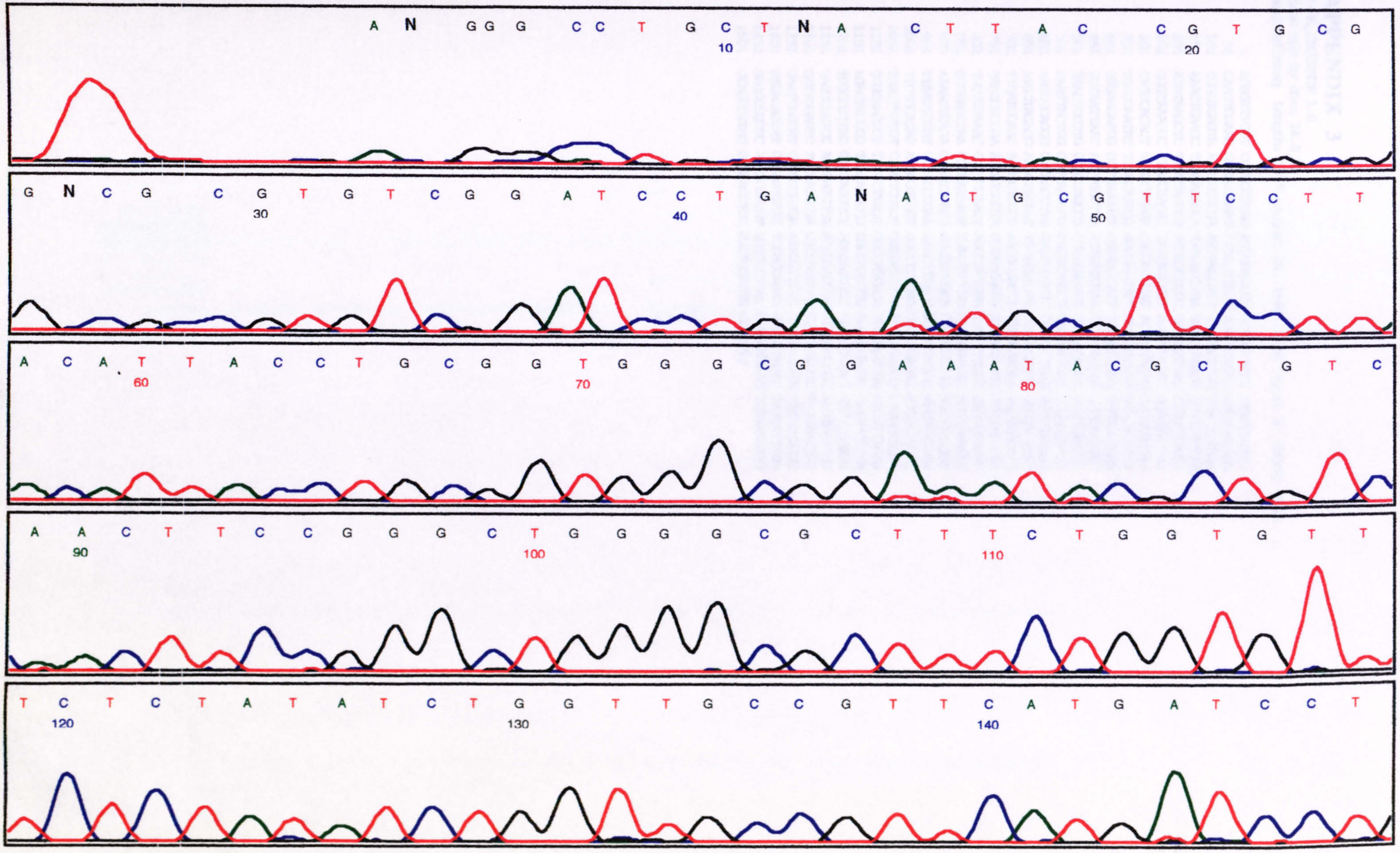
Model
Version 2.1.1

20•AG 27.1
10 MIN 2-20 OK

Lane 20

Signal G:92 A:314 T:142 C:126
DT6%Ac{A Set-AnyPrimer}
New Matrix 28 May 96
Points 798 to 8350 Base 1: 798

Tue, Apr 29, 1997 9:41 am
Mon, Apr 28, 1997 4:23 pm
Spacing: 9.30



APPENDIX 3

!!NA_SEQUENCE 1.0
from thb by disc 24.8.98
ard16sfinseq Length: 1537 August 24, 1998 18:40 Type: N Check: 9812 ..

```
   1  AGAGTTTGAT CCTGGCTCAG ATTGAACGCT GGIGGCATGC TTAACACATG
  51  CAAGTCGAAC GGTAGCAGAT CTAGCTTGCT AGATGCTGAC GAGTGGCGGA
 101  CGGGTGAGTA ACGCGTGGGA ATTTACCTTT TAGTGGGGGA TAACTTTAGG
 151  AAACTGAAGC TAATACCGCA TAAGACCTGA GGGTTAAAGA GGGCCTCTAT
 201  ATATAAGCTC TTGCTAGGAG ATGAGCCCGC GTTGGATTAG CTAGTTGGTA
 251  GGGTAAAGGC TTACCAAGGC GACGATCCAT AGCTGGTTTG AGAGAATGGC
 301  CAGCCACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG GAGGCAGCAG
 351  TGGGGAATAT TGGACAATGG GGGGAACCCT GATCCAGCAA TGCCACGTGT
 401  GTGAAGAAGG CCTTAGGGTT GTAAAGCACT TTCAGCGGGG AGGAAGGTAA
 451  GCTAATTAAT ACTTGGCTTA ATTGACGTTA CCTGCAGAAG AAGCACCGGC
 501  TAACTCCGTG CCAGCAGCCG CGGTAATACG GAGGGTGCGA GCGTTAATCG
 551  GAATTACTGG GCGTAAAGGG CGCGTAGGCG GAAGATTAAG TTGGATGTGA
 601  AATCCCAGGG CTCAACCTTG GAACTGCATC CGAAACTGGT ATTTTAGAGT
 651  ATGGTAGAGG AAAGTGGAAT TTCAGGTGTA GCGGTGAAAT GCGTAGATAT
 701  TTGAAGGAAC ACCGGTGGCG AAGGCGACTT TTTGGATCAA TACTGACGCT
 751  GAGGCGCGAA AGCATGGGTA GCGAACAGGA TTAGATACCC TGGTAGTCCA
 801  TGCTGTAAAC GATGTCAACT AGCCGTTGGA TTCCCTTGAG GAGTTTAGTG
 851  GCGTAGCTAA CGCGATAAGT TGACCGCCTG GGGAGTACGG CCGCAAGGTT
 901  AAAACTCAAA GGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT
 951  TTAATTCGAT GCAACGCGAA GAACCTTACC TGGTCTTGAC ATCCTAAGAA
1001  TTCTGCAGAG ATGCGGAAGT GCCTTCGGGA GCTTAGAGAC AGGTGCTGCA
1051  TGGCTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT CCCGCAACGA
1101  GCGCAACCCT TATCCTTAGT TGCCAGCACG TGATGGTGGG AACTCTAGGG
1151  AGACTGCCGG TGATAAACCG GAGGAAGGTG GGGACGACGT CAAGTCATCA
1201  TGGCCCTTAC GACCAGGGCT ACACACGTGC TACAATGGGG CGTACAGACG
1251  GAGGCGAAGC AGCGATGTGG AGCGAACCTG AGAAAGCGCC TCGTAGTCCG
1301  GATTGGAGTC TGCAACTCGA CTCCATGAAG TCGGAATCGC TAGTAATCGC
1351  GAATCAGCAT GTCGCGGTGA ATACGTTCCC GGGCCTTGTA CACACCGCCC
1401  GTCACACCAT GGGAGTGAAT TGCACCAGAA GGGGCTAGGC TAACTTTAGG
1451  GAGGCCGGTC ACCACGGTGT GGTTCATGAC TGGGGTGAAG TCGTAACAAG
1501  GTAGCCGTAG GGGAACCTGC GGCTGGATCA CCTCCTT
```

Appendix 4 Base differences between 16SrRNA genes of Piscirickettsia isolates, raw data from Table 13
Plurality: 2.00 Threshold: 1 AveWeight 1.00 AveMatch 1.00 AvMisMatch 0.00
PRETTY of: ard.msf{*} September 28, 1998 19:11 ..

```
                1                                                50  51                                                100
  ard.msf{ard1}
ard.msf{u55015}
ard.msf{u36941}
ard.msf{u36942}
ard.msf{u36915}            a
ard.msf{u36940}            a
      Consensus AGAGTTTGAT CCTGGCTCAG ATTGAACGCT GGTGGCATGC TTAACACATG  CAAGTCGAAC GGTAGCAGAT CTAGCTTGCT AGATGCTGAC GAGTGGCGGA

              101                                               150  151                                               200
  ard.msf{ard1}
ard.msf{u55015}
ard.msf{u36941}
ard.msf{u36942}
ard.msf{u36915}
ard.msf{u36940}
      Consensus CGGGTGAGTA ACGCGTGGGA ATTTACCTTT TAGTGGGGGA TAACTTTAGG  AAACTGAAGC TAATACCGCA TAAGACCTGA GGGTTAAAGA GGGCCTCTAT

              201                                               250  251                                               300
  ard.msf{ard1}
ard.msf{u55015} t
ard.msf{u36941} t
ard.msf{u36942}
ard.msf{u36915}                                                    .
ard.msf{u36940}                                                    a
      Consensus ATATAAGCTC TTGCTAGGAG ATGAGCCCGC GTTGGATTAG CTAGTTGGTA  GGGTAAAGGC TTACCAAGGC GACGATCCAT AGCTGGTTTG AGAGAATGGC

              301                                               350  351                                               400
  ard.msf{ard1}
ard.msf{u55015}
ard.msf{u36941}
ard.msf{u36942}
ard.msf{u36915}
ard.msf{u36940}                                                    a          ...  c at
      Consensus CAGCCACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG GAGGCAGCAG  TGGGGAATAT TGGACAATGG GGGGAACCCT GATCCAGCAA TGCCACGTGT

              401                                               450  451                                               500
  ard.msf{ard1}                                    .
ard.msf{u55015}                                    .
ard.msf{u36941}                                    .
ard.msf{u36942}                                    .
ard.msf{u36915}                                    g
ard.msf{u36940}                                    .
      Consensus GTGAAGAAGG CCTTAGGGTT GTAAAGCACT TTCAGCGGGG AGGA-AGGTA  AGCTAATTAA TACTTGGCTT AATTGACGTT ACCTGCAGAA GAAGCACCGG
```

```
                501                                                 550  551                                                 600
  ard.msf{ard1}
ard.msf{u55015}
ard.msf{u36941}
ard.msf{u36942}
ard.msf{u36915}   .
ard.msf{u36940}                    g                    c
      Consensus CTAACTCCGT GCCAGCAGCC GCGGTAATAC GGAGGGTGCG AGCGTTAATC  GGAATTACTG GGCGTAAAGG GCGCGTAGGC GGAAGATTAA GTTGGATGTG

                601                                                 650  651                                                 700
  ard.msf{ard1}                                                    t
ard.msf{u55015}
ard.msf{u36941}
ard.msf{u36942}           a
ard.msf{u36915}
ard.msf{u36940}                                                                        .
      Consensus AAATCCCAGG GCTCAACCTT GGAACTGCAT CCGAAACTGG TATTCTAGAG  TCTGAAGGAA CACCGGTGGC GAAGGCGACT TTCTGGATCA ATACTGACGC

                701                                                 750  751                                                 800
  ard.msf{ard1}  t                           t
ard.msf{u55015}
ard.msf{u36941}
ard.msf{u36942}
ard.msf{u36915}                    .
ard.msf{u36940}              c     .
      Consensus TCTGAAGGAA CACCGGTGGC GAAGGCGACT TTCTGGATCA ATACTGACGC  TGAGGCGCGA AAGCATGGGT AGCGAACAGG ATTAGATACC CTGGTAGTCC

                801                                                 850  851                                                 900
  ard.msf{ard1}                                                                    g                 g
ard.msf{u55015}                                                                    g                 g
ard.msf{u36941}                                                                    g                 g
ard.msf{u36942}                                                                  .     .             c
ard.msf{u36915}                                                                  ..                c c
ard.msf{u36940}                                                                                    c c
      Consensus ATGCTGTAAA CGATGTCAAC TAGCCGTTGG ATTCCCTTGA GGAGTTTAGT  GGCGTAGCTA ACGCGATAAG TTGACCCCCT GGGGAGTACG GCC-CAAGGT

                901                                                 950  951                                                1000
  ard.msf{ard1}                    a         g
ard.msf{u55015}
ard.msf{u36941}                              .                                   .
ard.msf{u36942}          c                                                       .
ard.msf{u36915}            .. g a tg                                             .
ard.msf{u36940}                    .           a ca g                    ..cg at
      Consensus TAAAACTCAA AGGAATTGTC GGGGGCCCCC ACAAGCGGTG GAGCATGTGG  TTTAATTCGA TGCAACGCGA AGAACCTTAC CTGGTCTTGA CATCCTAAGA
```

```
                1001                                                1050  1051                                                1100
  ard.msf{ard1}      g
ard.msf{u55015}
ard.msf{u36941}      .
ard.msf{u36942}                                                                                                          .
ard.msf{u36915}      .
ard.msf{u36940}  g tgat       att  t                                                                                     c
      Consensus  ATTCTCCAGA GATGCGGAAG TGCCTTCGGG AGCTTAGAGA CAGGTGCTGC  ATGGCTGTCG TCAGCTCGTG TCGTGAGATG TTGGGTTAAG TCCCGCAACG

                1101                                                1150  1151                                                1200
  ard.msf{ard1}
ard.msf{u55015}
ard.msf{u36941}
ard.msf{u36942}
ard.msf{u36915}
ard.msf{u36940}                                     a
      Consensus  AGCGCAACCC TTATCCTTAG TTGCCAGCAC GTGATGGTGG GAACTCTAGG  GAGACTGCCG GTGATAAACC GGAGGAAGGT GGGGACGACG TCAAGTCATC

                1201                                                1250  1251                                                1300
  ard.msf{ard1}
ard.msf{u55015}
ard.msf{u36941}
ard.msf{u36942}
ard.msf{u36915}
ard.msf{u36940}
      Consensus  ATGGCCCTTA CGACCAGGGC TACACACGTG CTACAATGGG GCGTACAGAC  GGAGGCGAAG CAGCGATGTG GAGCGAACCT GAGAAAGCGC CTCGTAGTCC

                1301                                                1350  1351                                                1400
  ard.msf{ard1}
ard.msf{u55015}
ard.msf{u36941}
ard.msf{u36942}
ard.msf{u36915}
ard.msf{u36940}
      Consensus  GGATTGGAGT CTGCAACTCG ACTCCATGAA GTCGGAATCG CTAGTAATCG  CGAATCAGCA TGTCGCGGTG AATACGTTCC CGGGCCTTGT ACACACCGCC

                1401                                                1450  1451                                                1500
  ard.msf{ard1}
ard.msf{u55015}    c
ard.msf{u36941}
ard.msf{u36942}
ard.msf{u36915}
ard.msf{u36940}
      Consensus  CGTCACACCA TGGGAGTGAA TTGCACCAGA AGGGGCTAGG CTAACTTTAG  GGAGGCCGGT CACCACGGTG TGGTTCATGA CTGGGGTGAA GTCGTAACAA
```

```
                1501                                                1538
  ard.msf{ard1}
ard.msf{u55015}                          g
ard.msf{u36941}
ard.msf{u36942}                        t t
ard.msf{u36915}                        t -
ard.msf{u36940}
     Consensus  GGTAGCCGTA GGGGAACCTG CGGCTGGATC ACCTCCTT
```

Appendix 5

Photocopy of publication

I would also like to suggest that the Ministry of Agriculture, Fisheries and Food be disbanded and divided into 'Fisheries', 'Food and Drugs' and 'Agriculture', as it appears that one organisation cannot do justice to consumers and purchasers at the same time, without compromising either one.

M. B. de C. Giles, *The Surgery, Balsam Fields, Wincanton, Somerset BA9 9HE*

SIR, – It is reassuring to learn that the Government intends to resist the pressures for extensive slaughter of cattle as a gesture of atonement to consumer confidence: whether the EU will permit such resistance remains uncertain. Those who would have us spend millions converting cattle into smoke, slaughtering living creatures, destroying livelihoods and creating unemployment in the 'good cause' of bowing to market forces, should consider whether such sums should be squandered to such ends.

If it is a marketing venture for British beef, how does it compare with subsidising the price for a few weeks until the next health panic – (?) brain damage from portable phones (*Sunday Times*, April 14) – occupies the media? If it is an investment in public health, how does it compare with a similar expenditure on renal or intensive care units, first class hospitals like Barts, or research on umpteen diseases far more important than CJD? If it goes 'beyond science' (that is, to the irrational), how much blood must be shed to placate disproportionate and misdirected fear? BSE is already vanishing fast.

If there was a link between BSE and a new form of CJD, which *The Lancet* article does not prove, it is a reflection of a situation which existed several years ago, not currently. Why, after 10 years of BSE, do abattoir workers seem to have escaped CJD, exposed as they are to casual inhalation, ingestion and abrasion – why, instead, are vicars peculiarly prominent among the occupational groups manifesting this disease (CJD Surveillance Data 1995)? The 10 new cases of CJD in young people (Will and others 1996) share unusual pathology. This includes marked damage to the basal ganglia, a feature previously associated with possible adherence of the infective agent to growth hormone or other hormones with hypothalamic receptors (Markus and others 1992), although administration of hormones is excluded as a cause in these patients. *The Lancet* patients share the methionine homozygote at codon 129, as do 83 per cent of sporadic CJD cases in the UK (Will and others 1996) and four out of nine cases of iatrogenic CJD in the USA and France, whereas in the UK, four out of seven iatrogenic CJD cases were valine homozygotes (Brown and others 1992).

Why have the media encouraged so much alarm about beef when the supposed risk, if it exists, arises from lower grade carcase products? Why is there so little awareness that over 80 per cent of beef herds have never had a case of BSE: what economic or epidemiological sense can slaughter make in such herds? 'Seven epidemiological studies conclude that aluminium in drinking water is a risk factor for the expression of the dementia characteristic of Alzheimer's disease ... no published study has failed to find a statistically significant relation between aluminium *in drinking water and Alzheimer's disease*' (McLachlan 1991). Where is the fuss? Yet if CJD increased 10-fold, it would still only be *1 per cent as likely to afflict beef-eaters* (or anyone else) as Alzheimer's disease. The excess deaths from a single British winter kill as *many people as 400 years of CJD. Millions* might be better spent on warmth for the elderly rather than ritual incineration to appease exaggerated fears. Above all, national responses to perceived dangers should be based on authoritative risk assessment, not their emotive power and not the purging fires of spin-doctors, witch-doctors or sound-byte experts.

Bob Michell, *Brewers Cottage, 59 Brewhouse Hill, Wheathampstead, Hertfordshire AL4 8AN*

References

BROWN, P., PREECE, M. A. & WILL, R. G. (1992) *Lancet* **340**, 24

CJD SURVEILLANCE DATA (1995) Creutzfeldt-Jakob Disease Surveillance in the United Kingdom. 4th Annual Report. National CJD Surveillance Unit, Western Infirmary, Edinburgh

McLACHLAN, D. R. (1991) Alzheimer's Disease and the Environment. Ed Lord Walton. Round Table Series. London, Royal Society of Medicine

MARKUS, H. S., DUCHEN, L. W., PARKIN, G. M., KURTZ, A. B., JACOBS, H. S., COSTA, D. C. & HARRISON, M. J. (1992) *Quarterly Journal of Medicine* **82**, 43

WILL, R. G., IRONSIDE, J. W., ZEIDLER, M., ESTIBEIRO, K., ALPEROVITCH, A., POSER, S., POCCHIARI, M. & HOFMAN, A. (1996) *Lancet* **347**, 921

SIR, – In my letter (*VR*, April 20, p 399) I incorrectly attributed a reference to the eating of beef to Sir Toby Belch in *Twelfth Night*. The remark was in fact made by Sir Andrew Aguecheek.

Mandy Kellagher, *Cutmere Cottage, Cutmere, Tideford, Cornwall PL12 5JU*

Rickettsia-like organism in farmed salmon

SIR, – We wish to report the isolation of a rickettsia-like organism (RLO) from farmed Atlantic salmon (*Salmo salar*) associated with neurological signs and mortality.

Two fallow marine farms in the same Scottish loch were each stocked in April 1995 with healthy smolts from several fresh water sources. In November 1995, when the water temperature was 11°C, abnormal swimming and skin damage was noted in several pens on both farms along with an increase in mortality. To date, several thousand fish have been lost. Gross pathology in affected fish was unremarkable and the only internal change was splenomegaly. All major organs were sampled for known bacterial and viral pathogens with negative results.

Histopathological changes were evident in the brain, and in one fish the spleen also. Associated with areas of encephalitis and vasculitis in the brain were numerous basophilic intracellular coccoid bodies which stained positive for RNA and weakly positive for DNA. Explants of brain tissue from clinically affected fish were cultured on monolayers of antibiotic-free Chinook salmon embryo (CHSE) cells and produced a characteristic and replicable cytopathic effect within nine days at 15°C. The organism failed to react in a latex agglutination test for *Piscirickettsia salmonis*. Electron micrographs of culture supernatant revealed clusters of organisms within vacuoles inside degenerate CHSE cells. The organism is approximately 1 µm in diameter with a characteristically rippled outer membrane (Fig 1); a cell undergoing division can also be seen.

Culture supernatant was injected into disease-free fish held in tanks supplied with sterile fresh water and held at 12 to 13°C. A mortality of 100 per cent was produced within 18 days of injection and the organism was reisolated from dead fish. It is not known whether these fish had exhibited neurological signs before death. The organism is sensitive in vitro to oxytetracycline and oxolinic acid but there has been no recurrence to date of significant clinical disease in the field though a few affected fish can still be found.

Comps and others (1996) have recently reported the isolation of an RLO from juvenile Mediterranean sea-bass associated with neurological signs and high mortality. *Piscirickettsia salmonis* is the cause of a serious disease of farmed Atlantic and Pacific salmon in Chile (Fryer and others 1992), and Rodger and Drinan (1993) have reported an RLO in Atlantic salmon in Ireland. It is likely, therefore, that other rickettsiae will be found in farmed fish and may become significant pathogens under intensive conditions. Further investigations are underway to identify fully the organism isolated in Scotland and a full account of this case and subsequent studies will be submitted for publication in due course.

FIG 1: Rickettsia-like organism isolated from Atlantic salmon (x 57,000)

A. N. Grant, A. G. Brown, D. I. Cox, *Marine Harvest McConnell, Farms Office, Blar Mhor Industrial Estate, Fort William PH33 7PT*

T. H. Birkbeck, A. A. Griffen, *Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ*

References

COMPS, M., RAYMOND, J. C. & PLASSIART, G. N. (1996) *Bulletin of the European Association of Fish Pathologists* **16**, 30

FRYER, J. L., LANNAN, C. N., GIOVANONNI, S. J. & WOOD, N. D. (1992) *International Journal of Systematic Bacteriology* **42**, 120

RODGER, H. D. & DRINAN, E. M. (1993) *Journal of Fish Diseases* **16**, 361

Appendix 6a

Raw data for Figure 1

```
P A U P *
Portable version 4.0.0d55 for Unix
Wed Sep  2 20:17:46 1998
        -----------------------------NOTICE---------------------------
          PAUP* is experimental in this release.
          Please check your results carefully!
        --------------------------------------------------------------
```

Tree description:

Unrooted tree(s) rooted using outgroup method
Optimality criterion = maximum parsimony
Character-status summary:
Of 1588 total characters:
All characters are of type 'unord'
All characters have equal weight
637 characters are constant
305 variable characters are parsimony-uninformative
Number of parsimony-informative characters = 646
Character-state optimization: Accelerated transformation (ACCTRAN)
Multistate taxa interpreted as uncertainty

Tree number 1 (rooted using default outgroup)

Branch lengths and linkages for tree #1 (unrooted)

Node	Connected to node	Assigned branch length	Minimum possible length	Maximum possible length	% similarity
m73219 (1)	41	6	2	10	0.06
m21290 (2)	41	17	4	20	0.17
40	41	144	85	153	1.44
25	40	56	40	95	0.56
24	25	51	31	72	0.51
m73221 (3)	24	21	18	23	0.21
u03777 (4)	24	19	17	27	0.19
m60313 (5)	25	52	34	71	0.52
39	40	69	42	99	0.69
27	39	87	60	131	0.87
26	27	11	10	18	0.11
m21789 (6)	26	5	5	7	0.05
m20499 (7)	26	2	2	2	0.02
m21293 (8)	27	7	3	8	0.07
38	39	71	23	102	0.71
29	38	78	39	124	0.78
28	29	35	20	57	0.35
m65249 (9)	28	16	10	36	0.16
u28268 (10)	28	15	9	21	0.15
x13695 (11)	29	33	26	52	0.33
37	38	101	39	135	1.01
34	37	105	57	160	1.05
33	34	127	78	151	1.27
32	33	43	22	59	0.43
31	32	39	20	62	0.39
30	31	37	25	50	0.37
e05133 (12)	30	16	10	21	0.16
u88545 (13)	30	21	16	27	0.21
m59292 (14)	31	37	28	48	0.37
x16895 (16)	32	81	60	114	0.81
x60405 (15)	33	50	37	90	0.50
m21291 (17)	34	140	115	167	1.40
m59157 (18)	34	123	101	153	1.23
x60783 (19)	34	139	113	165	1.39
m21292 (20)	34	156	138	190	1.56
36	37	91	28	134	0.91
35	36	86	33	123	0.86
m58822 (21)	35	152	113	202	1.52
x51601 (22)	35	172	137	216	1.72
d89067 (23)	36	184	153	264	1.84

Appendix 6b

Raw data for Figure 33

Branch lengths and linkages for tree #1 (unrooted)

Node	Connected to node	Branch length	%
ard1 (1)	10	0.00317	0.31
9	10	0.00071	0.07
u55015 (2)	9	0.00158	0.16
8	9	0.00133	0.13
u36942 (4)	8	0.00224	0.22
7	8	0.00102	0.10
u36915 (5)	7	0.00273	0.27
u36940 (6)	7	0.01700	1.7
u36941 (3)	10	0.00009	0.009