Phenotypic and Genotypic analysis of intestinal spirochaetes

Sarah Jayne Rayment

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Aston University

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Summary

Pulsed field gel electrophoresis of 82 intestinal spirochaete isolates showed specific differentiation of *Serpulina pilosicoli* and *Serpulina hyodysenteriae* although considerable heterogeneity was observed, especially amongst *S. pilosicoli* isolates. In several cases genotypically similar isolates originated from different animals suggesting that cross-species transmission may have occurred.

The Caco-2 and Caco-2/HT29 cell models have been proposed as potentially realistic models of intestinal infection. Quantitation of adhesion to the cells showed isolate 382/91 (from a bacteraemia) to adhere at significantly greater numbers than any other isolate tested. This isolate produced a PFGE profile which differed from other *S. pilosicoli* isolates and so would be of interest for further study. Comparison of bacteraemic and other *S. pilosicoli* isolates suggested that bacteraemic isolates were not more specifically adapted for adhesion to, or invasion of the epithelial cell layer than other *S. pilosicoli* isolates. Genotypically similar isolates from differing animal origins adhered to the Caco-2 model at similar levels.

Generation of a random genomic library of *S. pilosicoli* and screening with species specific monoclonal antibody has enabled the identification of a gene sequence encoding a protein which showed significant homology with an ancestral form of the enzyme pyruvate oxidoreductase. Immunoscreening with polyclonal serum identified the sequences of two gene clusters and a probable arylsulphatase. One gene cluster represented a ribosomal gene cluster which has a similar molecular arrangement to *Borrelia burgdorferi*, *Treponema pallidum* and *Thermatoga maritima*. The other gene cluster contained an ABC transporter protein, sorbitol dehydrogenase and phosphomannose isomerase.

An ELISA type assay was used to demonstrate that isolates of *S. pilosicoli* could adhere to components of the extracellular matrix such as collagen (type 1), fibronectin, laminin, and porcine gastric mucin.

Keywords: intestinal spirochaete, pulsed-field gel electrophoresis, genomic library, pyruvate oxidoreductase, arylsulphatase, sorbitol dehydrogenase, ABC transporter, ribosomal protein, extracellular matrix, adhesion, Caco-2, Co-culture.

I would like to dedicate this thesis to my Nan, Joan Daniell, who taught me some of the most valuable lessons I have ever learned.

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Abbreviations

AMPS	Ammonium persulphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
CLO	Cyst-like object
DIG	Digoxigenin
DMSO	Dimethyl sulphoxide
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbant assay
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
LB	Luria Bertoni
MEE	Multi-locus enzyme electrophoresis
MOI	Multiplicity of infection
NBT	Nitroblue tetrazolium
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
POR	Pyruvate oxidoreductase
REA	Restriction endonuclease analysis
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TBS	Tris buffered saline
TBST	TBS-Tween
T_M	Melting point temperature
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Chapter 1: General introduction

1.1 Historical perspective

Spirochaetes were placed as early as Ehrenberg's 1838 catalogue of organisms with what are now recognised as bacteria. The first isolation of intestinal spirochaetes was not reported until 1884 by Escherich who was able to distinguish them from *Vibrio cholerae* in the stools of cholera patients in Naples. This was confirmed in 1885 by Kuisl and 1886 by Giauxxa and Lustig when the first drawings were made and in 1891 spirochaetes were associated with animal disease. These organisms were later classified as *Serpulina hyodysenteriae* and this species was shown to cause swine dysentery. In contrast, as early as 1893, Kowalski suggested that intestinal spirochaetes were part of the normal intestinal flora. This idea was later supported by Mathan and Mathan (1985) who found intestinal spirochaetes in a number of healthy individuals in southern India.

The first description of intestinal spirochaetes based on microscopy was made by Le Dantec in 1903 who examined a specimen from a dysentery patient in France who had returned from a visit to China. In 1909, Werner identified two morphologically distinct types of spirochaete in his own stools. The first type was a loosely coiled form which he called *Spirochaeta eurygyrata* and the second type was a tightly coiled form termed *Spirochaeta stenogyrata*. The latter term largely disappeared from use but several subsequent reports mentioned *S. eurygyrata*. Fantham (1916) and Macfie (1917) both reported cases of *S. eurygyrata* in developing countries and Macfie and Carter (1917) reported intestinal spirochaetes in all native African and 56.2% of English patient specimens examined.

Few subsequent reports of intestinal spirochaetes were made and the term *S. eurygyrata* was eventually replaced by use of the loose description "intestinal spirochaetes". The advent of routine bacteriology stimulated some interest in intestinal spirochaetes including Harland and Lee (1967) who first described a condition which they called "intestinal spirochaetosis" when they observed the attachment of intestinal spirochaetes to colonic epithelium. Difficulties in culturing continued to hamper investigations until Kaplan and Takeuchi (1979) were able to propagate a pure isolate and detail its morphological characteristics using electron microscopy.

Characterisation of local human isolates began in Italy with organisms which were morphologically identical (Sanna *et al.*, 1984). Whilst electron microscopy and enzyme comparisons demonstrated that the isolates were similar (Coene *et al.*, 1989), other properties such as volatile fatty acid production (Jones *et al.*, 1986) and DNA hybridization (Dettori *et al.*, 1987) showed heterogeneity, with some isolates appearing similar to animal isolates.

1.2 Taxonomic and phylogenetic relationships of intestinal spirochaetes

Early systems of classification for intestinal spirochaetes largely relied upon morphology although more recently, taxonomists have drawn upon techniques such as biochemical testing, enzyme profiling and DNA homology to aid spirochaete classification.

Treponema hyodysenteriae was first named as the agent of swine dysentery in 1972 (Harris *et al.*, 1972) and this was followed by the classification of *Treponema innocens* which is a non-pathogenic species found in pigs (Kinyon and Harris, 1979). In 1991, DNA-DNA relative reassociation, restriction endonuclease analysis (REA)

and 16S rRNA sequence analysis were used to compare *T. hyodysenteriae* and *T. innocens* to other *Treponema* species and demonstrated that they showed less than 5% DNA homology with other *Treponema* species suggesting that *T. hyodysenteriae* and *T. innocens* belonged to a separate genus (Stanton *et al.*, 1991). *T. hyodysenteriae* and *T. innocens* were renamed *Serpula hyodysenteriae* and *Serpula innocens* respectively. An examination of their G+C content also confirmed that the intestinal spirochaetes belonged to a different genus (25.8mol% compared to 53.0mol% in *Treponema pallidum*). The genus was later amended from *Serpula* to *Serpulina* as the genus *Serpula* was already used in fungal taxonomy (Stanton, 1992). The genetic relatedness of *S. hyodysenteriae* and *S. innocens* to species of *Spirochaeta*, *Treponema*, *Borrelia*, *Leptospira* and *Leptonema* has been determined using 16S rRNA sequences (Paster, *et al.*, 1991). This study showed that intestinal spirochaetes were most closely related to the spirochaetal clusters containing *Borrelia* and *Leptospira/Leptonema* and this was confirmed by Ochiai *et al.* (1997).

An early indication of heterogeneity amongst intestinal spirochaetes was demonstrated in DNA sequence homology testing of a reference strain of *S. hyodysenteriae* with 21 unidentified human isolates which showed sequence homology varying from 30% to 100% with one another (Coene *et al.*, 1989). Heterogeneity was also observed within the species *S. hyodysenteriae* when analyzed using REA and DNA hybridisation (ter Huurne *et al.*, 1992). In this study, REA profiles showed considerable variation, implying the presence of more than one genotype of *S. hyodysenteriae* in the Netherlands. Multilocus enzyme electrophoresis (MEE) and REA were compared as methods for subspecific differentiation of *S. hyodysenteriae* and both methods were more useful for assessing genetic relatedness than serotyping as isolates with similar electrophoretic types and REA patterns possessed the same

serotype but isolates of one serological group exhibited markedly different profiles using MEE and REA (Lee *et al.*, 1993a).

In 1982, the first intestinal spirochaete isolated from humans was classified as *Brachyspira aalborgi* (Hovind-Hougen *et al.*, 1982). It was distinctive in its morphology and in that it possessed fewer enzymes (such as phosphatases, esterases, glucosidases and galactosidases) than other intestinal spirochaetes. In addition, the culture of these organisms required anaerobic incubation on plates for up to 14 days compared to the 4-5 days required by other intestinal spirochaetes. 16S rRNA coding regions have been compared for various intestinal spirochaetes including *S. hyodysenteriae*, *S. innocens*, *B. aalborgi* and *E. coli*. This study confirmed that *B. aalborgi* was distinct but related to other *Serpulina* species and demonstrated heterogeneity among human isolates (Hookey *et al.*, 1994). Further studies using 16S rRNA analysis have confirmed that *B. aalborgi* was most closely related to *Serpulina* species (96-97% homology) and it has been proposed that the *Serpulina* species should be transferred to the genus *Brachyspira* (Ochiai *et al.*, 1997).

MEE demonstrated heterogeneity amongst human, porcine and canine isolates (Lee and Hampson, 1994) and a dendrogram of MEE relatedness produced 3 main clusters which corresponded to different genera. One cluster contained isolates from humans, pigs and dogs clustered together including a closely related canine and human isolate from the same community. This suggested that transmission of intestinal spirochaetes between humans and dogs may be feasible for the isolates in this cluster. This group was assigned to the genus *Anguillina* (the remaining clusters represented *Serpulina* and *Brachyspira* isolates). Weakly β-haemolytic spirochaetes from pigs, dogs and humans (including P43/6/78^T) have been identified by the polymerase chain reaction (PCR) and DNA-DNA reassociation to be distinct from other *Serpulina*

species and were provisionally named "*Anguillina coli*" (Duhamel *et al.*, 1995). *A. coli* has now been confirmed as the species *Serpulina pilosicoli*. The reference strain of this species (P43/6/78^T) was isolated from a pig and was shown to be genetically distinct from both *S. hyodysenteriae* B78^T and *S. innocens* B256^T (Trott *et al.*, 1996a). P43/6/78^T was also shown to be morphologically and biochemically distinct from B78^T in gelatinase activity, hippurate hydrolysis, enzyme profiles, substrate utilization, antibiotic susceptibility and analysis of fermentation products (Table 1.1). Using DNA-DNA relative reassociation experiments, P43/6/78^T was shown to have only 25-32% homology to B78^T and 24-25% homology to B256^T thus demonstrating sufficient similarity to belong to the same genus but not the same species. The phenotypic characteristics of *S. pilosicoli* isolates from pigs and humans were identical except that human isolates utilize the pentose sugar D-xylose (Trott *et al.*, 1996c).

Heterogeneity amongst human isolates has also been observed using SDS-PAGE gels visualised with Coomassie blue staining or ^{35}S-methionine labelling (Barrett *et al.*, 1996). Whilst the two techniques showed different clustering patterns of isolates from homosexual men in the UK and subjects in the Oman, both demonstrated considerable variation between isolates.

DNA-DNA hybridisation data, restriction fragment length polymorphism-PCR and biochemical analysis has now enabled the identification of two further species of intestinal spirochaete. These new species are *Serpulina intermedia* (reference strain PWS/A^T) and *Serpulina murdochii* (reference strain 56-150^T) (Stanton *et al.*, 1997). An amplified PCR product from the gene *smpA* which encodes a 16kDa lipoprotein that is highly antigenic in *S. hyodysenteriae* was used to probe isolates from

Table 1.1: Properties of intestinal spirochaetes based on sugar utilization, biochemical properties and haemolysis on Trypticase soy blood agar (Derived from Taylor and Trott, 1997)

Property	*S. pilosicoli*	*S. hyodysenteriae*	*S. innocens*	*S. intermedia*	*S. murdochii*
Number of periplasmic flagella	8-12	22-28	20-26	24-28	22-26
Haemolysis	weak	strong	weak	weak	weak
Indole	–	+	–	+	–
Hippurate	+	–	–	–	–
α-glucosidase	–	+	–	+	–
α-galactosidase	+	–	+	–	–
Cellobiose	+	–	+	–	+
L-fucose	+	–	+	–	–
D-ribose	+	–	–	–	–

S. innocens, *S. intermedia*, *S. murdochii* and *S. pilosicoli* (Turner *et al.*, 1995). *S. intermedia* and *S. murdochii* both showed weak hybridisation at low stringency and *S. pilosicoli* did not hybridise to the probe under the conditions used. *S. innocens* showed hybridisation at low stringency with all isolates and with one isolate at high stringency. This suggested that in *Serpulina* species the gene is either absent or varies considerably from the *smpA* found in *S. hyodysenteriae*.

MEE and 16S rRNA studies have clustered intestinal spirochaetes into 7 groups (Stanton *et al.*, 1996). The first group contained all *S. hyodysenteriae* isolates which were differentiated into four sub-groups. The second group consisted of members of "*S. intermedius*" (now accepted as *S. intermedia*). The third group were *S. innocens* strains. Group four consisted of one isolate from a chicken: isolate C1. This isolate is believed to represent a new species named *Serpulina alvinipulli* (Stanton *et al.*, 1998). Further study also identified a pathogen in a chicken with diarrhoea which was morphologically identical to *S. hyodysenteriae* and *S. innocens* but distinct by rRNA gene restriction analysis and MEE (Swayne *et al.*, 1995). The fifth group contained isolates which have now been confirmed as *S. murdochii* and the sixth group consisted of *S. pilosicoli* (formerly known as *A. coli*). The final group was *B. aalborgi*. Studies carried out simultaneously to that of Stanton and co-workers used 16S rRNA gene analysis on spirochaetes isolated from pigs in Sweden to confirm a number of their clusters (Pettersson *et al.*, 1996). In agreement with the study of Stanton *et al.* (1996), the Swedish group identified clusters consisting of *S. hyodysenteriae* isolates and a closely related group of less pathogenic weakly β-haemolytic isolates (*S. intermedia*). A third group contained isolates of *S. innocens* and a final cluster of isolates (group 6 in the Stanton *et al.*, 1996 study) contained

P43/6/78^T-like isolates. It was suggested that these isolates belonged to a different species based on differences in the structure of the 16S rRNA V2 region.

Another species of intestinal spirochaete has been proposed although further experiments will be required to confirm its taxonomy. This species has been provisionally named "*Serpulina canis*" (Duhamel *et. al.*, 1998) and all known isolates from this species were obtained from dogs. When examined by MEE these isolates formed a discrete cluster which was most closely related to *S. innocens* but exhibited a unique ribotyping pattern. One isolate which was previously believed to be *S. pilosicoli* has been shown to be "*S. canis*" and failed to adhere to chick cecal enterocytes (Muniappa *et al.*, 1996) suggesting that "*S. canis*" may be a non-pathogenic species. This was further supported, by the observation that most isolates of "*S. canis*" have been obtained specifically from healthy dogs (Duhamel *et. al.*, 1998).

1.3 Morphological characteristics of intestinal spirochaetes

1.3.1 Cell morphology

Intestinal spirochaetes are Gram negative bacteria which share many features with other members of *Spirochetales*: they all contain a central protoplasmic cylinder which is enclosed by a cytoplasmic membrane and axial filaments (flagella) which are found between the cytoplasmic membrane and the cell wall. The axial filaments are wound around the helix and end at a terminal disc (Takeuchi and Jarvis, 1974). There is inter-species variation in the number of flagella but all isolates have equal numbers attached at each end and overlapping in the centre such as the 4-8-4 arrangement in

B. aalborgi (4 flagella attached at each end, 8 flagella in the centre) (Hovind-Hougen *et al.*, 1982).

In 1909, Werner's classification of two species of spirochaetes was based on size and the number of coils it possessed. The organisms classified as *S. eurygyrata* were described as loosely coiled and 4.6-7.3μm long: *S. stenogyrata* was more tightly coiled and 3.5-6.3μm in length. Kaplan and Takeuchi (1979) reported that the spirochaetes they examined had an average length of 7-8μm, with a diameter of 0.2-0.4μm and 4-5 flagella. Further electron microscope studies have confirmed that intestinal spirochaetes have a diameter of 0.2-0.4μm and are 6-10μm in length with 2-6 spirals. Spirochaetes identified as *S. hyodysenteriae* were morphologically indistinguishable from *S. innocens* (Kinyon and Harris, 1979) and this has also been demonstrated for *S. intermedia* and *S. murdochii* (Stanton *et. al.*, 1997). These workers described *S. hyodysenteriae*, *S. intermedia* and *S. murdochii* as all being 0.35-0.4μm in width, varying between 5-10μm in length and possessing 12-14 flagella at each end (11-13 in the case of *S. murdochii*).

Isolates of *S. pilosicoli* were found to be shorter, narrower (0.25-0.3μm diameter), with fewer flagella (4-5 at each end) and more pointed at the tips (Trott *et al.*, 1996a). Like *S. pilosicoli*, *B. aalborgi* had 4 flagella at each tapering end, but was shorter than *S. pilosicoli* (from 2-6μm in length compared to 5-7μm for *S. pilosicoli*) and narrower (0.2μm diameter or less) (Hovind-Hougen *et al.*, 1982).

Figure 1.1 shows a culture of *S. hyodysenteriae* viewed by dark field light microscopy as described in section 2.2.2.

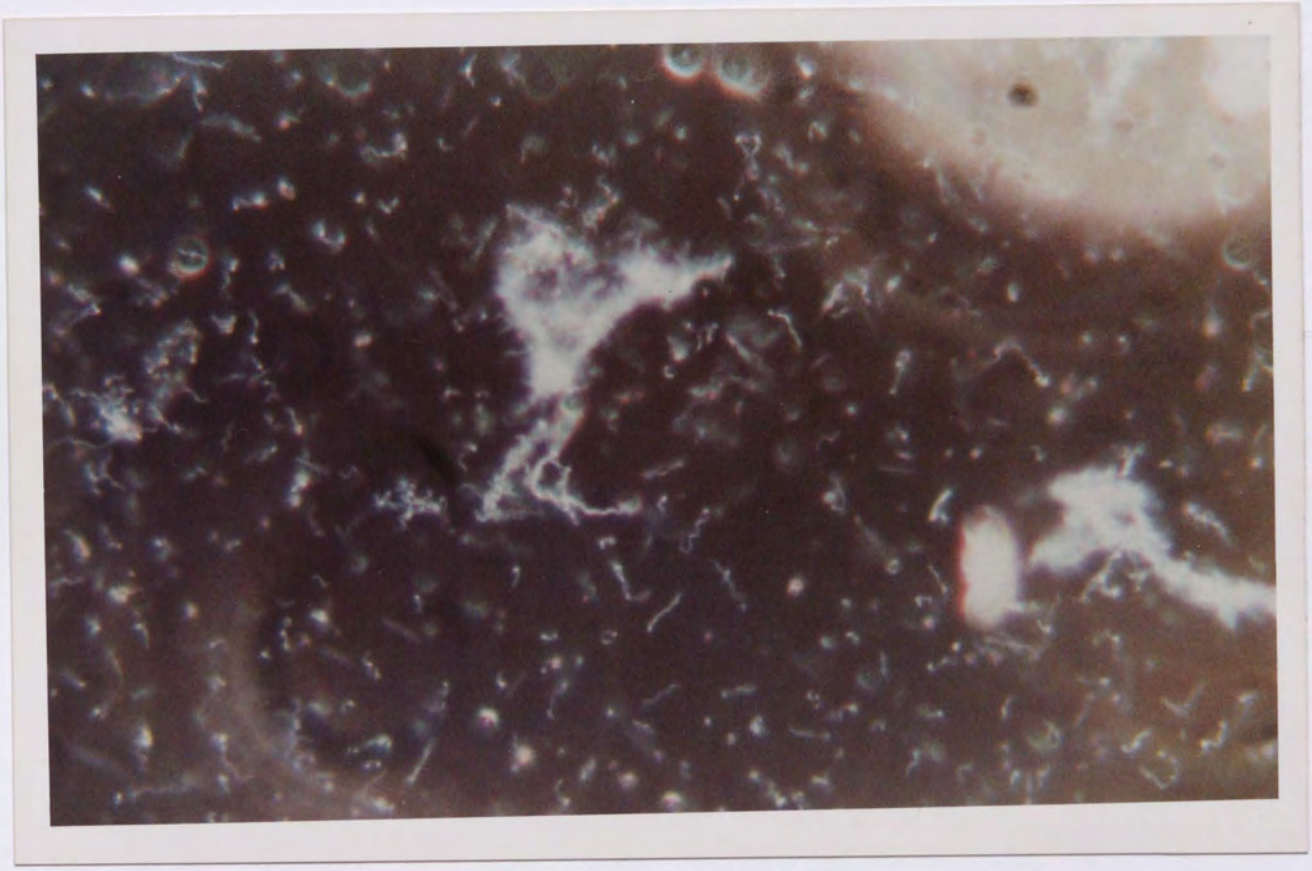


Figure 1.1: Examination of *S. hyodysenteriae* culture by dark field light microscopy at a magnification of x400.

1.3.2 Formation of cyst-like objects (CLO)

Several species of spirochaete and other enteric pathogens such as *Helicobacter pylori* and *Campylobacter* species have been observed to form a CLO or coccoid form. These include the oral spirochaete *Treponema denticola* and spirochaetes in the genus *Borrelia*. It has been speculated that the CLO may represent a stage in the normal life-cycle of the organism, a reproductive structure or some form of spore which is used to survive unfavourable conditions such as the cyst production of *Azospirillum brasilense* in prolonged culture (Sadasivan and Neyra, 1987).

H. pylori is an organism which makes coccoid forms in prolonged culture. Electron microscope studies showed that these CLO retain their double membrane system, polar membrane and invagination structures suggesting that they are not purely a degenerative structure (Benaïssa *et al.*, 1996). Furthermore western blotting of coccoid form proteins against patients' serum suggested that there were certain antigens which were specific to the CLO which were expressed *in vivo*.

Coccoid forms exist in the *Campylobacter* species *Campylobacter jejuni* and *Campylobacter upsaliensis*. In the case of *C. jejuni*, it was demonstrated that as the age of the CLO increased, so PFGE profiles showed degradation of the DNA and, proteins and nucleic acids were released into the media suggesting that the coccoid form was degenerative (Hudock *et al.*, 1998). Similarly, agarose gel electrophoresis of rRNA from *C. upsaliensis* showed marked degradation in the coccoid form, and this together with the lack of oxygen uptake and enzyme activity suggested that the coccoid form was a non-viable structure (Santiwatanakul and Krieg, 1998).

CLOs have been observed in the oral spirochaetes *T. denticola* and *Treponema macrodentium* by scanning electron microscopy and freeze fracture techniques which

revealed them attached to a number of spirochaetes by free fibrils (Umemoto and Namikawa, 1980; Umemoto *et al.*, 1982).

CLOs were first observed in intestinal spirochaetes in 1917 by Macfie but have been more recently investigated by electron microscopy on isolates collected from the Oman (Barber and Barrett, 1995). They observed two different forms of CLO. The first type consisted of single granules (which often contained flagella) between the outer and cytoplasmic membranes. The second type were larger cyst-like structures which contained single granules, mature spirochaetes and filamentous material. Cyst-like structures can be easily seen by dark field light microscopy (figure 1.2). Further work has shown that DNA and carbohydrates were present in both mature spirochaetes and CLOs but the exact role of the CLO remained unclear (Barber *et al.*, 1995).

1.4 Pathogenicity

1.4.1 S. hyodysenteriae

1.4.1.1 Clinical symptoms and animal models of infection

Swine dysentery, caused by *S. hyodysenteriae*, affects pigs after weaning and is a mucohaemorrhagic disease which is characterised by inflammation, excess mucus production and necrosis. Furthermore, it can lead to haemorrhage, dehydration, weight loss and eventually death due either to dehydration, acidosis or hypokalemia.

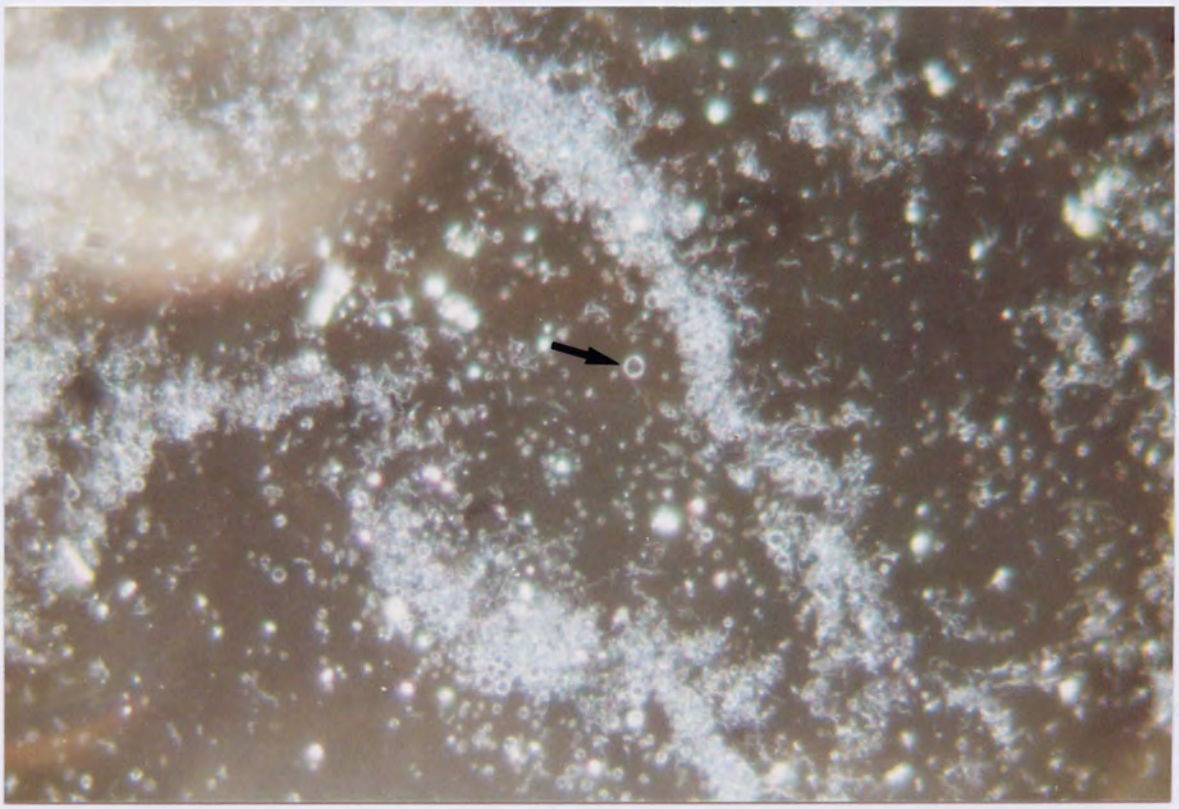


Figure 1.2 : Dark field light microscope view showing a culture of spirochaetal and coccoid forms at a magnification of 400. An example of a coccoid form is indicated by the arrow.

Experiments using pigs are expensive and so two animal models have frequently been used to study swine dysentery. These are a murine model and a chicken model. Different inbred strains of mice which have been experimentally infected with isolates of *S. hyodysenteriae* have shown differing levels of susceptibility although all strains showed cecal lesions. The most susceptible mouse strain was C3H/HeN and it appeared that an important factor for disease susceptibility was the host response to lipopolysaccharide (Nibbelink and Wannemuehler, 1991).

An alternative model is the use of chicks which possess two large ceca compared to the one small cecum in mice. Chicks experimentally infected with *S. hyodysenteriae* showed a variety of symptoms including extensive cell shedding, hyperplasia of mucosal epithelial cells, elongation of the crypt and edema in the lamina propria. It was concluded that the diarrhoea model represented a synergy between the impaired absorptive function and increase in secretion (Sueyoshi and Adachi, 1990).

The mouse model has been used to study the effect of dietary zinc on the susceptibility of animals to infection as it has been demonstrated to reduce diarrhoea and mortality associated with naturally acquired collibacillosis or *S. hyodysenteriae* challenge in pigs. It was demonstrated that increasing amounts of zinc reduced the severity of lesions observed in infected animals. Despite the reduction in lesion severity, the recovery rate of these animals was reduced (Zhang *et al.*, 1995).

Dietary experiments have also been conducted directly in pigs. Soluble non-starch polysaccharides (NSP) were shown to reduce susceptibility of pigs to experimental infection with *S. hyodysenteriae* and diets which had reduced levels of fermentable substrates demonstrated increased protection with those exceeding 1g NSP/100g of feed (cooked white rice diet) being most successful (Pluske *et al.*, 1996). This observation was confirmed in the study of Siba *et al.* (1996) which found a diet of

rice and animal proteins reduced fermentation and prevented colonisation with *S. hyodysenteriae*.

1.4.1.2 Virulence characteristics

S. hyodysenteriae possesses a number of characteristics which may be important in its virulence. In the gut lumen, most organisms which cause an enteric disease adhere to the gut surface and this is facilitated by motility. Intestinal spirochaetes possess a corkscrew-like motility which is generated by the movement of the periplasmic flagella which are wound around the helical core. The importance of chemotaxis was demonstrated by Milner and Sellwood (1994) who observed that spirochaetes which were unable to respond to chemotactic stimuli were avirulent. Chemotaxis to fucose and serine (components of hog gastric mucin) has been observed in *S. hyodysenteriae* which was at its most motile in mid- to late log phase (Kennedy and Yancey, 1996). Intestinal spirochaetes including *S. hyodysenteriae* produced an outer membrane serine protease which may be important for colonisation. Adherent spirochaetes may enhance chemotaxis of luminal spirochaetes to the gut surface by breaking down mucin to components such as fucose and serine (which are chemotactic stimuli) using protease enzymes (Muniappa and Duhamel, 1997). Generation of mutants in the *flaA* and *flaB* genes has confirmed the importance of motility (Rosey *et al.*, 1996). The polypeptides encoded by these genes were components of periplasmic flagella and mice infected by bacteria with mutations in both genes were shown to be severely attenuated (Rosey *et al.*, 1996). The production of mutants in a single gene indicated that *flaB* produced a more pronounced reduction in virulence and the ability to colonise, than *flaA* (Kennedy *et al.*, 1997).

In common with other enteric pathogens such as *V. cholerae*, *S. hyodysenteriae* produces toxins, such as a haemolysin which is active at 27-40°C and pH3-9, and was suggested to lyse cells through pore formation (Hyatt and Joens, 1997). The importance of this haemolysin was observed when mutants (lacking the haemolysin) were introduced into mice (ter Huurne *et al.*, 1992a). Animals infected with the mutants produced fewer cecal lesions demonstrating that the haemolysin was likely to be an important virulence factor. This has been confirmed by the injection of purified haemolysin into pig ileal loops after which the proximal part of the absorptive villi was shed, villi became shortened and the basement membrane folding increased (Bland *et al.*, 1995). Preparations of endotoxin demonstrated the ability to induce tumour necrosis factor (TNF) and interleukin-6 (IL-6) bioactivity in mice but only IL-6 activity in pigs (Nibbelink *et al.*, 1997). This demonstrated that the endotoxin is able to induce the production of proinflammatory cytokines *in vivo* although the magnitude of the reaction was 5-10 times lower than the reaction to *E. coli* or *Salmonella typhimurium* LPS. *S. hyodysenteriae* LPS stimulated TNF activity in mice but to a lesser extent than the endotoxin.

Transmission of virulence characteristics may be important for the spread of favourable mutations. Agarose gel electrophoresis demonstrated that intestinal spirochaetes produced 6.5 kbp fragments of extracellular DNA which were packaged in vesicles (Turner and Sellwood, 1997). Vesicles containing DNA have been observed in a number of other Gram negative organisms including *Borrelia burgdorferi*, *S. typhimurium* and *Shigella flexneri* and, it was predicted that these fragments were exported for genetic exchange between compatible organisms (Dorward and Garon, 1990). In addition, a bacteriophage (VSH-1) was recently discovered which was able

to carry DNA of up to 7.5 kbp between spirochaetes thus permitting mutations to be transmitted between spirochaetal populations (Humphrey *et al.*, 1997).

1.4.1.3 Diagnosis

Rapid diagnosis of swine dysentery cannot rely on culture techniques as spirochaetes require incubation for 4-5 days and any mortality is usually observed approximately 5 days after the onset of clinical symptoms. For this reason, two main methods have evolved for rapid diagnosis.

ELISA assays for *S. hyodysenteriae* provide one method of detection. Two different assays have been developed, the first involved detection of anti-*S. hyodysenteriae* antibodies in the serum of infected pigs (Wright *et al.*, 1989). The second assay used a monoclonal antibody to the 16kDa lipoprotein encoded by *smpA* to detect spirochaetes in faecal samples (Sellwood *et al.*, 1995). The limit of detection was $>10^6$ spirochaetes/g of faeces which, even at these levels could not be detected after the onset of clinical symptoms possibly due to down regulation of gene expression of the 16kDa lipoprotein. A more sensitive method for detection of spirochaetes in faeces was PCR which detected 1-10 seeded organisms per 0.1g of faeces (Elder *et al.*, 1994) or 10^4 seeded organisms/0.1g faeces (Harel and Forget, 1995).

1.4.1.4 Cross-species transmission

A number of isolates of *S. hyodysenteriae* have been obtained from animals other than swine. Mice and rats are carriers of *S. hyodysenteriae* (Hampson *et al.*, 1997) and may be important in transmission between or in reinfection of animals in a herd. *S. hyodysenteriae* has also been isolated from rheas with necrotizing typhlocolitis

and the spirochaetes were identified using 16S rRNA sequencing, ribotyping, and biochemical tests (Jensen *et al.*, 1996). Subsequently, a number of isolates from rheas with necrotizing typhlocolitis and one from a rat were found clustered with *S. hyodysenteriae* strains using MEE (Trott *et al.*, 1996b).

Further evidence for cross-species transmission was obtained using isolates from chickens (which had indirect contact with infected pigs) which were demonstrated to be infected with spirochaetes of unknown species and showed retarded growth rate and delayed onset of egg production (Griffiths *et al.*, 1987). *S. hyodysenteriae* has been identified in dogs which had indirect contact with infected pigs but once removed from the infected area, no spirochaetes were isolated suggesting that the dog was not a carrier but had acquired the spirochaetes by eating contaminated faeces (Songer *et al.*, 1978).

1.4.2 S. pilosicoli

S. pilosicoli has been implicated in a condition known as intestinal spirochaetosis where watery diarrhoea has been induced in a variety of animals including pigs (Lee *et al.*, 1993b), dogs (Duhamel *et al.*, 1995), birds (McLaren *et al.*, 1994) and humans (Lee and Hampson, 1994).

1.4.2.1 Clinical characteristics of human intestinal spirochaetosis

Faecal culture and histopathology have been the principal methods used to study intestinal spirochaetosis. Spirochaetes appear as a thin blue line at the brush border of the mucosal surface of colonic and rectal tissue using a light microscope when a haematoxylin and eosin stain is used. They are more clearly seen using electron

microscopy where spirochaetes are observed between and parallel to microvilli. Such adhesion to the intestinal epithelium is supported by the presence of NADH oxidase (an enzyme which is found mainly in host-associated bacteria) in isolates of *S. hyodysenteriae*, *S. innocens* and *S. pilosicoli* (Stanton *et al.*, 1995).

Most studies of *S. pilosicoli* have centered either on the collection and identification of isolates or upon the examination of biopsy samples without identification of spirochaetes and comparison to any clinical symptoms. This has made elucidating whether *S. pilosicoli* is pathogenic in humans a complicated process.

In some cases where intestinal spirochaetes have been cultured from stool samples it has been proposed that the spirochaetes are pathogenic, as exemplified by a study of Aboriginal communities where patients shedding intestinal spirochaetes more frequently had abnormal rather than normal stools (Lee and Hampson, 1992). One study used both faecal culture and a rectal biopsy sample to identify intestinal spirochaetes in a patient with persistent diarrhoea. Intestinal spirochaetes were observed adhering to the epithelial cells causing blunting and destruction of microvilli. Treatment with metronidazole both relieved the symptoms and removed the spirochaetes suggesting that the spirochaetes had been responsible for the patient's diarrhoeal symptoms (Rodgers *et al.*, 1986).

Barrett (1990) did not observe a correlation between isolation of intestinal spirochaetes from stool samples and the symptoms observed by the patient. A positive correlation was observed between the age of the patient and isolation of bacteria from stool samples. This was confirmed by a study of the Australian Aboriginal community (Lee and Hampson, 1992). Intestinal spirochaetes have been frequently found in HIV patients (Kostman *et al.*, 1995) and the observation that they were often associated with immunocompromised groups has lead to the theory that intestinal spirochaetosis

may be an opportunistic infection in humans. Intestinal spirochaetes may be able to colonise the intestinal tract but not cause symptoms unless they are able to heavily colonise the intestinal epithelium. Mathan and Mathan (1985) observed spirochaetes in the intestines of healthy volunteers in southern India and suggested that such colonisation may be part of the normal intestinal flora for people living in the area. The mature luminal columnar cells were shorter with irregular grouped smaller microvilli whilst fewer goblet cells were observed. The lamina propria showed an increase in the presence and activity of plasma cells, and the numbers of subluminal macrophages and degranulated eosinophils and mast cells. Evidence of vascular damage was also observed. This was interpreted as a colonopathy which is unique to the tropics and which may be more susceptible to colonisation by spirochaetes (9/14 biopsies were infected) (Mathan and Mathan, 1985). It has been suggested that microvillus structure is an important factor in colonisation and this was demonstrated by a study of two patients with tubulovillous adenomas who were colonised by intestinal spirochaetes. The spirochaetes were able to adhere to normal colonic epithelium (non-neoplastic) but not to neoplastic areas. Neoplastic areas were characterised by shortened irregular microvilli and in neoplastic areas where near-normal microvilli were seen, spirochaetes were able to adhere (Coyne *et al.*, 1995).

Several studies have suggested that in addition to colonisation of the large intestine, spirochaetes may be able to spread into the blood. A number of studies have also observed intracellular intestinal spirochaetes such as in examination of rectal biopsy material (Rodgers *et al.*, 1986). Whole spirochaetes were observed within epithelial cells and sub-epithelial macrophages of two patients with gastrointestinal symptoms. Further investigation showed an increase in IgE plasma cells in the lamina propria and degranulated intra-epithelial mast cells suggesting that penetration of the

epithelial barrier results in the described immune response (Gebbers *et al.*, 1987). A similar case was reported in a patient suffering with Crohn's disease (Antonakopoulos *et al.*, 1982).

Six isolates from France were obtained from the blood of patients suffering from a variety of symptoms which affected the integrity of the gastrointestinal tract. It was unclear whether the spirochaetes were invasive or whether damage to the mucosal surface facilitated their entry into the blood (Fournie-Amazouz *et al.*, 1995). When compared to other *S. pilosicoli* isolates by MEE, the French isolates were not found to cluster together in dendrograms suggesting that they lacked specific adaptations for invasion (Trott *et al.*, 1997a). Further evidence suggesting that intestinal spirochaetes were invasive was found in three HIV patients with what was described as "invasive intestinal spirochaetosis" (Kostman *et al.*, 1995). Two cases of colitis were reported which were characterised by diffuse ulcers and pustules, superficial cell necrosis and infiltration of the mucosa and lamina propria. Silver staining revealed spirochaetes adhering to the epithelium including areas in the crypts. Kostman *et al.* (1995) also described a third case of a patient with colestatic hepatitis in which spirochaetes were located in the bile canaliculi suggesting that the spirochaetes had disseminated to the liver from the bowel via the blood.

The presence of intestinal spirochaetes (mostly unidentified) has been demonstrated in other regions of the gastrointestinal tract apart from the large intestine, including the appendix and the small intestine. Spirochaetes colonised the appendix of 2-4% of European subjects examined but were seen less frequently in cases of acute appendicitis (0.7%) than in cases where the appendix was removed *per occasionem* (1.9%). A number of patients with suspected acute appendicitis were later found to have histologically normal appendices (a condition known as

pseudoappendicitis) and of these 12.6% were found to be colonised with *B. aalborgi*-like spirochaetes (Henrik-Nielsen *et al.*, 1985).

One study showed spirochaetes colonising biopsy material taken from below the pyloric sphincter in patients suffering from conditions including gastrointestinal disease, rheumatoid arthritis and ankylosing spondylitis. These workers demonstrated the presence of spirochaetes in 50% of the arthritis sufferers, the patient with ankylosing spondylitis and four of the five patients with gastrointestinal symptoms but no attempts to correlate spirochaetes in the small intestine with the pathogenesis of rheumatoid arthritis were made as the number of patients studied was too small (Potter *et al.*, 1995).

1.4.2.2 Clinical characteristics of intestinal spirochaetosis in animals

The clinical picture of intestinal spirochaetosis in animals is better understood than in humans as many experiments have been performed on animals with a naturally acquired intestinal flora or specific pathogen free animals whose intestinal flora has been defined and artificially acquired.

Experimental infection of pigs which have a naturally acquired intestinal flora with porcine isolates of *S. pilosicoli* has been shown to induce diarrheic faeces, a depression in weight gain and caused lesions in the large intestine (Thomson *et al.*, 1997). Newly weaned pigs infected with human and porcine isolates of *S. pilosicoli* similarly developed watery, mucoid diarrhoea and histological examinations showed end-on attachment of spirochaetes and lesion development in the caecum and colon (Trott *et al.*, 1996d). Furthermore, an investigation of pig units with colitis where *S. pilosicoli* was suspected as the primary agent, found that the pigs suffered with diarrhoea, grew poorly (5-15% reduction) and lesions were observed in the proximal

part of the colon. In only 1% of cases was the colitis fatal suggesting that *S. pilosicoli* caused a mild colitis which was sufficient to depress weight gain but not to be lethal (Thomson *et al.*, 1998).

As in the case of *S. hyodysenteriae*, chickens and mice have been used as models for infection with *S. pilosicoli*. When naturally infected with *S. pilosicoli*, chickens suffer a range of symptoms similar to those which occur in pigs. Symptoms vary in severity but include wet faeces, pasty vents, delayed onset of egg laying, faeces stained eggs and a reduction in the mean egg weight and carotenoid content (Swayne and McLaren, 1997). When one day old specific pathogen free chicks were infected with human and porcine isolates, the spirochaetes were found to colonise the intestinal surface, cause clinical diarrhoea and a depressed growth rate (Trott *et al.*, 1995). Transmission electron microscopy of cecal tissue taken from chickens experimentally infected with human and canine isolates revealed end-on attachment of the spirochaetes, effacement of brush border microvilli and some evidence of invasion beyond the mucosal barrier (Muniappa *et al.*, 1996). C3H/HeJ and C3H/HeOuJ mice infected with avian and porcine isolates of *S. pilosicoli* showed characteristic end-on attachment and effacement of microvilli, and developed a serum immune response (Sacco *et al.*, 1997).

1.4.2.3 Diagnosis

Two attempts have been made to identify *S. pilosicoli* by PCR using 16S rDNA sequences as the source of primers although different regions of the gene were used in each case. PCR has been used successfully to identify bacteria when as few as one genome (one bacterium) is present. In the first PCR trial, the lower level of detection was 250 bacteria from cultured plates or primary cultures from faecal

samples (Park *et al.*, 1995). The second trial used PCR as a complement to biochemical testing for bacterial identification and was able to detect a lower limit of 124 bacteria but failed to identify spirochaetes in faecal samples from infected animals (Fellström *et al.*, 1997). Whilst the current studies have a high threshold for detection of cultured *S. pilosicoli*, no PCR test for *S. pilosicoli* has yet been reported which is sufficiently sensitive to detect spirochaetes in faecal or blood samples.

Following the lack of success with PCR, monoclonal and polyclonal antibodies against *S. pilosicoli* outer envelope proteins have been developed as a potentially useful reagents for the detection and identification of *S. pilosicoli*. (Tenaya *et al.*, 1998). These reagents have not yet been developed into a commercially available assay.

1.4.2.4 Cross species transmission

Whilst successful experimental infection of chickens with isolates from humans, pigs and dogs, may suggest that spirochaetes from these sources are able to colonise different host animals, studies of phylogenetic relationships of isolates has not supported this view. The one exception to this is potential cross-species transmission between dogs and humans. Genotypic analysis has demonstrated that a number of similar profiles were found amongst isolates from canines and humans, whilst other genotypes were only found amongst dogs, suggesting that cross-species transmission may be possible (Duhamel *et al.*, 1995; Koopman, *et al.*, 1993). As neither *S. pilosicoli* nor "*S. canis*" had been identified at the time of these observations, it is possible that the shared genotypes represent isolates of *S. pilosicoli* and those unique profiles represent "*S. canis*".

1.4.3 S. intermedia, S. murdochii, S. alvinipulli and "S. canis"

The pathogenicity of the most recently identified species *S. intermedia*, *S. murdochii*, *S. alvinipulli* and "*S. canis*" has yet to be determined although the C1 isolate of *S. alvinipulli* may be enteropathogenic (Swayne *et al.*, 1995). The lack of adherence of an *S. canis* isolate to chicken cecal enterocytes and its isolation from healthy animals has indicated that this may be a non-pathogenic species (Duhamel *et al.*, 1998).

Most isolates of *S. intermedia* and *S. murdochii* have been found in pigs although isolates have been obtained from other animals such as MEE studies which showed that an isolate obtained from a rat clustered with *S. murdochii* isolates in dendrograms although the significance of this will not become clear until the pathogenicity of *S. murdochii* has been determined (Trott *et al.*, 1996b). Using MEE, isolates from chickens have clustered with *S. murdochii* and *S. intermedia*. The chicken isolate which clustered with *S. intermedia* isolates was demonstrated to be pathogenic in chickens suggesting that the species *S. intermedia* may be pathogenic in other species (McLaren *et al.*, 1997).

1.4.4 S. innocens

S. innocens is a species which is mostly found in pigs but has been isolated from other animals such as chickens (McLaren, *et al.*, 1997). Experimental infection of gnotobiotic pigs (animals with no intestinal flora except for the anaerobic bacteria *Bacteroides vulgatus*) demonstrated that *S. innocens* was able to cause mucoid faeces and typhlocolitis, although it failed to induce symptoms in a normal animal (Neef *et al.*,

1994). This confirms the observation of Kinyon and Harris (1979) that the organism is non-pathogenic in normal pigs.

1.4.5 Brachyspira aalborgi

B. aalborgi has only been isolated from one host, humans. Sections of colonic epithelium which showed heavy infestation with *B. aalborgi* showed no signs of tissue damage or inflammation suggesting that these organisms were non-pathogenic and possibly commensal (Hovind-Hougen *et al.*, 1982).

1.5 Epidemiology

Early microscope studies (Werner, 1909; Fantham, 1916 and Macfie, 1917) suggested that human intestinal spirochaetes were prevalent in populations in Africa, Europe and America. One study reported that all natives and European visitors to the Gold Coast were colonised to some extent (Macfie, 1917). More recent studies which have combined microscopy and faecal culture have suggested that the prevalence of spirochaetes is now less than 1% in European populations and 18% in Rwanda (Goossens *et al.*, 1983).

As swine dysentery usually affects pigs after weaning, it has a great effect on their weight gain and consequently can considerably increase production costs for farmers taking pigs to market or for meat production. *S. hyodysenteriae* is spread within herds by the ingestion of contaminated faeces and this can be enhanced by mice and rats which become carriers (Hampson *et al.*, 1997). These carriers may increase the risk of re-infection once swine dysentery is removed from the herd although they

are unlikely to have a role in infection between herds which is most likely to be caused by movement of pigs harbouring the bacteria (Hampson *et al.*, 1997). Another source may be contaminated feed, animal transporters, farm equipment or clothing.

Swine dysentery has been reported in all pig producing countries and whilst it has become a less common infection in the USA and Australia, in the UK it remains the second most commonly diagnosed disease after enteric colibacillosis (Hampson *et al.*, 1997). *S. hyodysenteriae* isolates (231) were examined by MEE to investigate population structure. The index of association for the number of electrophoretic types demonstrated that there was a significant degree of recombination whilst the index of association for the number of isolates suggested a clonal (epidemic) population. They suggested that as in other bacterial populations, recombination shaped the population and dissemination of clones with enhanced survival or virulence characteristics generated an epidemic population (Trott *et al.*, 1997b).

Porcine intestinal spirochaetosis is also a significant factor in intensive swine farming by multi-site or all in/all out (AI/AO) pig-flow systems. Reduced performance has been reported in all swine producing countries and in one study, 5 of the 10 "finisher" farms in the USA tested by rectal swabs showed 50% of their animals to be infected with *S. pilosicoli*. Continuous flow production systems are likely to spread *S. pilosicoli* from older to younger pigs whilst AI/AO and multi-site systems are more likely to transmit spirochaetes through carrier pigs or a contaminated environment (Duhamel, 1998).

Histopathological investigations have examined the prevalence of human intestinal spirochaetes and have observed that several populations were at an increased risk of infection with intestinal spirochaetes. These groups included homosexual males (McMillan and Lee, 1981; Surawicz *et al.*, 1987), immunocompromised hosts such as

AIDS patients (Kostman *et al.*, 1995) and young children (Barrett, 1990; Lee and Hampson, 1992), and people in developing countries (Barrett, 1990; Trott *et al.*, 1996b). The highest incidence of infection with intestinal spirochaetes was observed in Scotland where 36% of homosexual male subjects examined were colonised (McMillan and Lee, 1981). Another study of homosexual men found 30% of subjects were infected with intestinal spirochaetes and showed a positive correlation between infection with intestinal spirochaetes and *Neisseria gonorrhoeae* suggesting that infection with one species increases the suitability of the mucosal surface for infection with the other (Surawicz *et al.*, 1987). Immunocompromised hosts such as three patients with advanced HIV have also been described with "invasive intestinal spirochaetosis" (Section 1.4.2.1) (Kostman *et al.*, 1995) and studies in the Sultanate of Oman showed a positive correlation between human intestinal spirochaetosis and subjects which were 2-18 years old (Barrett, 1990). An increased prevalence of human intestinal spirochaetes was observed in Australian aboriginal communities where 32.6% of faecal samples contained spirochaetes compared to the 1.2% colonisation observed in non-aboriginal children and other individuals within Western Australia and the Northern Territories (Lee and Hampson, 1992). This study confirmed the findings of the Omani investigation which demonstrated a positive correlation between age (2-18 years) and presence of spirochaetes in faeces. Lee and Hampson (1992) also suggested that intestinal spirochaetes may be present for considerable periods of time (they were isolated from one subject in two consecutive years but not the third year), an observation also made in experiments with *S. hyodysenteriae* in pigs (Neef *et al.*, 1994). A survey of five villages in Papua New Guinea found 22.8% of individuals to harbour intestinal spirochaetes and reexamination of a number of individuals six weeks later demonstrated that the infection had persisted in 93.1% of cases (Trott *et al.*,

1997b). They also found intestinal spirochaetes in 5.3% of village dogs and one of two ducks tested suggesting that cross-species transmission was possible although no spirochaetes were obtained from village pigs.

Intestinal spirochaetes were isolated in six blood samples from France (Fournie-Amazouz, *et al.* 1995). The prevalence of spirochaetes in the blood has not been documented as blood samples are not routinely screened for intestinal spirochaetes.

1.6 Aims of the study

The initial objective of this project was to characterise a number of previously unidentified intestinal spirochaete isolates by phenotype (immunoblotting with species specific monoclonal antibodies) and genotype (pulsed field gel electrophoresis). This demonstrated that there was little correlation between genotype and phenotypic characteristics such as the ability to produce a bacteraemia and, subsequent investigations examined phenotypic properties which may be involved in pathogenicity. This involved the use of an *in vitro* model of the intestinal epithelium (Caco-2 cell line) to investigate the adherence of genotypically diverse isolates (including the bacteraemic isolates). The nature of intestinal spirochaete adhesins which interact with the host epithelium was investigated by examination of similarities between intestinal spirochaete DNA and known adhesin sequences, and spirochaete interaction with extracellular matrix proteins. A genomic expression library was constructed to identify immunogenic proteins which may be involved in the host's response to spirochaetes.

Chapter 2: Genotypic analysis by Pulsed Field Gel Electrophoresis

2.1 Introduction

2.1.1 Pulsed field gel electrophoresis

Conventional agarose electrophoresis can separate fragments of up to approximately 50kbp but to resolve larger fragments, electrophoresis with pulsed electric fields was developed. Whole bacterial cells may be digested *in situ* by embedding in agarose which prevents the large DNA molecules from being sheared prior to restriction digestion. Restriction endonucleases are chosen which cut the genome at infrequent restriction sites producing a relatively small number of fragments with distinct electrophoretic mobilities which may be separated with good resolution.

Pulsed field gel electrophoresis (PFGE) is a useful tool for several reasons. It has enabled the physical mapping of bacterial chromosomes, sizing of bacterial genomes and creation of libraries from defined subregions of the genome. This approach has been used in the physical mapping of *Pseudomonas fluorescens* SBW25 chromosome (Rainey and Bailey, 1996), *Pseudomonas aeruginosa* PAO (Schmidt *et al.*, 1996) and *S. hyodysenteriae* $B78^T$ (Zuerner and Stanton, 1994). PFGE may be used to map transposon insertions in uncloned loci or as a highly effective tool in molecular epidemiological studies. In this respect, it is a superior technique to phenotypic methods for discriminating within many species.

Tenover *et al.* (1995) developed a set of criteria which facilitate the interpretation of PFGE profiles in an epidemiological context. Isolates which present identical profiles are defined as "genetically related" isolates (clones) which are likely to be the same strain. Closely related isolates differ by one genetic event (one or two fragments differ due to a single genetic event such as a point mutation, insertion, deletion or chromosomal inversion). Isolates separated by two independent genetic events (4-6 fragments) are considered to be "possibly related" strains. The strains are considered unrelated if they are separated by three or more genetic events (seven or more fragments in the profile are different) (Tenover *et al.*, 1995). If the strains have multiple or distinctive bands in common, they may have a common ancestry (Maslow *et al.*, 1993).

PFGE has been used as a method for examining differences between intestinal spirochaete strains (Atyeo, *et al.*, 1996). The study of Atyeo *et al.* (1996) examined *S. pilosicoli* isolates obtained from a variety of geographical locations and a number of different animals including pigs, dogs, humans, a duck and a rhea. The isolates were digested with the restriction enzyme, *MluI*. Using the unweighted pair group method of arithmetic averages (UPGMA), a dendrogram was generated which illustrated that there was considerable heterogeneity among isolates of *S. pilosicoli*, although in some instances isolates from the same herd or area produced identical profiles suggesting that they represented the same strain. Some isolates from the same animal but different geographical area were also found to be closely related suggesting that cross-species transmission was feasible.

2.1.2 Western blotting

The use of monoclonal antibodies has been applied to intestinal spirochaetes to aid characterisation of isolates. Achacha and Mittal (1995) produced four genus specific monoclonal antibodies which reacted with heat stable proteins of 26-45kDa in the *S. hyodysenteriae* and *S. innocens* strains tested. Thomas and Sellwood (1992) made a monoclonal antibody directed against a 16kDa outer envelope antigen from *S. hyodysenteriae*. Another monoclonal antibody designated BJL/SH1 reacted with a 30kDa outer membrane antigen of *S. hyodysenteriae* (Lee and Hampson, 1996). A monoclonal antibody has also been raised to a 29kDa outer membrane protein in the *S. pilosicoli* strain 3295 which was isolated from a pig with intestinal spirochaetosis (Lee and Hampson, 1995). This antibody was designated BJL/AC1 and was used together with BJL/SH1 to probe the isolates in this study.

2.1.3 Aims

The purpose of this study was to characterise a number of isolates from human, porcine, canine, murine, feline and avian origins using PFGE and Western blotting. This was undertaken in order to look for epidemiologically related isolates, particularly amongst those from a single geographic origin - in this case, Oman.

2.2 Materials and methods

2.2.1 Sources of isolates

82 isolates from a variety of animal and geographic origins were examined. The sources, animal origin and clinical symptoms for each isolate are detailed in Tables 2.1 and 2.2. 54 isolates were obtained from humans, 18 from pigs, 6 from dogs, 2 from chickens, one from a cat and one from a mouse. The isolates originated from the Muscat region of Oman (n=34), Australia (n=4), UK (n=15), Italy (n=2), France (n=6), USA (n=3), Denmark (n=1) and The Netherlands (n=17).

A number of isolates have previously been characterised and these include B78^T (59), B256^T and P43/6/78^T (51) which are the reference strains of *S. hyodysenteriae*, *S. innocens* and *S. pilosicoli* respectively. B204 (61) and B78 (59) were confirmed as *S. hyodysenteriae* by species-specific PCR (Elder *et al.*, 1994). Isolates 81/80 (5), 28/94 (18), PE90 (30), 128/90 (31), RA87 (40), P43/6/78 (51) and 382/91 have been confirmed as *S. pilosicoli* by biochemical, morphological and MEE analysis (Trott, *et al.*, 1997).

Table 2.1: Isolates included in this table feature in Figure 2.4 with their *number in the figure and †designated name

No. *	Designation †	Patient	Symptoms	Source	Reference/ Supplier
1	1	Human: Male aged 2	Diarrhoea, giardiasis	Muscat, Oman	Barrett, 1990
2	26	Human: Male aged 10	Diarrhoea, vomiting (1 day)	Muscat	Barrett, 1990
3	3	Human: Male aged 50	Abdominal pain	Muscat	Barrett, 1990
4	4	Human: Male aged 50	Giardiasis	Muscat	Barrett, 1990
5	81/80	Human: Male aged 69	Stroke/hemiplegia	France	Fournie-Amazouz *et al*, 1995

6	12	Human: Female aged 12	Abdominal pain (years)	Muscat	Barrett, 1990
7	HRM3	Human: Male aged 40	Constipation/ diarrhoea	Rome	Sanna *et al*, 1984
8	19	Human: Female aged 5	Diarrhoea	Muscat	Barrett, 1990
9	47	Human: Female aged 40	Abdominal pain for 6 months	Muscat	Barrett, 1990
10	39	Human: Male aged 13	Abdominal pain	Muscat	Barrett, 1990
11	40	Human: Female aged 25	Flatulence	Muscat	Barrett, 1990
12	43	Human: Male aged 46	Abdominal pain	Muscat	Barrett, 1990
13	3941	Human	Not known	Muscat, no records kept	Barrett, 1990
14	64	Human: Male aged 27	Abdominal pain/ backache (3 years)	Muscat	Barrett, 1990
15	6	Human: Male aged 50	Abdominal pain/giardiasis	Muscat	Barrett, 1990
16	17	Human: Female aged 35	Not known	Muscat	Barrett, 1990
17	13	Human: Female aged 2	Diarrhoea for 2 weeks	Muscat	Barrett, 1990
18	28/94	Human: Male aged 55	Alcoholic with acute intoxication with ethylene glycol	France	Fournie-Amazouz *et al*, 1995
19	HRM16	Human: Male aged 22	Not known	Rome	Sanna *et al*, 1984
20	29	Human: Male aged 2	Abdominal pain (1 month)	Muscat	Barrett, 1990
21	37	Human: Female aged 30	Headaches (6 years)	Muscat	Barrett, 1990
22	18	Human: Female aged 26	Diarrhoea and vomiting	Muscat	Barrett, 1990
23	20	Human: Male aged 24	Diarrhoea/ abdominal pain	Muscat	Barrett, 1990
24	31	Human: Female aged 10	Chronic diarrhoea	Muscat	Barrett, 1990
25	25	Human: Male aged 4	Diarrhoea for 2 weeks	Muscat	Barrett, 1990
26	32	Human	Diarrhoea	AIDS patient	Supplied by S. Barrett
27	28	Human: Female aged 45	Not given	Muscat	Barrett, 1990
28	30	Human: Male aged 60	Not given	Muscat	Barrett, 1990
29	14	Human: Female aged 20	Abdominal pain for 1 week	Muscat	Barrett, 1990
30	PE90	Human: Female aged 77	Stroke/hemiplegia	France	Fournie-Amazouz *et al*, 1995
31	128/90	Human: Male aged 69	Acute peritonitis	France	Fournie-Amazouz *et al*, 1995
32	PWS/B	Pig	Post weaning scour	UK	Supplied by IAH, Compton[†]

33	10	Human: Female aged 12	Acute lymphocytic leukaemia, abdominal pain, giardiasis	Muscat	Barrett, 1990
34	A3888	Dog	Diarrhoea	Netherlands	Ch 6 PhD thesis M B H Koopman
35	8	Human: Female aged 12	Enuresis	Muscat	Barrett, 1990
36	9	Human: Female aged 4	Abdominal pain (1 year)	Muscat	Barrett, 1990
37	M15183	Human: Male aged 29	Diarrhoea/ abdominal pain	London, homosexual AIDS patient	Supplied by S. Barrett
38	A5660	Dog	Diarrhoea	Netherlands	Ch 6 PhD thesis M B H Koopman
39	60	Human: Male aged 3	Diarrhoea and vomiting	Muscat	Barrett, 1990
40	RA87	Human: Male aged 62	Severe arteriopathy	France	Fournie-Amazouz *et al*, 1995
41	36	Human: Female aged 24	Abdominal pain/ *S. flexneri*	Muscat	Barrett, 1990
42	Korlos	Human	Not known	Australian Aborigine	Lee and Hampson, 1992
43	23	Human: Male aged 35	Abdominal pain	Muscat	Barrett, 1990
44	A3077	Dog	Diarrhoea	Netherlands	Ch 6 PhD thesis M B H Koopman
45	FT6	Human	Diarrhoea	London, AIDS patient	Supplied by S. Barrett
46	A5687	Dog	Diarrhoea	Netherlands	Ch 6 PhD thesis M B H Koopman
47	Gel-2	Human: Male aged 31	Diarrhoea	London, homosexual AIDS patient	Supplied by S. Barrett
48	Gel	Human: Male aged 31	Not known	London, homosexual AIDS patient	Supplied by S. Barrett
49	38	Human: Female aged 45	Abdominal pain/headache	Muscat	Barrett, 1990
50	Matthews	Human: child	Diarrhoea	Australian Aborigine	Lee and Hampson, 1992
51	P43/6/78	Pig	Diarrhoea	UK	ATCC51139
52	FT9	Human	Not known	London	Supplied by S. Barrett
53	FT7	Human	Not known	London	Supplied by S. Barrett
54	Wesley	Human: child	Diarrhoea	Australian Aborigine	Lee and Hampson, 1994

55	Jeremiah	Human: child	Diarrhoea	Australian Aborigine	Lee and Hampson, 1994
56	44	Human: Male aged 29	Diarrhoea	Muscat	Barrett, 1990
57	57	Human: Male (child)	Not known	Muscat	Barrett, 1990
58	35	Human: Female aged 28	Fever, typhoid	Muscat	Barrett, 1990
59	B78	Pig	Swine dysentery	USA	ATCC27164
60	JWPM	Pig	Swine dysentery	Netherlands	Lemcke and Bew, 1984
61	B204	Pig	Swine dysentery	USA	Supplied by Prof. van de Zeijst
62	A1 (ST.4)	Pig	Swine dysentery	Netherlands	Supplied by Prof. van der Zeijst
63	11416	Chicken	Not known	Holland	Supplied by IAH, Compton
64	P18A	Pig	Swine dysentery	UK	Supplied by IAH, Compton
65	WT10/82	Mouse	Not known	Isolated from farm with swine dysentery, UK	Supplied by IAH, Compton
66	C5	Pig	Diseased	Netherlands	Ch 3 PhD thesis A. ter Huurne 1993
67	A4	Pig	Diseased	Netherlands	Ch 3 PhD thesis A. ter Huurne 1993
68	AF6/80	Pig	Swine dysentery	UK	Supplied by IAH, Compton

‡ Institute of Animal Health, Compton

Table 2.2 Isolates used in this study which were not included in Figure 2.4.

Designation	**Patient**	**Symptoms**	**Source**	**Reference/ Supplier**
Brachyspira aalborgi	Human: Adult	No symptoms	Denmark	Hovind-Hougen *et al*, 1982
VDMC18251	Dog	Not known	Netherlands	Ch 6 PhD thesis M B H Koopman
VMDC18252	Dog	Not known	Netherlands	Ch 6 PhD thesis M B H Koopman
Heal	Chicken	Not known	UK	Supplied by IAH, Compton
D11	Pig	Diseased	Netherlands	Ch 3 PhD thesis A. ter Huurne 1993
Z10	Pig	Not known	Netherlands	Ch 3 PhD thesis A. ter Huurne 1993

Z2	Pig	Not known	Netherlands	Ch3 PhD thesis A. ter Huurne 1993
D7	Pig	Diseased	Netherlands	Ch 3 PhD thesis A. ter Huurne 1993
382/91	Human: Male aged 52	Myeloma/alcoholic	France	Fournie-Amazouz *et al*, 1995
FF3	Cat	Not known	UK	Supplied by IAH, Compton
PWS/A	Pig	Post weaning scour	UK	ATCC51140
A2	Pig	Diseased	Netherlands	Ch 3 PhD thesis A. ter Huurne 1993
B256	Pig	No symptoms	USA	ATCC 29796, Kinyon and Harris, 1978
A3	Pig	Diseased	Netherlands	Ch 3 PhD thesis A. ter Huurne 1993

2.2.2 Culturing techniques

Spirochaetes were cultured on plates of Columbia agar base (Oxoid) supplemented with 5% (v/v) horse blood. When necessary, an antibiotic mixture of 200μg/ml spectinomycin, 3.2μg/ml vancomycin and 3.2μg/ml colistin (final concentration in plates) was used to remove contamination. Plates were incubated in an anaerobic environment for five days at 37°C then suspensions prepared for examination using dark field light microscopy at x400 magnification. Pure cultures were then transferred to liquid cultures of Tryptone Soya Broth (TSB, Oxoid) with 7.5% horse serum (Oxoid), 0.25% (v/v) glucose and 0.05% (v/v) cysteine by loop inoculation. The liquid cultures were incubated for 48 hours at 37°C prior to examination by dark field light microscopy at x400 magnification.

2.2.3 Maintenance of bacterial stocks

Stocks of bacteria were made by placing a heavy inoculum from uncontaminated plates into 1ml TSB/15% (v/v) glycerol. An alternative method of making frozen stocks was to take 850µl of an uncontaminated broth culture and add 150µl of sterile glycerol. Stocks were stored at -70°C or in liquid nitrogen.

2.2.4 PFGE analysis of intestinal spirochaete genomic DNA

2.2.4.1 Preparation of genomic DNA

Spirochaetes were harvested from broth cultures (20ml) by centrifugation for 10 minutes at 3000rpm (Beckman J2-21, JA25.50 rotor). The supernatant was discarded and the pellet resuspended in 1ml Net-100 (0.1M NaCl; 0.1M EDTA pH8.0; 0.01M Tris-HCl pH8.0). The spirochaetes were centrifuged (13000rpm, Eppendorf 5415C centrifuge, 5 minutes) in pre-weighed eppendorf tubes, the supernatant removed and resuspended in Net-100 to a final concentration of 40mg/ml. Equal volumes of prewarmed (50°C, 5 minutes) bacterial suspension and molten 0.9% (w/v) chromosomal grade agarose (BioRad) in Net-100 were mixed and immediately dispensed into a perspex block mould. Once solidified, the agarose blocks were incubated in lysis solution (6mM Tris-HCl pH7.6; 1M NaCl; 100mM EDTA pH8.0; 0.5% (w/v) sarcosyl; 1mg/ml lysozyme) for 24 hours at 37°C. The lysis solution was drained, replaced with ESP (0.5M EDTA pH 9.0; 1% sarcosyl; 1.5mg/ml proteinase K) and incubated at 50°C for 48 hours.

The ESP was drained and the blocks treated with 3ml TE (10mM Tris-HCl pH 8.0; 1mM EDTA pH 8.0) and 30µl of 0.1M Phenylmethylsulfonyl fluoride (PMSF) in isopropanol for two hours with constant rolling. This was repeated, then the blocks were subjected to three 20 minute incubations in TE only, with rolling. Alternatively to avoid the use of PMSF, blocks were rolled for two hours in 3ml TE. This process was repeated followed by a further rolling incubation in TE for one hour. Samples were rolled for 16-18 hours in fresh TE, and this was then replaced with 5ml of fresh TE. The blocks were stored at 4°C (Maslow *et al.*, 1993).

2.2.4.2 Digestion of genomic DNA

A sliver of agarose (1mm x 1mm x 9mm) was cut from the test block and incubated in restriction endonuclease buffer (50mM Potassium acetate; 20mM Tris acetate; 10mM Magnesium acetate; 1mM DTT) on ice for 15 minutes. The buffer was removed and replaced by 40 units of restriction endonuclease in buffer at a final reaction volume of 200µl. Two enzymes were used: *SacII* which has the restriction site 5'- C C G C / G G - 3' (Stratagene) and *SmaI* which has the restriction site 5' - C C C / G G G - 3' (Boehringer Mannheim).

The enzyme mixture was equilibrated at 0-4°C for 15 minutes before incubating for 16-18 hours at 37°C for *SacII* digests or at 30°C in the case of *SmaI* digests. Every isolate studied was digested with both enzymes in duplicate. In cases where the banding patterns were not clearly visible, slivers of agarose with dimensions of 2mm x 1mm x 9mm were used.

2.2.4.3 Separation of fragments and visualisation

1% (w/v) molecular biology grade agarose (BioRad) was melted in 0.5x TBE, then cast as a gel. Two litres of 0.5x TBE buffer were pre-cooled in the CHEF electrophoresis cell (BioRad) to 10°C. The restriction enzyme solution in which the agarose slivers were incubated was removed, 200µl of ES added (0.5M EDTA pH9.0; 1% (w/v) sarcosyl) and incubated at 50°C for 15 minutes. The ES was replaced with 1ml TE and after 15 minutes at 20-25°C, the samples were loaded into the gel. Standards were loaded onto each gel: a 2mm sliver of Lambda concatamer molecular weight standard (BioRad) with a range of 48.5kbp to 970kbp was used. The samples were sealed into the wells with molten 0.5% (w/v) agarose in 0.5x TBE. The gel was electrophoresed for 22 hours with a ramped pulse time of 10 to 60 seconds at 6 volts/cm, then for a further 2 hours with a ramp of 70 to 90 seconds at 6 volts/cm. Finally, the gel was stained with ethidium bromide (0.5µg/ml) for 30 minutes then destained in distilled water for at least one hour. The gels were visualised under ultraviolet light (254nm), using a scanner (UVP products).

2.2.5 Fragment analysis

2.2.5.1 Sizing bands

The fragments visualised in each gel were used in the Intermediate 1d Gelworks programme (UVP Products). The programme was used to compare the position of

fragments in the test lanes with those in the molecular standard lane (which were of a known size) to determine the size of fragments in the test isolates.

2.2.5.2 Isolate comparison

The fragment sizes assigned by the Gelworks 1d package were used to confirm the number of fragments each isolate had in common. In cases of fragments between the size of 40 and 300kbp, fragments were considered to be identical if they were within 5kbp in value. If the fragments were 300 - 800kbp the fragments were considered identical if they differed by less than 20kbp. Above this range there were few fragments and any fragments in this range were examined by inspection.

Isolates which exhibited 5 or less fragments in total for digests with *SacII* and *SmaI* were removed from the study as they contained too few fragments for meaningful statistical comparisons.

2.2.5.3 Analysis of macrorestriction fragments using UPGMA

The comparison of isolates yielded the number of bands that each pair of strains had in common. Dice co-efficients (S_D) were calculated to determine the degree of relatedness of pairs of strains. S_D is equal to twice the number of bands that two isolates have in common divided by the total number of bands in each isolate (Grothues and Tummler, 1991).

$S_D = 2n_i / (n_a + n_b)$

where n_i = no. bands which are common to profile A and B

n_a = no. bands in profile A

n_b = no. bands in profile B

A mean value of S_D was calculated for all isolates digested with both restriction enzymes and used in the statistical package Unistat to create a dendrogram based on UPGMA.

2.2.6 Characterisation of intestinal spirochaetes by Western Blotting of whole cell preparations to species-specific monoclonal antibodies

2.2.6.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of soluble spirochaete proteins was carried out in the Mini Protean system (BioRad) using a 12% separating gel and a 3% stacking gel (table 2.3) (Laemmli, 1970). An equal volume of the whole cell suspension (40mg/ml in Net-100) was mixed with an equal volume of sample buffer and boiled for 5 minutes. Gels were electrophoresed at 200V.

Table 2.3: Components for preparing SDS-PAGE gels

Components	Separating gel (12%)	Stacking gel (3%)	Sample buffer
Stock 1	10ml		
Stock 2		2.5ml	
10% (w/v) SDS	0.5ml	0.15ml	5ml
1.5M Tris pH8.8	6ml		
0.5M Tris pH6.8		3.75ml	2.5ml
Distilled water	3ml	8ml	5ml
10% (w/v) AMPS	70µl	50µl	
TEMED	50µl	40µl	
Glycerol			2.5ml
2-mercaptoethanol			0.25ml
5% bromophenol blue			0.2ml

Stock 1 - 22% w/v acrylamide and 0.57% (w/v) N,N'-methylene-bis-acrylamide (BIS)
Stock 2 - 30% w/v acrylamide and 0.78% (w/v) N,N'-methylene-bis-acrylamide (BIS)

Gels were electrophoresed with nine samples on each gel and one lane containing a standard. If gels were to be stained, 8µl of Dalton mark VII molecular weight standard (Sigma) (Bovine alpha - lactalbumin 14.2kDa, soy bean trypsin inhibitor 20.1kDa, bovine typsinogen (PMSF treated) 24kDa, bovine erythrocyte carbonic anhydrase 29kDa, rabbit muscle glyceraldehyde-3 phosphate dehydrogenase 36kDa, egg albumin 45kDa, bovine albumin 66kDa) was loaded. If gels were electrophoresed for Western blotting, 3µl of BioRad low range prestained SDS-PAGE marker (Phosphorylase B 105kDa, bovine serum albumin 82kDa, ovalbumin 49kDa, carbonic anhydrase 33.3kDa, soybean trypsin inhibitor 28.6kDa and lysosyme 19.4kDa) was loaded. The optimal sample loading was determined using Coomassie blue: 5µl was optimal for staining gels, for Western blotting it was 7µl.

After electrophoresis, the proteins on the gels could be stained or transferred to a nitrocellulose membrane and used for probing with antisera. Gels for staining were incubated for one hour in Coomassie brilliant blue R-250 0.01% (w/v) in 50% methanol/20% (v/v) acetic acid then transferred to 10% methanol/20% (v/v) acetic acid for destaining. After destaining for one hour with at least one change of solution, gels were visualised using a scanner (UVP Products).

2.2.6.2 Immunoblotting

Proteins for probing with antisera were transferred from SDS-PAGE gels to nitrocellulose membranes (0.45μm pore size, BioRad) using a Mini Trans-blot cell (BioRad) (Towbin *et al.*, 1979). The transfer was carried out in transblot running buffer (25mM Tris, 192mM glycine and 20 % v/v methanol, pH8.3) on ice for one hour at 100V.

To block the unbound sites, the membrane was washed in TBS containing 0.3% Tween (TBST) for one hour with gentle agitation. Monoclonal antibody was applied to the membrane and incubated overnight at 4°C. BJL/SH1, species-specific for *S. hyodysenteriae* (Lee and Hampson, 1996) and BJL/AC1 which is species-specific for *S. pilosicoli* (Lee and Hampson, 1995) were used at a dilution of 1:250 and 1:500 in TBST respectively.

The membranes were washed three times in TBST before incubation in TBST containing 0.25μg/ml goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma) with agitation. Finally, blots were developed with 0.01% H_2O_2 (v/v) and 25μg/ml (w/v) 4-chloro-1-naphthol in 10mM Tris HCl pH7.4. Colour development was accelerated by

warming at 37°C and stopped by washing with distilled water. Control membranes were incubated in TBST without monoclonal antibodies and developed as previously described.

Isolates which initially were not reactive with either monoclonal antibody, were retested with the BJL/AC1 monoclonal antibody at a dilution of 1:250. Isolates which did not react with BJL/AC1 at a dilution of 1:250 were probed with two other monoclonal antibodies: H9724 is a monoclonal antibody specific for flagellin, a 41kDa protein present in *Borrelia* species and 2B11 which reacts with a 44kDa protein of *T. pallidum*. Positive controls were included, namely B31 (the reference strain of *B. burgdorferi sensu stricto*, ATCC 35210) for H9724 and a recombinant protein derived from *E. coli* expressing the *T. pallidum* 44kDa protein gene for 2B11.

2.3 Results

2.3.1 Pulsed field gel electrophoresis

2.3.1.1 Macrorestriction fragment profiles

SacII and *SmaI* were selected for PFGE as they restricted the intestinal spirochaete genome into an average of 5-7 fragments. Five isolates from Table 2.2 (382/91, *B. aalborgi*, Z10, ?, ?) contained too few fragments for statistical analysis and so these were removed from this part of the study. These isolates contained a total of less than 5 fragments and included *B. aalborgi* (Hovind-Hougen *et al.*, 1982) and the bacteraemic isolate 382/91 (Fournie-Amazouz, *et al.*, 1995). For each isolate tested, profiles were duplicated on separate gels to ensure reproducibility. Of all the profiles examined, only two isolates shared identical restriction fragment profiles with both restriction endonucleases. These were isolates Gel-2 (47) and Gel (48) which were obtained from the same homosexual male in the UK but at different times.

The importance of using two restriction enzymes for discriminating between isolates is shown in Figure 2.1. This shows two isolates (B78 (59) and JWPM (60)) which had identical profiles using *SmaI* but were differentiated using *SacII*. The same was true of another pair of isolates: AF6/80 (68) and WT10/82 (65) which exhibited the same *SmaI* profile but could be differentiated by 2 bands in their *SacII* profiles. Although a small number of isolates showed similar patterns, most isolates displayed considerable heterogeneity (Figure 2.2).

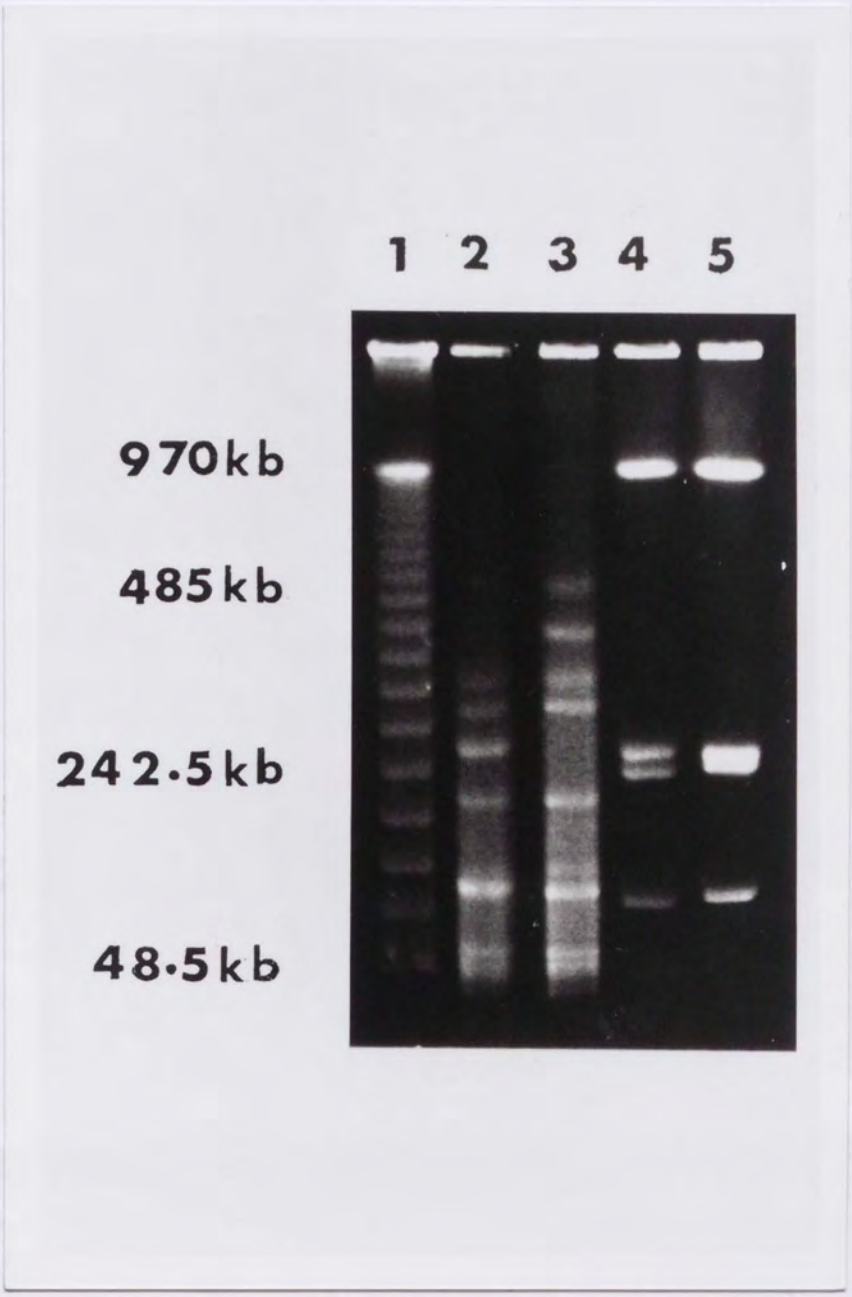


Figure 2.1: Discrimination of isolates B78 (59, lanes 2 and 4) and JWPM (60, lanes 3 and 5) using PFGE. Profiles were identical when using *SmaI* (lanes 4 and 5) but were differentiated using *SacII* (lanes 2 and 3). Lane 1 contained a λ concatamer marker and the fragment sizes in kbp are indicated on the left.

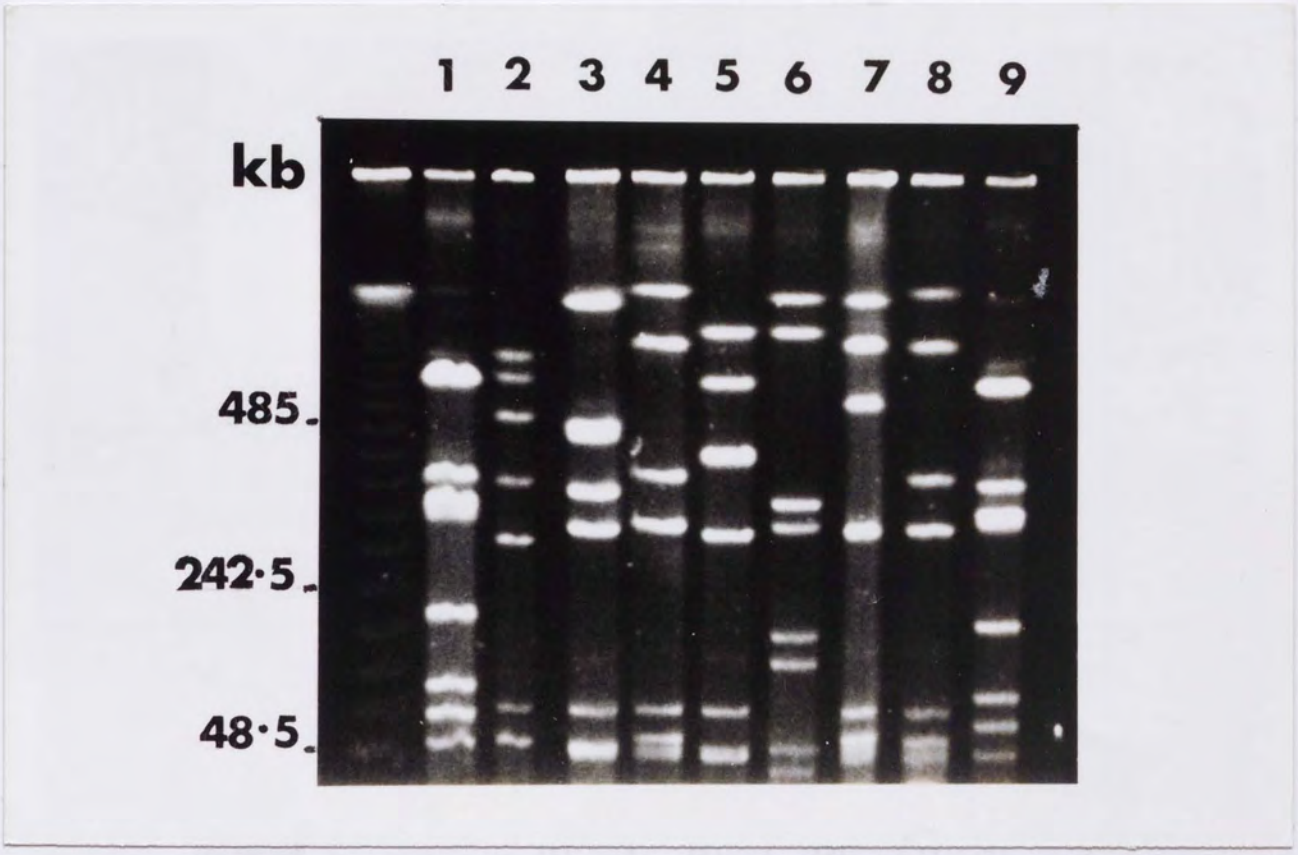


Figure 2.2: PFGE gel illustrating heterogeneity shown by isolates from the Muscat region of Oman, digested with *SmaI*. Lanes 1-9 correspond to isolates 1, 3, 4, 6, 9, 10, 12, 17 and 26 from table 2.1. The sizes of λ concatamers are shown on the left in kbp.

The approximate size of the intestinal spirochaete genome was estimated by two methods. The sum total of the restriction fragment sizes estimated the genome to be approximately 3Mb. The PFGE of genomic DNA from representative isolates digested with *NotI* which has a single restriction site (Zuerner and Stanton, 1994) showed it to be approximately 2.6-3.0 Mb.

2.3.1.2 Determination of fragment sizes

The Gelworks 1d programme determined the fragment sizes by comparison of the lambda concatamer marker with fragments (represented by peaks) in test lanes. A representative example of a profile is illustrated in Figure 2.3.

Figure 2.3: Densitometric analysis of restriction fragments produced by Wesley (54) after digestion with *SacII* using the Gelworks 1d programme. Fragment sizes are shown in bp.

2.3.1.3 Division of *S. pilosicoli* isolates into two clusters

The dendrogram generated from the UPGMA of typable isolates in Table 2.1 is shown in Figure 2.4. Two clusters were obtained, the first of these was the largest and consisted of isolates no. 1-58. The second cluster which was separated from the first at a distance of 1.75 Euclids contained isolates 59-68.

2.3.1.4 Division of *S. hyodysenteriae*, *S. pilosicoli* and unidentified isolates into 4 clusters.

The addition of unidentified isolates to the UPGMA analysis resulted in a dendrogram which was differentiated into four clusters (Figure 2.5). As in Figure 2.4, the first cluster was the largest and consisted of isolates 1-58 (excluding 44, 52 and 53).

Clusters 2 and 3 were separated from cluster 1 at a distance of 1.75 Euclids and from each other at 1.6 Euclids. Eight isolates from Figure 2.4 (59-62 and 65-68) and a porcine isolate (D11) were contained in cluster 2. Isolate no. 44, 52 and 53 grouped with other isolates from a variety of origins in cluster 3. Cluster 4 consisted of 4 isolates, $B256^T$ and three other isolates (A2, A3 and D7) which were separated from the other clusters at a distance of 1.875 Euclids.

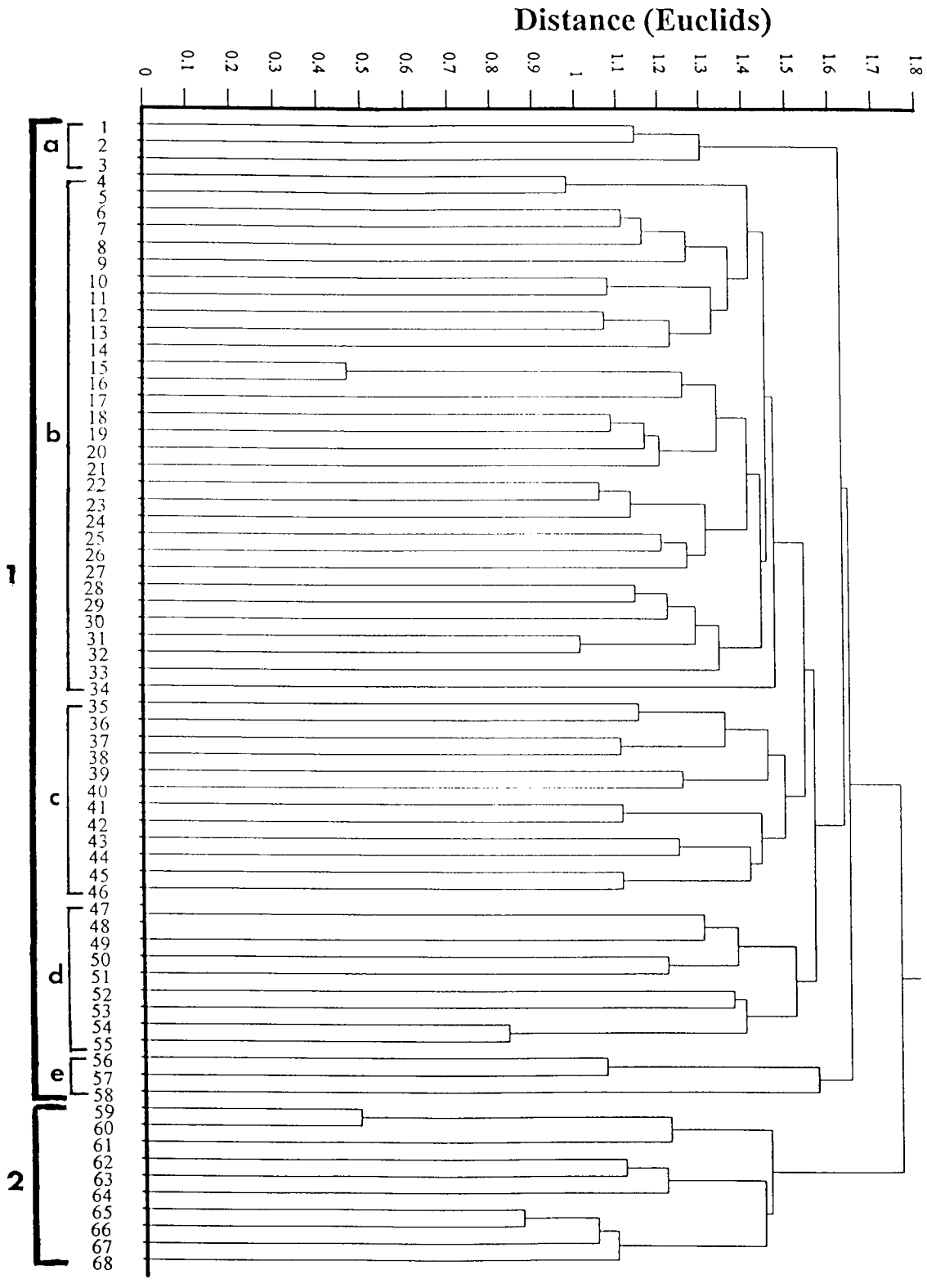


Figure 2.4: Dendrogram of relatedness (*SacII/SmaI*) for isolates in table 2.1 showing specific differentiation into two clusters consisting of *S. pilosicoli* isolates (cluster 1) and *S. hyodysenteriae* isolates (cluster 2).

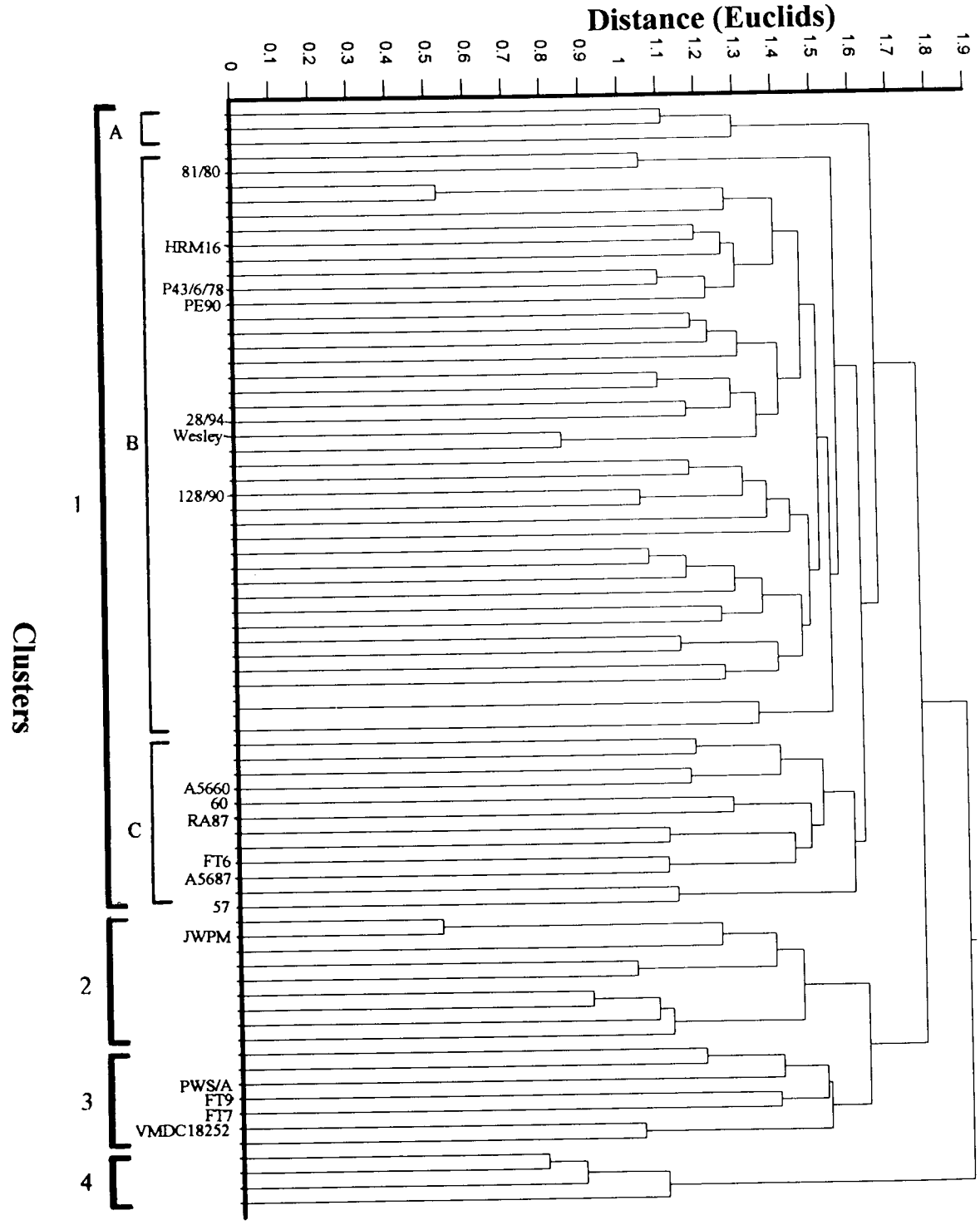


Figure 2.5: Differentiation of isolates (*SacII/SmaI*) from tables 2.1 and 2.2 into four clusters. *S. pilosicoli* isolates were located in clusters 1 and 3, *S. hyodysenteriae* isolates in cluster 2 and unidentified isolates in clusters 2, 3 and 4. The positions of isolates used in Chapter 3 are indicated on the dendrogram of relatedness.

2.3.2. Western blotting

2.3.2.1 Reactivity of isolates with monoclonal antibody probes

Of the 82 isolates examined in this study, whole cell preparations of 80 isolates were probed with both of the monoclonal antibodies. P43/6/78^T (no.51) and B256^T were not tested as they had been probed during the production of both antibodies (Lee and Hampson, 1995; Lee and Hampson, 1996). B256^T is the type strain of *S. innocens* and so did not react with either monoclonal antibody whereas P43/6/78^T, the type strain of *S. pilosicoli* reacted with BJL/AC1.

Of the remaining isolates, numbers 59-68 from table 2.1 and isolate Z10 (table 2.2) reacted with the monoclonal antibody BJL/SH1 for *S. hyodysenteriae*. Isolates B78^T (59), B204 (61) and P18A (64) have previously been identified as *S. hyodysenteriae* using BJL/SH1 (Lee and Hampson, 1996). Whilst most isolates identified as *S. hyodysenteriae* were obtained from pigs, WT10/82 (65) was acquired from a mouse, and 11416 (63) from a chicken. A representative immunoblot showing the positive reaction of BJL/SH1 with isolate B204 (61) is shown in figure 2.6.

A further 59 isolates were characterised as *S. pilosicoli* by BJL/AC1. These consisted of isolates 1-59 in table 2.1 (excluding 51) and 382/91 which was not included in figure 2.4 due to an insufficient number of DNA fragments. The isolates identified as *S. pilosicoli* represented isolates from a number of animal species: 52 were from humans (34 from the Muscat region of Oman), 4 from dogs and 2 from pigs. Figure 2.7 shows the reactivity of the canine isolate A5687 with BJL/AC1.

A further 11 isolates which were tested, did not react with either monoclonal antibody. These were *B. aalborgi*, VMDC 18251, VMDC 18252, Heal, D11, Z2, D7, FF3, PWS/A, A2 and A3. Of these, PWS/A had previously been tested with both monoclonal antibodies and *B. aalborgi* with BJL/AC1 (Lee and Hampson, 1995; Lee and Hampson, 1996). When probed with the *Borrelia* specific flagellin monoclonal antibody H9724 and the *T. pallidum* monoclonal antibody 2B11 none of the unidentified intestinal spirochaete isolates produced a positive reaction.

During immunoblotting, a number of occasions arose when in addition to the expected monoclonal specific band at 29kDa (BJL/AC1), additional bands were observed at 24kDa or, 58kDa. Control immunoblots developed without incubation with a monoclonal antibody resulted in the visualisation of a band at 24kDa suggesting that this band was produced by non-specific binding of the goat anti-mouse IgG conjugate.

The 58kDa band was not observed in the control blots or immunoblots probed with BJL/SH1. Since the observation was only made in two isolates which reacted with BJL/AC1, it may represent dimeric forms of the protein with which the antibody reacted in these isolates.

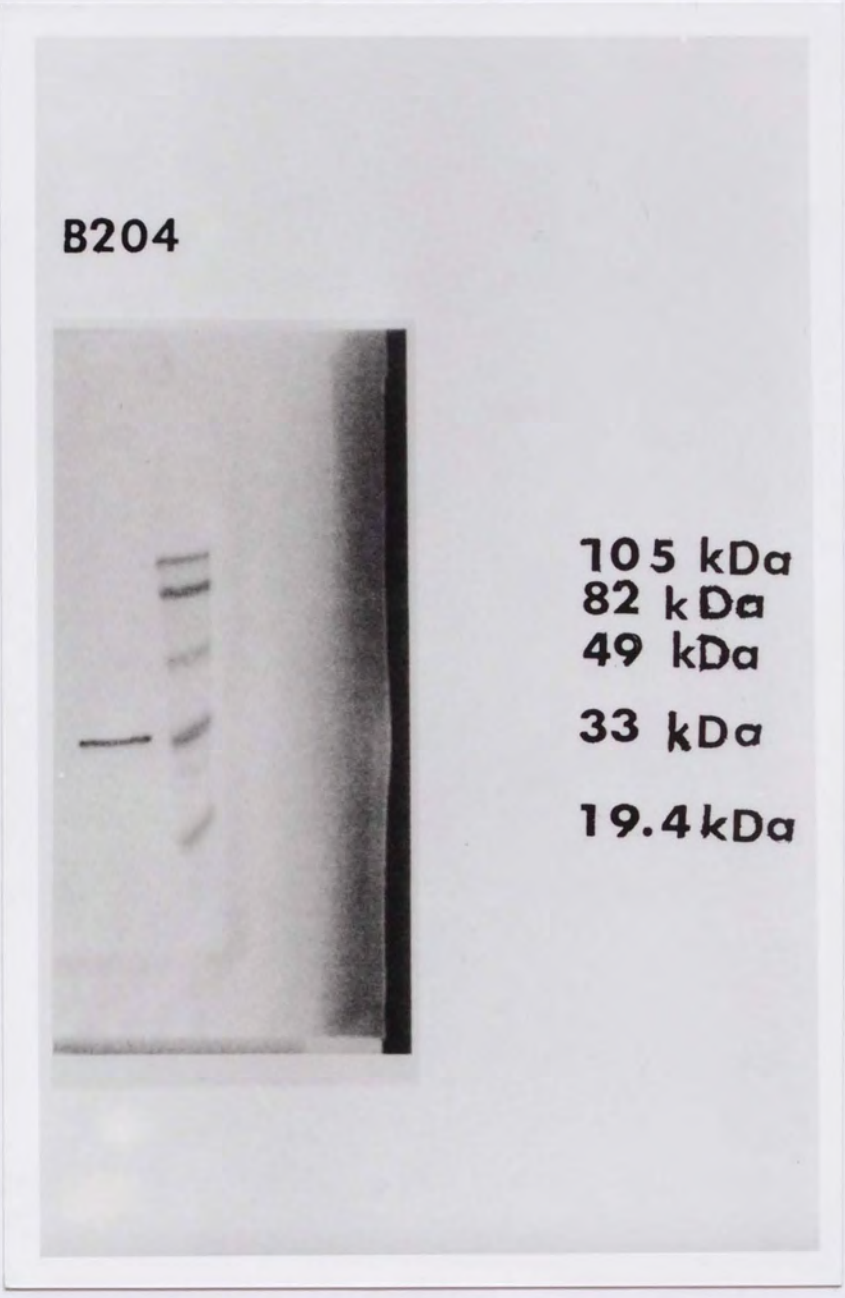


Figure 2.6: Western blot illustrating isolate B204 (61) probed with species specific monoclonal antibody BJL/SH1 (specific for a 30kDa protein of *S. hyodysenteriae*). Molecular weight standards are shown on the right in kDa.

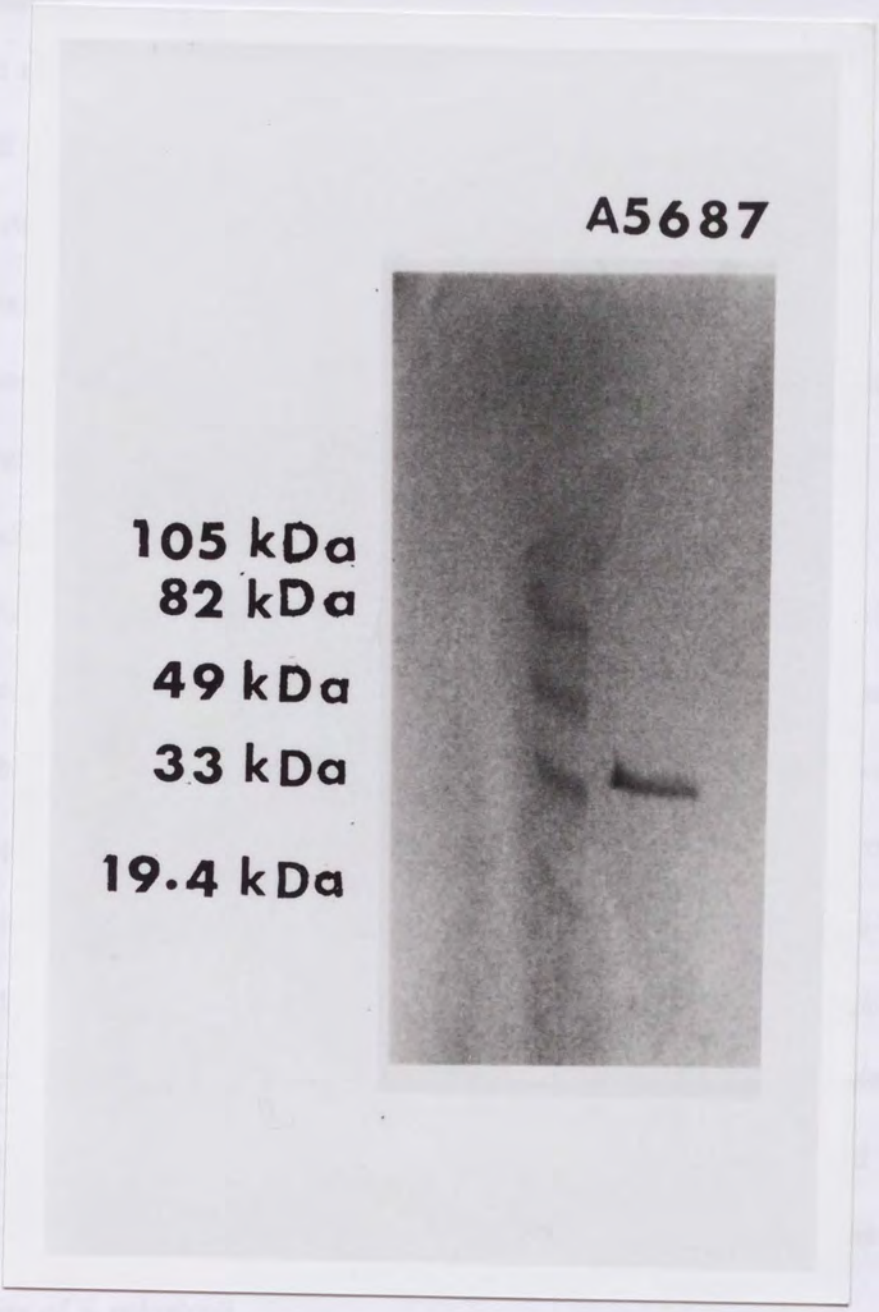


Figure 2.7: Western blot illustrating isolate A5687 (46) probed with species specific monoclonal antibody BJL/AC1 (specific for a 29kDa protein of *S. pilosicoli*). Molecular weight standards are shown on the left in kDa.

2.4. Discussion

The dendrogram in Figure 2.4 separates clusters 1 and 2 at a distance of 1.75 Euclids. All isolates in cluster 1 were identified by immunoblotting as *S. pilosicoli* and those in cluster 2 as *S. hyodysenteriae* suggesting that specific differentiation of intestinal spirochaetes is possible. Cluster 1 was subdivided into 5 groups (a-e). Omani isolates were found in each group, which demonstrates the genetic diversity of isolates which are epidemiologically related (isolated from a discrete area within a defined time frame). Previous studies have demonstrated the heterogeneity of intestinal spirochaetes, particularly *S. pilosicoli* using PFGE (Atyeo *et al.*, 1996), MEE (Lee and Hampson, 1994; Duhamel *et al.*, 1995) and SDS-PAGE (Barrett *et al.*, 1996). The Omani isolates did not appear to be genetically closely related as there was little correlation between genotype and geographical area. Two groups (a and e) consisted entirely of isolates from Oman and of these, group 1a contained isolate 26 (2) which was found in a separate branch when compared to other intestinal spirochaetes using 16S rRNA sequences (Hookey *et al.*, 1994). The largest group in cluster 1 was group b which consisted entirely of human isolates with the exception of PWS/B (32) and A3888 (34). Groups c and d were from diverse origins (pigs, dogs and humans) further demonstrating the subspecific heterogeneity of *S. pilosicoli*.

The French bacteraemic isolates did not cluster together within the *S. pilosicoli* groups suggesting that the bacteraemic property may not be correlated with genotype although the number of isolates available for study was small. This was also observed when bacteraemic isolates were compared with other *S. pilosicoli* isolates by MEE (Trott *et al.*, 1997a).

Two isolates of *S. pilosicoli* were found to have identical genotypes : Gel-2 (47) and Gel (48). These isolates were obtained from the same homosexual male at different times suggesting that the infection was stable and persistent, as observed in mice (*S. hyodysenteriae*) (Clark, 1984), pigs (Neef *et al.*, 1994) and aboriginal children (Lee and Hampson, 1992). It is also possible they were infected by more than one bacterial population although only one was selected during the isolation procedure. Another three isolates in the study were from homosexual males (M15183 (37), FT9 (52) and FT7 (53)) and each of these showed a different genotype. To establish whether homosexual men were predisposed to infection with certain genotypes, further investigation would be required with a larger pool of isolates.

No correlation between genotype and disease manifestation was observed in this study. Similarity was observed between genotypes of some human and canine isolates such as FT6 (45) and A5687 (46) which were divergent at a distance of 1.1 Euclids. This suggested that they shared a common ancestor and that cross species transmission may be feasible as proposed after similar observations were made in MEE profiles (Koopman *et al.*, 1993; Lee and Hampson, 1994; Duhamel *et al.*, 1995).

Cluster 2 consisted of isolates identified as *S. hyodysenteriae*. These isolates showed less diversity than *S. pilosicoli*, which is illustrated by B78 (59) and JWPM (60) which were only differentiated from each other by digestion with one enzyme (Figure 2.1).

Cluster 1 of Figure 2.5 which consisted entirely of *S. pilosicoli* isolates as determined by western blotting could be sub-divided (A-D). Group 1A contained the same *S. pilosicoli* isolates as in Figure 2.4 cluster 1a. Group 1B consisted of 20 human isolates and one porcine isolate ($P43/6/78^T$ reference strain of *S. pilosicoli*) and of the human

isolates, three were French, two Australian, two Italian and the rest Omani. Group 1C was made up of 19 isolates which were all of human origin with the exception of PWS/B (porcine) and A3888 (canine). All human isolates in this cluster were obtained from the Muscat region of Oman with the exception of the French isolate 128/90. Ten human and two canine isolates from a mixture of origins were found in group 1D. Isolates which were not identified by western blotting with BJL/AC1 and BJL/SH1 were included in Figure 2.5 and were found in clusters 2-4 but did not form species specific clusters. It is likely that the numbers of unidentified isolates were too few for specific differentiation and that the isolates were from more than one species. The three porcine isolates which clustered with $B256^T$ may belong to *S. innocens* whereas the unidentified isolates in cluster 3 which were porcine, avian and canine may be representatives of more than one species. The porcine isolate was the reference strain for *S. intermedia* but the chicken and canine isolates may belong to *S. alvinipulli*, "*S. canis*", *S. intermedia, S. murdochii* or species which have yet to be defined.

In conclusion, intestinal spirochaetes and especially *S. pilosicoli* showed considerable heterogeneity using genotyping methods. This is consistent with other PFGE studies of intestinal spirochaetes as well as MEE and SDS-PAGE data.

Chapter 3: Adhesion to intestinal epithelial cell lines

3.1 Introduction

3.1.1 Use of cell culture as an in vitro model of the human intestinal epithelium

Human adenocarcinoma cell lines such as Caco-2 and HT29 show varying degrees of differentiation but are a reproducible source of intestinal epithelial cells. A study of twenty cell lines classified them into four categories according to their differentiation (Chantret *et al.*, 1988). The only cell line which fitted into type 1 was Caco-2 cells which spontaneously differentiate into polarized cells with dome formation, a well developed brush border and expression of a number of hydrolases. HT29 cells were assigned to the second category as they were able to differentiate but not spontaneously (Chantret *et al.*, 1988). Differentiation in HT29 cells was observed when glucose in the media was substituted for galactose (Pinto *et al.*, 1982). The number of surface expressed peptidases has been compared for Caco-2 and HT29 cells (Caco-2 cells express 8, compared to the 3 peptidases found in HT29 cells) (Howell *et al.*, 1992).

Several features of the Caco-2 model make it suitable for studies involving drug uptake, transport and metabolism and this includes the number of peptidases expressed, spontaneous differentiation and the formation of tight junctions which prevents the passive movement of the drug concerned. This model has been used to study the uptake of the β-lactam antibiotic ceftibuten (Muranushi *et al.*, 1994) and other cephalosporins (Dantzig and Bergin, 1990). It has been used to study the transport of compounds such as peptidomimetic thrombin inhibitors (Walter *et al*,

1995a), peptidomimetic renin inhibitors (Walter *et al.*, 1995b) and thyrotropin-releasing hormone (Walter and Kissel, 1994). The effect of permeation enhancers (Werner *et al.*, 1996; Jørgensen *et al.*, 1993), stereoisomerism of oligopeptides (Tamura *et al.*, 1996), and the degree of amino-acid methylation (Conradi *et al.*, 1992) on transport have also been investigated using the Caco-2 model. Caco-2 cells have been developed that express high levels of cytochrome P450 (a drug metabolising enzyme that is expressed at high levels *in vivo*) which has enabled more studies to evaluate drug metabolism as part of their drug absorption model (Crespi *et al.*, 1996).

3.1.2 Caco-2 as a model of bacterial adhesion and invasion

The Caco-2 cell model has been utilized in a number of ways to investigate the interaction of hosts with bacteria. Many studies have compared the adhesion and invasion of host cells with virulent and avirulent strains. Some workers have utilized this adherence system to identify moieties involved in the adhesion process or to investigate the competitive adhesion of different species of bacteria.

When tested with a range of epithelial cell lines, enterotoxigenic *Escherichia coli* (ETEC) adhered only to Caco-2 cells suggesting that Caco-2 cells behave in the same way as human enterocytes in this infection model (Darfeuille-Michaud *et al.*, 1990). Studies have been undertaken using a number of *Aeromonas* species. Adhesion and invasion studies were conducted on several *Aeromonas* species using the Caco-2 model (Nishikawa *et al.*, 1994) and comparison of the adherence of *Aeromonas* species to Hep-2 (human laryngeal epithelial cells) and Caco-2 cells demonstrated that both models correlated well with enteropathogenicity (Kirov, *et al.*, 1995). This was confirmed for *A. caviae* which showed similar patterns of adhesion for both cell lines (Thornley *et al.*, 1996). The adhesion of non-01 *V. cholerae* to Caco-2 cells correlated

well with investigations in humans, suggesting that it was an appropriate model of adhesion (Panigrahi *et al.*, 1990). Caco-2 cells have also been used to demonstrate the penetration and persistence of *Bordetella bronchiseptica* (Schipper *et al.*, 1994) and, adhesion and invasion of *C. jejuni* (Russell and Blake, 1994) which was subsequently shown to be dependent on swimming behaviour and environmental conditions such as viscosity and pH (Szymanski *et al.*, 1995). The adhesion of beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* species has utilized the Caco-2 model. Three species of *Lactobacillus* were tested for their adherence to the Caco-2 and Int-407 cell lines. It was demonstrated that adhesion to Caco-2 cells was protein mediated to brush border microvilli whereas bacteria adhering to Int-407 were trapped in a carbohydrate based fimbrial extracellular matrix material (Sarem *et al.*, 1996). A good correlation between the adhesion of *Bifidobacteria* species *in vivo* (which are believed to have a probiotic effect in the large intestine) and observations made using the Caco-2 model suggested that the cell culture model can be applied to both the large and small intestine (Crociani *et al.*, 1995).

Investigations into bacterial adhesive factors have studied the inhibition of bacterial adhesion to cells after pretreatment with chemicals, serum or bacterial factors such as putative fimbrial or pili subunits. Antibodies against *V. cholerae* outer membrane protein OmpU completely inhibited bacterial adhesion to a range of cell lines (including Caco-2) and colonisation of new born mice (Sperandio *et al.*, 1995). Antibodies raised against an ETEC 16kDa fimbrial protein and Fab fragments against KPF-28 (a fimbrial protein of *K. pneumoniae*) were also demonstrated to inhibit bacterial adhesion to Caco-2 cells (Grewal *et al.*, 1997; Di Martino *et al.*, 1996). CF29K and CS31A, colonisation factors of *K. pneumoniae* and ETEC were shown to be involved in adhesion to Caco-2 cells (Di Martino *et al.*, 1995; Bernet-Camard *et al.*,

1995). In the case of ETEC, this included the HT29 glc$^-$ cell sub-population (selected from parent population by glucose deprivation) provided glucose was present in the media (Bernet-Camard *et al.*, 1995). A plasmid mediated colonisation factor of *E. coli* has been demonstrated to be responsible for the diffuse adherence pattern observed with Caco-2 cells (Jallet *et al.*, 1994). The Caco-2 cell epithelial model has also been used to investigate the adherence properties of clones from a λ zap library of *Clostridium difficile* which had been screened with polyclonal serum raised against heat-shocked bacteria (Karjalainen *et al.*, 1994).

The competitive adhesion of beneficial bacteria, such as *Lactobacillus* species, over enteropathogens has been studied using the Caco-2 epithelial model. *Lactobacillus acidophilus* inhibited the adhesion of ETEC, enteropathogenic *E. coli*, and *S. typhimurium* as well as invasion by enteropathogenic *E. coli*, *Yersinia pseudotuberculosis* and *S. typhimurium* (Bernet *et al.*, 1994). Co-culture of Caco-2 cells and HT29 subclone HT29-MTX has been used to show that there was a good correlation between passive transport of drugs *in vitro* and *in vivo*, although active transport was less *in vitro* than *in vivo*.

3.1.3 Adherence of spirochaetes to epithelial cell lines

T. denticola produces a major surface protein (MSP) which was believed to have a cytopathic effect on epithelial cells. This was demonstrated by the ability of purified MSP to depolarize HeLa cells and cause an increase in membrane conductance suggesting the formation of ion channels (Mathers *et al.*, 1996).

The attachment of *S. hyodysenteriae* and *S. innocens* have been studied using Henle cells (an intestinal epithelial cell line). Motility and viability affected the

frequency of adhesion which reached a maximum of 20 adherent spirochaetes per cell after 90 minutes for *S. hyodysenteriae*. In contrast, *S. innocens* showed a maximum adherence of 10 spirochaetes per cell after 90 minutes. Adhesion was inhibited by pretreatment of the cells with hyper-immune or convalescent serum and with compounds which contained sialic acid suggesting that it may be an important part of the adhesin responsible for attachment to Henle cells (Bowden *et al.*, 1989).

3.1.4 Aims

This part of the project had several objectives. Figure 2.4 showed that isolates from different animal origins were genotypically similar and so the first objective of the study was to compare the adhesion of these isolates and genotypically different isolates using the Caco-2 cell line as a model of the human intestinal epithelium.

The second objective was to compare the Caco-2 cell culture model with that of the Caco-2/HT29 co-culture model for the adhesion of intestinal spirochaetes.

The final aim was to investigate the invasive properties of the French bacteraemic isolates. Figure 2.4 showed that the bacteraemic property was not correlated with genotype so this part of the study was to investigate whether penetration of the Caco-2 model was possible and if so, to compare the pattern of invasion observed for bacteraemic and other *S. pilosicoli* isolates.

3.2 Materials and Methods

3.2.1 Cell Lines

The cell lines used for the adhesion and invasion assays are described in Table 3.1.

Table 3.1: Cell lines and range of passages used in cell culture assays

NAME OF CELL LINE	PASSAGE NO.	SOURCE	REFERENCE
Caco-2	26 - 40	ECACC*, No. 86010202	Fogh *et al.*, 1977
HT29	140-145	ECACC, No. 91072201	Fogh and Trempe 1975

* European Collection of Cell Cultures.

3.2.2 Maintenance of Cell lines

3.2.2.1 Maintenance of Caco-2 cells

Caco-2 cells were routinely cultured in 75cm^2 flasks (Falcon/Costar) with maintenance media. This consisted of Dulbecco's modified eagle media with 0.11g/L sodium pyruvate pyroxidase (DMEM, Life Technologies) supplemented with the following (final concentrations): 10% (v/v) Foetal Bovine serum (FBS) (Life Technologies), 2mM Glutamine (Life Technologies), 100 units Penicillin/ Streptomycin (Life Technologies), 1% Non-essential amino acids (Life Technologies) and 5mM

HEPES (Sigma). Every 2-3 days, spent Caco-2 cell media was removed using a vacuum pump and replaced with fresh maintenance media which had been pre-warmed to 37°C. Cells which were approaching confluence as determined by phase inversion light microscopy were passaged as described in section 3.2.2.3.

3.2.2.2 Maintenance of HT29 cells

HT29 cells were routinely cultured in McCoy's media (Sigma) supplemented with the following (final concentration): 10% FBS, 2mM Glutamine, 100 units Penicillin/Streptomycin. As previously described, the maintenance media was replaced after every 2-3 days with media pre-warmed to 37°C, until the HT29 cells were approaching confluence.

3.2.2.3 Passage of cell lines

The process of passaging ("splitting") cells involved the removal of the cells from the flask to which they were attached and seeding at a lower density to allow their continued proliferation. In the case of the Caco-2 cell line which consists of more than one cell type, confluence permits one cell type to become predominant. Maintenance media was removed from flasks and replaced with 5ml of PBS/EDTA (1x, GibcoBRL). Rotation of the solution around the base of the flask ensured that all cells were soaked in PBS-EDTA to remove remaining media and cells were then incubated at 22-25°C for 5 minutes. The PBS/EDTA was removed and replaced with 2.5ml of trypsin-EDTA (1x, GibcoBRL) which detached cells from the surface of the flask. After all cells had been covered for approximately 30 seconds, excess trypsin-EDTA was removed and the cells were incubated for 5 minutes at 22-25°C. The flask

was manually agitated to ensure that the cells had separated from the surface of the flask and to prevent further action of the trypsin on the cells, 10ml of maintenance media was added to the flask and thoroughly mixed with the trypsinised cells. The cells were counted using a haemocytometer and seeded in $75cm^2$ flasks (Falcon or Costar) at $3x10^5$ cells per flask. After addition of cells, the volume of media was made up to 10ml using pre-warmed maintenance media.

3.2.2.4 Freezing cells

Cells were frozen by passaging the cells as described above (section 3.2.2.3). Cells from one $75cm^2$ flask were resuspended in 2ml freezing media (maintenance media containing 20% (v/v) FBS instead of 10% (v/v) FBS and supplemented with 10% (v/v) DMSO) which had been stored at 0-4°C. Cells were incubated at 0-4°C for 15 minutes before storage in liquid nitrogen or at -70°C.

3.2.2.5 Resuscitation of a cell line from frozen stocks

Frozen cell culture stocks were thawed rapidly at 37°C and the cells added to 10ml of pre-warmed maintenance media. This mixture was centrifuged at 1000rpm (MSE Mistral 3000i) for 5 minutes to pellet the cells and remove the DMSO from the media. The supernatant was replaced with 7.5ml maintenance media before seeding in a $25cm^2$ flask (Falcon). Cells which were seeded from frozen stocks were maintained in the same media until they required passaging.

3.2.3 Bacterial adhesion to intestinal epithelial cell lines

3.2.3.1 Collagen coating of 24-well plates

A circular 13mm diameter sterile glass coverslip (Chance Propper) was placed in each well of deep-well 24-well plates (Falcon). Type 1 rat tail collagen (Collaborative Biomedical products) was diluted to 50μg/ml in 0.02M acetic acid and 1ml added to each well. The plates were then incubated at 22-25°C for 1 hour. Excess collagen was removed and the plates washed three times in PBS (without Calcium/Magnesium, GibcoBRL).

3.2.3.2 Preparation of cells for adhesion assay

Caco-2 cells were seeded at $3x10^5$ cells per collagen-coated 24-well plate and incubated at 37°C with 5% CO_2 for 21-24 days (15-18 days post confluence) changing media on alternate days. Cells were washed three times in media without penicillin/ streptomycin prior to experimentation.

3.2.3.3 Isolates cultured for adhesion and invasion assays

19 isolates from Table 2.1 and 2.2 were selected for adhesion and invasion assays. Of these, 16 were characterised as *S. pilosicoli* using the monoclonal antibody BJL/AC1, and one was identified as *S. hyodysenteriae* using the monoclonal antibody BJL/SH1 (section 2.2.6). Two of the isolates selected did not react with either monoclonal antibody, and of these PWS/A^T is the reference strain of *S. intermedia.*

Table 3.2: Intestinal spirochaete isolates used during the adhesion and invasion assays. A dendrogram based on genotyping of these isolates, and reactivity with the monoclonal antibodies BJL/SH1 and BJL/AC1 is shown in Figure 2.5.

ISOLATE	SPECIES
HRM16	*S. pilosicoli*
FT6	*S. pilosicoli*
60	*S. pilosicoli*
57	*S. pilosicoli*
A5660	*S. pilosicoli*
A5687	*S. pilosicoli*
28/94	*S. pilosicoli*
128/90	*S. pilosicoli*
RA87	*S. pilosicoli*
382/91	*S. pilosicoli*
PE90	*S. pilosicoli*
81/80	*S. pilosicoli*
FT7	*S. pilosicoli*
FT9	*S. pilosicoli*
Wesley	*S. pilosicoli*
P43/6/78^T (ATCC 51139)	*S. pilosicoli*
PWS/A^T (ATCC 51140)	*S. intermedia*
VMDC 18252	Unknown
JWPM	*S. hyodysenteriae*

3.2.3.4 Preparation of bacteria for adhesion assays

Bacteria were cultured as described in section 2.2.2. Broth cultures were inoculated with $2x10^7$ bacteria and samples counted at regular intervals until the bacteria reached approximately mid-log phase ($2x10^7$ bacteria/ml). Bacteria were centrifuged at 3000rpm (Beckman J2-20; JA14 rotor) for 10 minutes, then resuspended in 1ml maintenance media without penicillin/streptomycin and re-centrifuged (Eppendorf 5415C centrifuge, 13000rpm, 5 minutes). The spirochaetes were resuspended to a final concentration of $4x10^8$ spirochaetes/ml. For initial experiments, HRM16 (Table 3.2) was used in exponential (mid-log phase) and stationary phase cultures (after growth for 48 hours), inoculating wells with $1x10^6$(MOI of 0.1), $1x10^7$ (MOI of 1) and $1x10^8$ (MOI of 10) bacteria in 0.25ml maintenance media without antibiotics. In each experiment, bacterial samples were added in triplicate and three wells which were not infected were used as controls. Each isolate was tested in two separate experiments to ensure reproducibility. After inoculation of the well plates, the cells were incubated with spirochaetes for 1 hour at 37°C with 5% CO_2. Cells were then washed once with DMEM and three times with PBS to remove non-adherent bacteria.

3.2.3.5 Scanning electron microscopy

For each bacterial isolate tested, samples were prepared for scanning electron microscopy. Coverslips were treated with 0.1M Cacodylate buffer/2.5% glutaraldehyde for at least 30 minutes, then washed with 0.1M Cacodylate buffer for 1 hour. Samples were dehydrated in an ethanol series (20%, 30%, 40%, 50%, 60%, 70%, 90%, 95% and 100% (v/v)), then dried with hexamethyldisilazane (TAAB laboratories) before gold sputter coating (Denton vacuum Desk II) and examination

with a scanning electron microscope (Joel JSM5300LV) using an accelerating voltage of 20kV.

The use of hexamethyldisilazane has been shown to be a suitable replacement for liquid CO_2 in critical point drying. Identical ultrastructural characteristics were observed in hepatic endothelial cells by atomic force microscopy as well as scanning and transmission electron microscopy (Braet *et al.*, 1997). In addition, this method was demonstrated to be a suitable alternative to critical point drying for samples mounted on Falcon inserts (Cooley *et al.*, 1994).

3.2.3.6 Quantitation of adhesion

Quantitation of the adherence of intestinal spirochaetes was evaluated using immunofluorescence. Samples of each isolate were fixed in cold acetone for 10 minutes, then allowed to air dry. Fixed samples were stored until required, when they were washed three times in PBS/1% (w/v) bovine serum albumin (BSA). Samples were incubated with polyclonal serum (as described in section 4.2.3.1) diluted 1/2 in PBS/1% (w/v) BSA for 1 hour at 37°C in moist conditions. These were washed three times in PBS/1% (w/v) BSA followed by incubation with Protein A-FITC conjugated antibody (0.1mg/ml) at 37°C for 1 hour in moist dark conditions. Samples were washed a further three times in PBS/1% (w/v) BSA, once in sterile water then allowed to air dry. For each sample, the number of spirochaetes adhering to 100 Caco-2 cells over 20 random fields of view was counted using a light microscope (Zeiss Axioskop) at a magnification of x400 and excitation at 450-490nm.

3.2.3.7 Caco-2/HT29 Co-culture adhesion assay

Trypsinised Caco-2 cells and HT29 cells were mixed at a ratio of 1:1 as described for HT29-MTX/Caco-2 transport studies (Walter *et al.*, 1996). Mixed cells were seeded at 3 x 10^5 cells per plate (coated as described in section 3.2.3.1). Co-cultures were maintained in Caco-2 maintenance media as described in section 3.2.3.2.

Bacteria were prepared as described in section 3.2.3.4. The isolates used in this part of the study were RA87, 28/94, 81/80, Wesley, FT6, A5687 and P43/6/78^T (Table 3.2). The adhesion assay for intestinal spirochaetes to Caco-2/HT29 co-cultures was carried out as described in section 3.2.3.4. Samples for evaluation by scanning electron microscopy and for quantitation of adhesion were prepared as described in sections 3.2.3.5 and 3.2.3.6 respectively.

3.2.3.8 Statistical Analysis

The mean adherence of intestinal spirochaetes, standard deviation and standard error of the mean (SEM) were calculated for each isolate. One way analysis of variance (ANOVA) was used to identify significant relationships between isolates.

3.2.4 Invasion of Caco-2 cells by intestinal spirochaetes

3.2.4.1 Preparation of cells and bacteria for invasion assay

Trypsinised Caco-2 cells were seeded at 3 x 10^5 cells per plate onto 6 well plates containing PET (polyethylene terephthalate) membrane inserts with a 3μm pore size (Falcon) and incubated at 37°C with 5% CO_2 until 15-18 days post confluence. Maintenance media in plates was replaced after 2-3 days from both the apical and basal

side. On the day of experimentation, media was removed and the cells washed three times (from both sides) in media lacking penicillin/streptomycin.

Bacteria were prepared as described in section 3.2.3.4 except that the final concentration was 1 x 10^8 spirochaetes/ml. Isolates used in the invasion assay were: RA87, 28/94, 81/80, 382/91, 60, PE90, Wesley and P43/6/78^T (Table 3.2).

3.2.4.2 Invasion assay

For each strain examined, 1ml of bacterial suspension replaced the media in the apical side of two wells. Two wells in each plate were left uninfected as controls and the plates were incubated at 37°C with 5% CO_2 for 6 hours. Each isolate was tested in duplicate and the experiment repeated with Caco-2 cells of differing passages. After 1, 2, 4, and 6 hours, six 10μl samples were taken from the basal side of each well, spotted onto teflon-coated multi-spot slides (C. A Hendley (Essex) Ltd) and allowed to air dry. The slides were then prepared as described in section 3.2.3.6 to observe whether spirochaetes had penetrated the cell layer on the apical side. Each slide was evaluated by two independent individuals to eliminate bias. After 6 hours, the wells were washed once in DMEM and three times in Hanks balanced salt solution (Life Technologies). One insert for each isolate and control was prepared for scanning electron microscopy as described in section 3.2.3.5. The remaining membranes were then stained with acridine orange (Sigma) (section 3.2.4.3).

3.2.4.3 Staining of intracellular bacteria with acridine orange

Membranes were removed from the insert using a scalpel and placed on glass slides. The staining followed the procedure used by Miliotis (1991). Briefly, Acridine orange 0.05% (w/v) in Gey's solution (Life Technologies) was added to each

membrane and incubated for approximately 45 seconds. Excess stain was removed by washing with Hanks balanced salt solution and staining of extracellular bacteria quenched by incubation of the membranes with crystal violet (10% (w/v) crystal violet in 95% ethanol diluted 1/5 (v/v) with 1% aqueous ammonium oxalate) for 45 seconds. Hanks balanced salt solution was used to remove excess crystal violet and membranes were visualised using UV (excitation 450-490nm) light microscopy at a magnification of x400.

3.3 Results

3.3.1 Caco-2 adhesion assay

3.3.1.1 Optimisation of inoculum

HRM16 was selected to optimise the experimental inoculum as it was found in the largest subgroup of the *S. pilosicoli* cluster of the dendrograms based on PFGE (Figures 2.4 and 2.5) and in Figure 2.4, it was found in the centre of this group. Both exponential and stationary phase cultures adhered to Caco-2 cells (see Table 3.3).

Table 3.3: Mean number of adherent bacteria/100 Caco-2 cells with standard deviation for stationary and exponential phase cultures of isolate HRM16 at each inoculum ($1x10^6$, $1x10^7$ and $1x10^8$).

Inoculum	Growth phase	Mean adherent bacteria (± SD)
$1x10^6$	Stationary	26 ± 6
$1x10^6$	Exponential	41 ± 18
$1x10^7$	Stationary	42 ± 23
$1x10^7$	Exponential	69 ± 18
$1x10^8$	Stationary	74 ± 47
$1x10^8$	Exponential	109 ± 69

Comparison of the adhesion observed for exponential and stationary phase cultures at each inoculum is found in Figure 3.1.

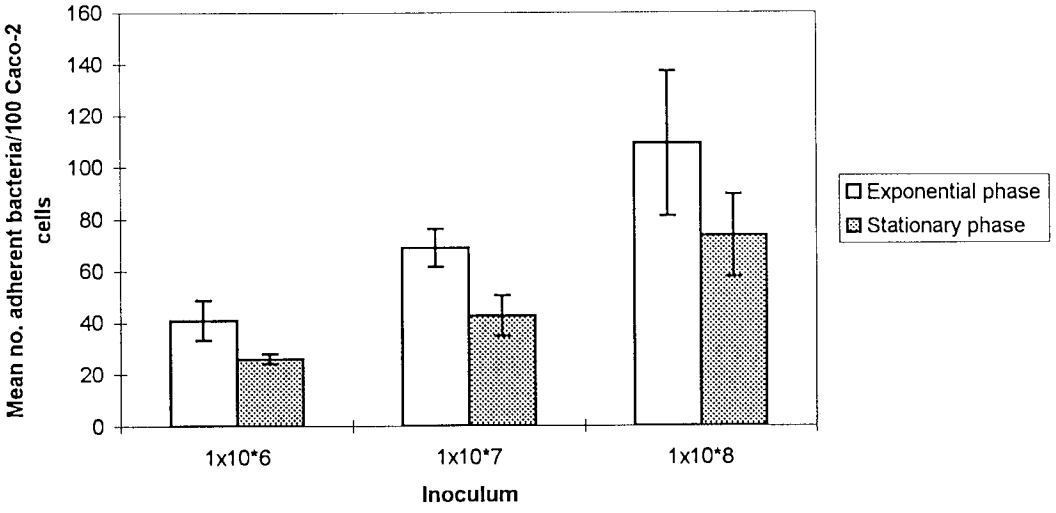


Figure 3.1: Adherence of exponential and stationary phase cultures for each inoculum with standard error of the mean (SEM). 10*6/10*7/10*8 represent $10^6/10^7/10^8$ spirochaetes.

One way analysis of variance showed no significant difference between adhesion of exponential and stationary phase cultures.

3.3.1.2 Visualisation of Caco-2 adhesion by scanning electron microscopy and immunofluorescence

Broth cultured intestinal spirochaetes were distinguished by immunofluorescence (Figure 3.2). Caco-2 cell monolayers were viewed using immunofluorescence (section 3.2.3.6), and adherent intestinal spirochaetes could be differentiated (Figure 3.3), and were frequently observed adhering at cell junctions. Isolates such as 382/91 which adhered in high numbers, frequently formed "networks"

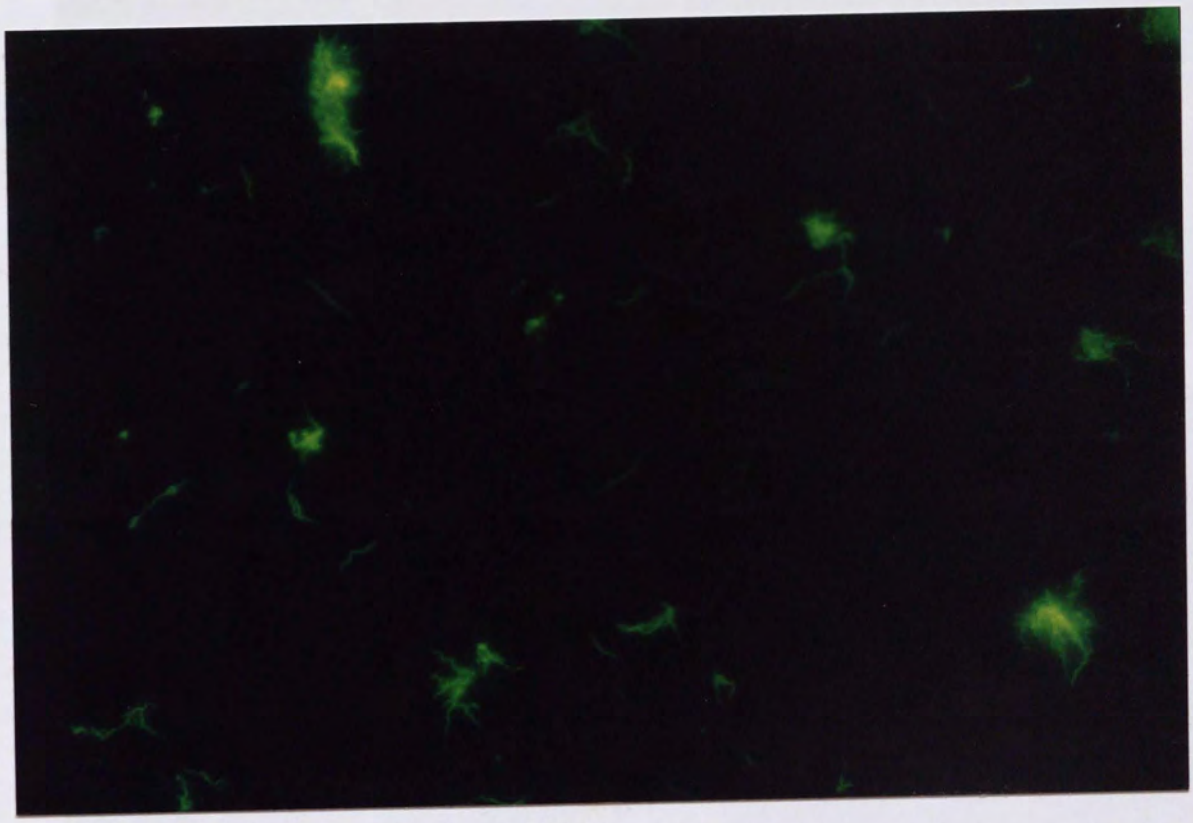
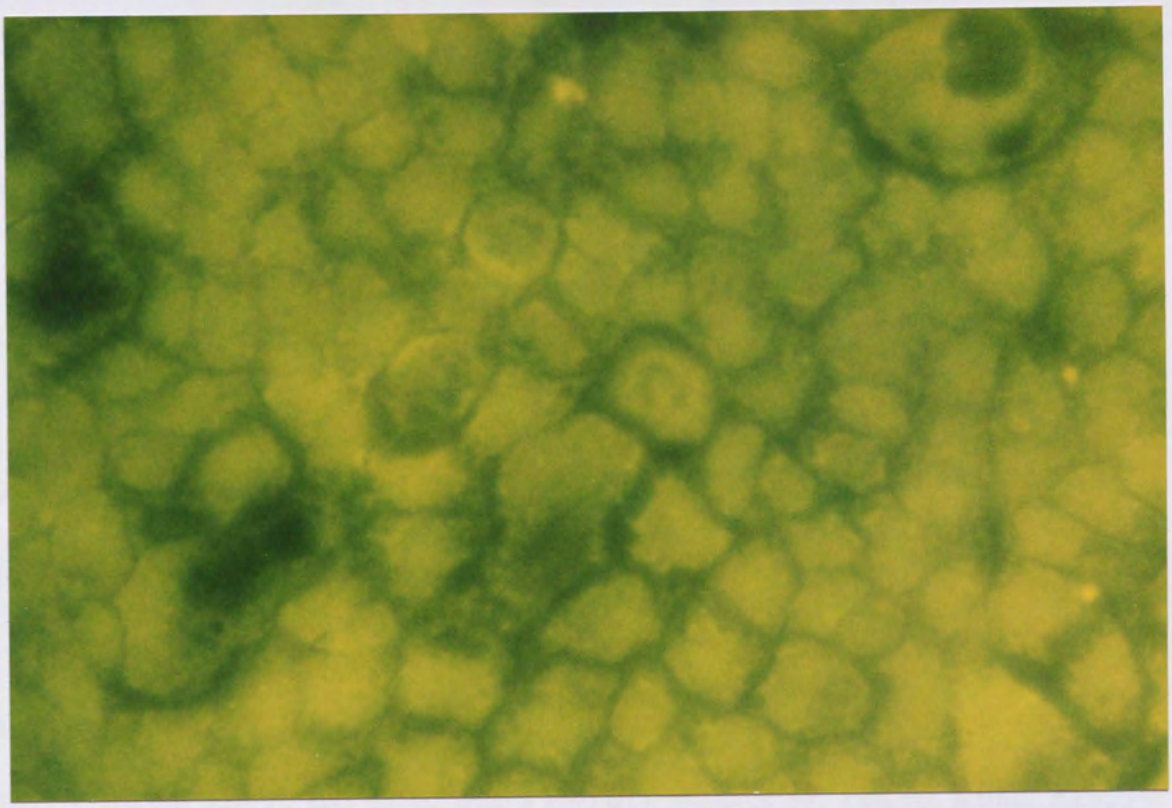


Figure 3.2: Stationary phase broth cultured *S. hyodysenteriae* isolate JWPM examined by immunofluorescence as described in section 3.2.3.6. Magnification x400.

(a)



(b)

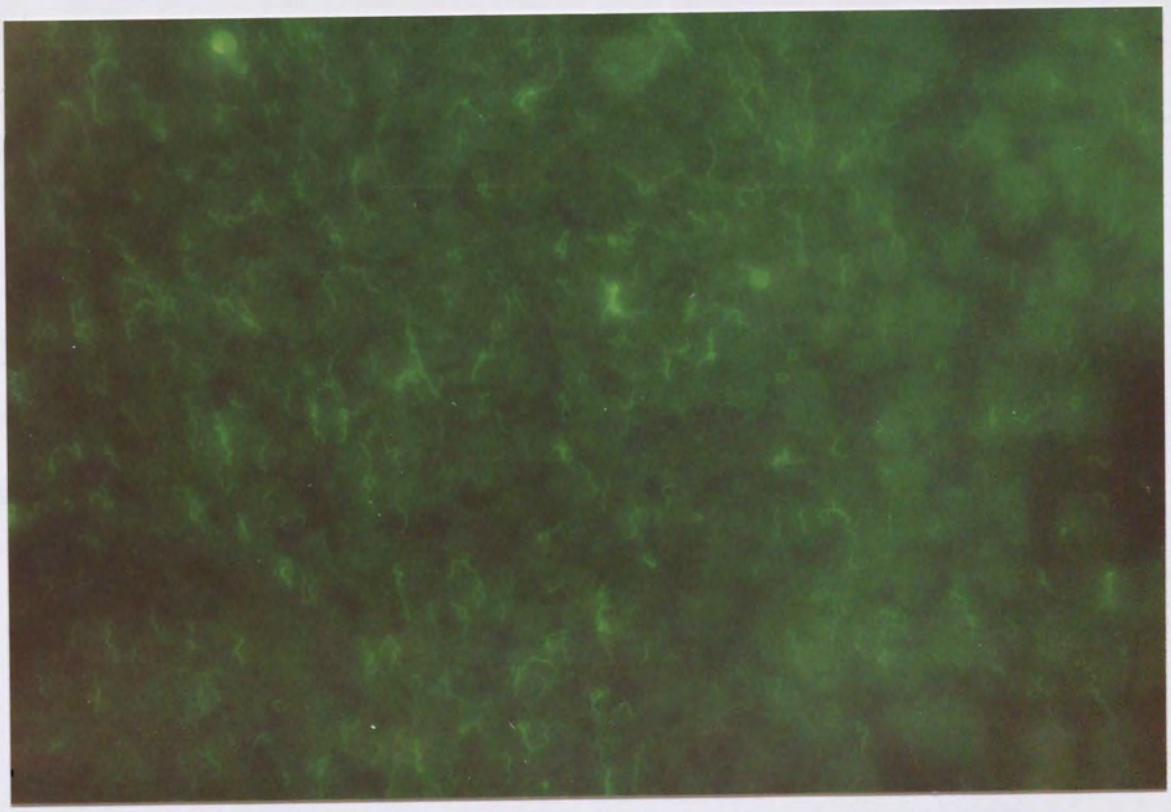


Figure 3.3: Immunofluorescent spirochaetes adhering to the Caco-2 cell layer (Magnification x400). Uninfected Caco-2 cell layer (control) (a) and isolate FT6 adhering to Caco-2 cells (b).

of spirochaetes which could be not be readily quantified (Figure 3.4). Scanning electron microscopy showed that the intestinal spirochaetes exhibited end-on attachment to Caco-2 cells, often at cell junctions (Figure 3.5).

3.3.1.3 Quantification of adhesion of intestinal spirochaetes to Caco-2 cells

Table 3.4 shows the results of quantification of adhesion observed in adhesion assays. The data in table 3.4 is shown in Figure 3.6 according to the position of the isolate in the dendrogram (Figure 2.5).

One way analysis of variance performed on test isolates showed that the adhesion of 382/91 to Caco-2 cells was significantly higher than any other isolate ($P<0.001$, except 128/90 and 81/80 where $P<0.01$). Isolates 57, 128/90, 81/80, FT9, FT7 and A5687 (which were identified as *S. pilosicoli* by reactivity with BJL/AC1) showed high levels of adherence to Caco-2 cells (significantly higher than most other intestinal spirochaete isolates). The *S. hyodysenteriae*, *S. intermedia* and unidentified isolates included in this study, showed low levels of adhesion to Caco-2 cells which were not significantly different from each other. These isolates showed significantly lower levels of adhesion than all *S. pilosicoli* isolates except PE90, A5660 and HRM16 (VMDC18252 showed significantly lower levels of adhesion than HRM16).

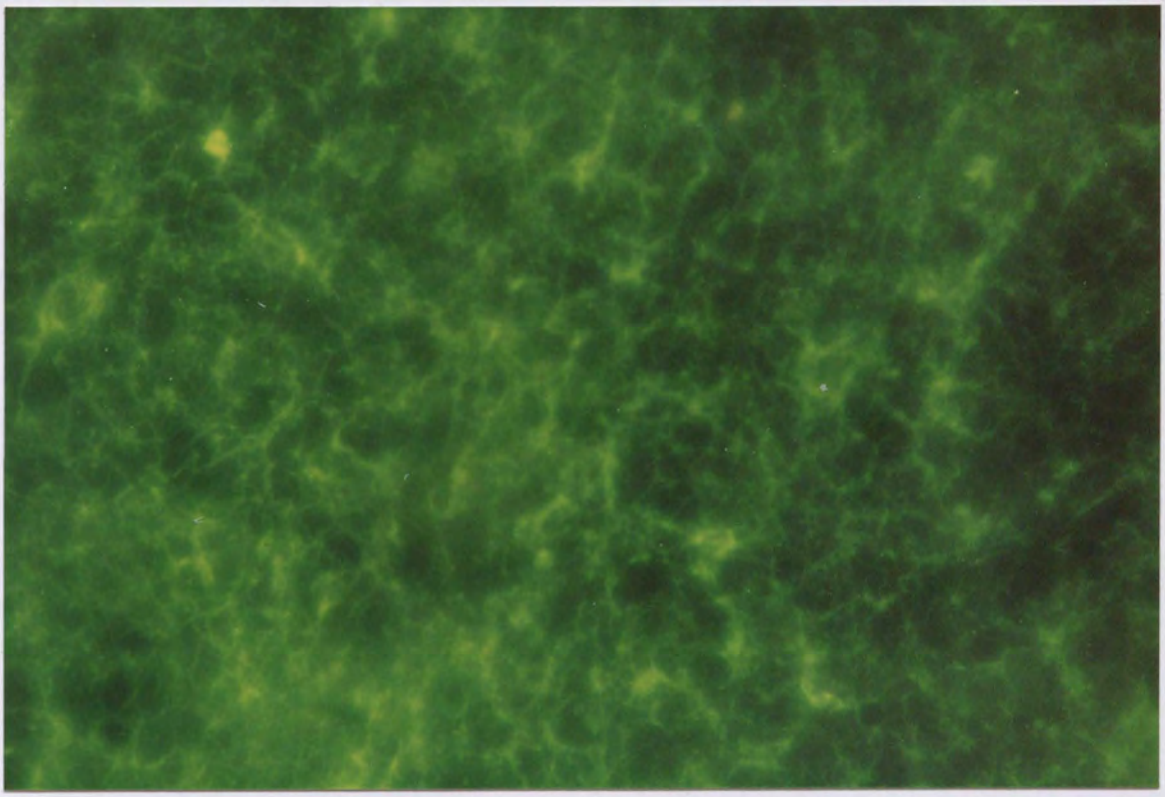
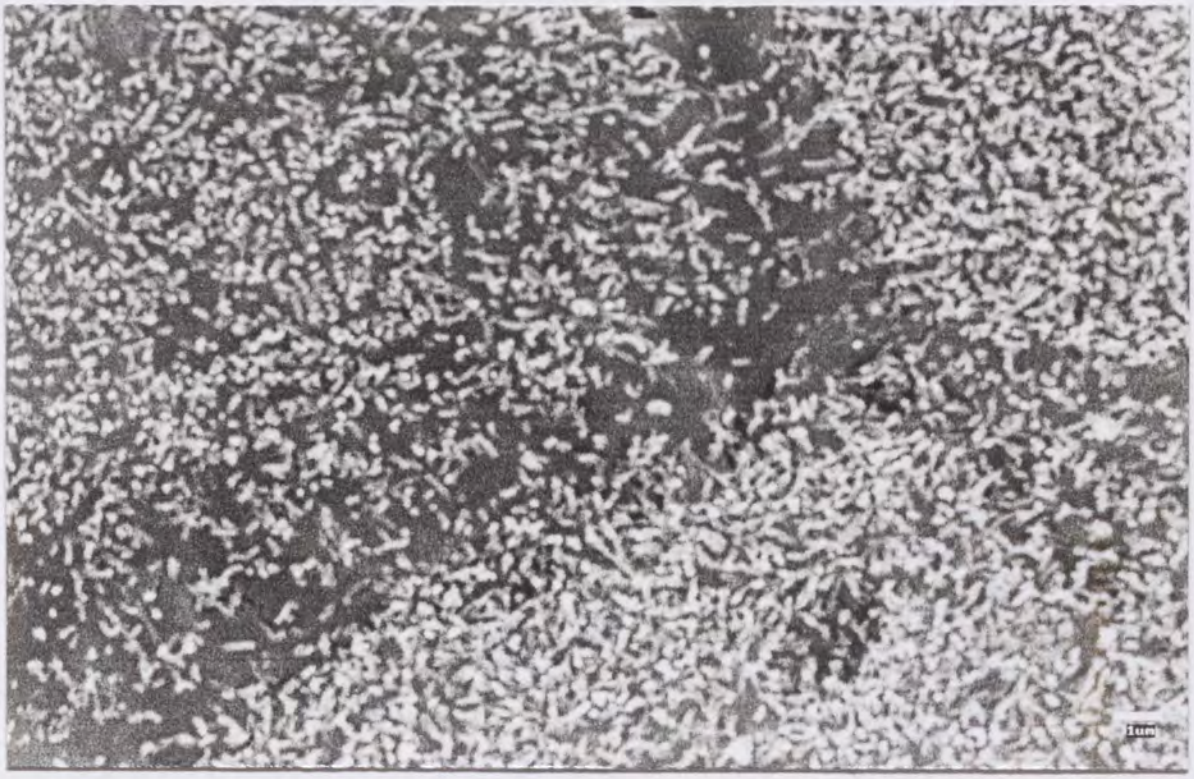


Figure 3.4: Adherence of isolate 382/91 to Caco-2 cells visualised by immunofluorescence. Spirochaetes formed a "network" around cells which was difficult to quantify. Magnification x400.

(a)



(b)

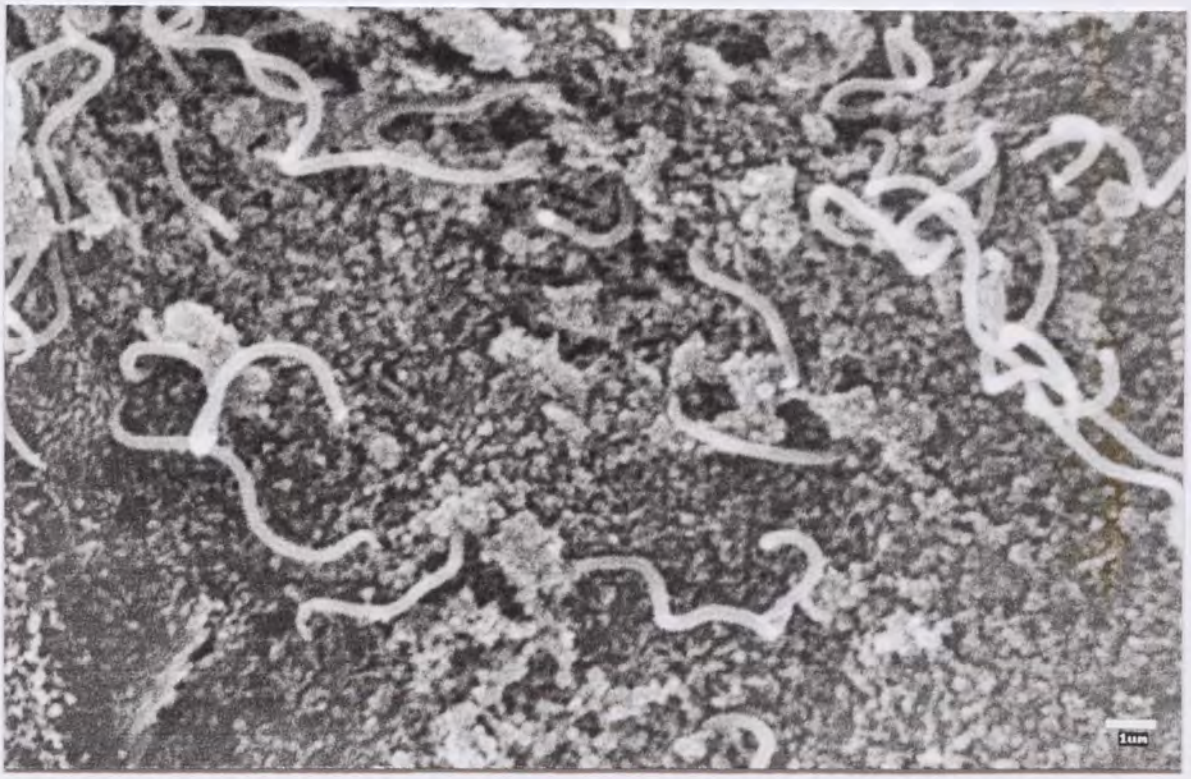


Figure 3.5: Scanning electron micrographs showing the junction between three similar cells in an uninfected Caco-2 cell layer (a) and the junction between two cells which was infected with P43/6/78^{T}(b). The spirochaetes may be observed adhering at cell junctions. Magnification x5000.

Table 3.4: Mean number of adherent bacteria/100 Caco-2 cells shown with standard deviation for each test isolate.

Strain	Mean adherent bacteria (± SD)
HRM16	126 ± 75
FT6	128 ± 27
60	138 ± 25
A5660	85 ± 26
A5687	202 ± 108
57	218 ± 44
28/94	164 ± 23
128/90	247 ± 55
RA87	159 ± 84
PE90	81 ± 22

Strain	Mean adherent bacteria (± SD)
80/81	235 ± 37
382/91	338 ± 38
P43/6/78	193 ± 80
FT7	209 ± 33
FT9	240 ± 54
Wesley	132 ± 37
PWS/A	43 ± 4
VMDC18252	35 ± 9
JWPM	44 ± 13

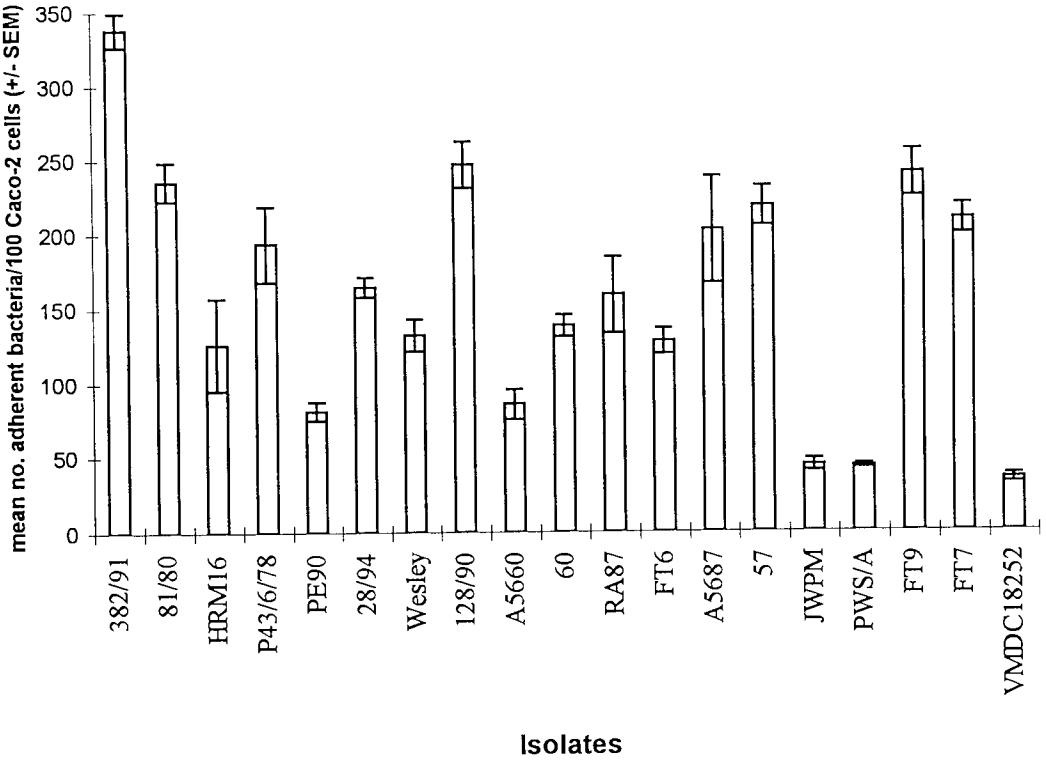


Figure 3.6: Adherence of *S. pilosicoli* isolates, an *S. hyodysenteriae* isolate (JWPM), an *S. intermedia* isolate (PWS/A^T) and an unidentified canine isolate (VMDC18252) to Caco-2 cells by immunofluorescence showing mean value and standard error of the mean (SEM).

Figure 2.4 showed genotypically similar isolates were obtained from different animal origins suggesting that cross-species transmission was possible. The closely related isolates FT6 (human isolate) and A5687 (canine isolate) showed levels of adhesion which were not significantly different ($P>0.05$). The relationship of 60 (human isolate) and A5660 (canine isolate) which were closely related was also not significantly different ($P>0.05$).

3.3.2 Co-culture adhesion assay

3.3.2.1 Evaluation of adhesion by scanning electron microscopy and light microscopy

When examined by immunofluorescence light microscopy (section 3.2.3.6), spirochaetes were distinguished on Caco-2/HT29 cells (Figure 3.7). Heavy colonisation resulted in a similar adherence pattern to that observed in Caco-2 adhesion assays (section 3.3.1.2) and examination by scanning electron microscopy showed attachment of spirochaetes which were entangled with each other (Figure 3.8).

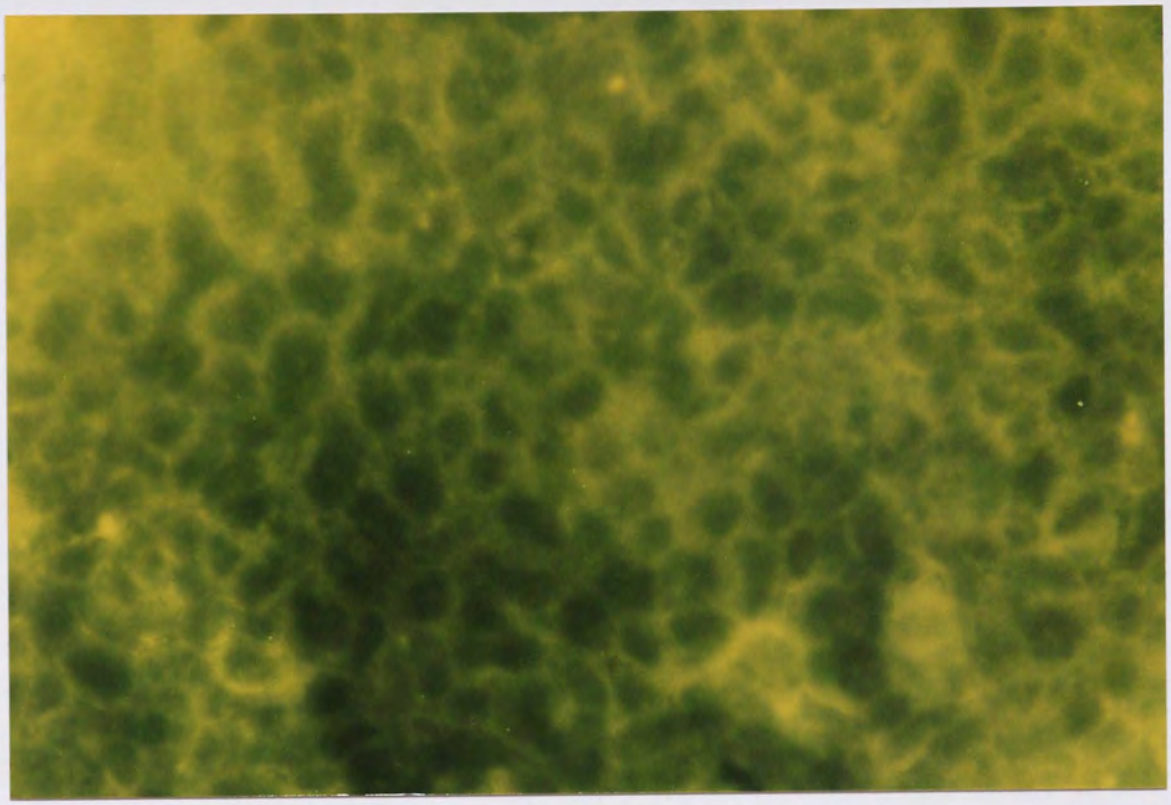
3.3.2.2 Quantitation of adhesion

Table 3.5 shows the levels of adhesion observed for isolates used in the Caco-2/HT29 adhesion assay.

Table 3.5: Mean number of adherent bacteria/100 cells with standard deviation for isolates tested with Caco-2/HT29 coculture.

Strain	Mean adherent bacteria (± SD)
RA87	192 ± 68
Wesley	118 ± 12
FT6	244 ± 53
A5687	≥400
P43/6/78	122 ± 21
80/81	≥347
28/94	231 ± 98

(a)



(b)

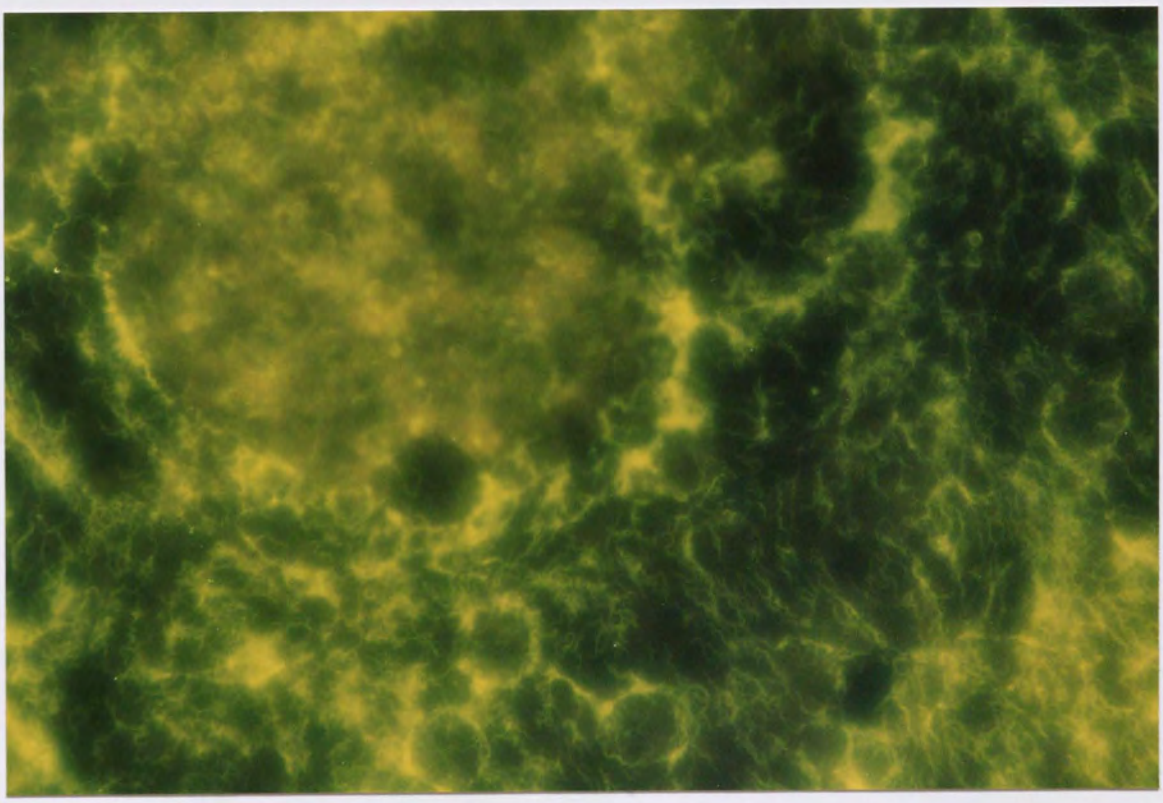
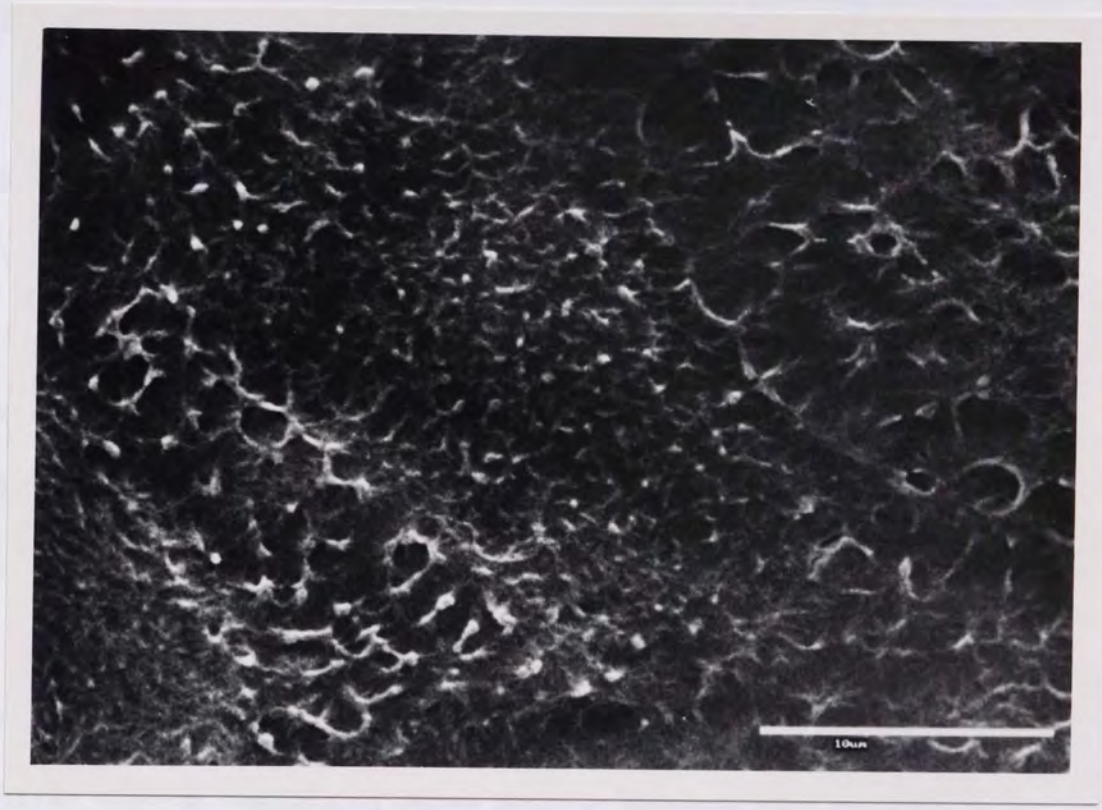


Figure 3.7: Heavy colonisation of Caco-2/HT29 cells with A5687 (b) can be differentiated from uninfected cells (a). Magnification x400.

(a)



(b)

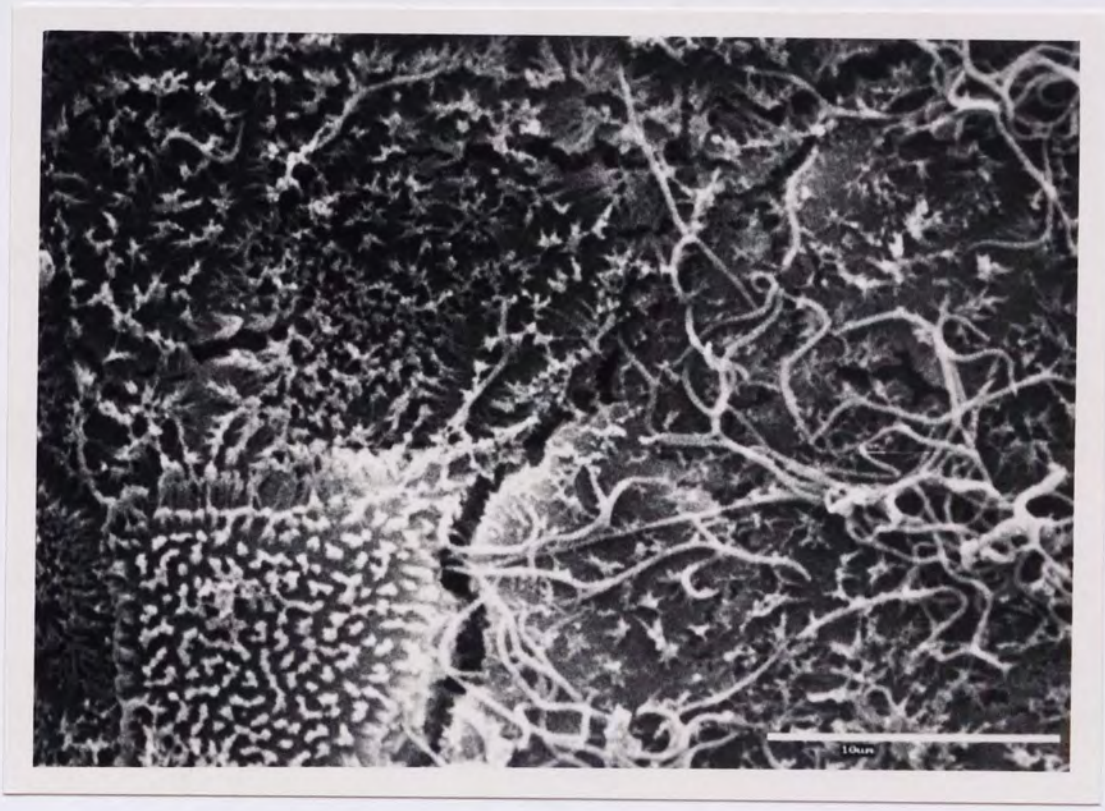


Figure 3.8: Spirochaetes (A5687) colonising Caco-2/HT29 cultures in large numbers appeared to be "entangled" when observed by scanning electron microscopy (b). Spirochaetes were absent from the uninfected cells (a). Magnification x3500.

In samples of A5687 and 81/80, the number of adherent bacteria exceeded the number that could be counted and so these samples were given the value ≥400, as the maximum number which could be quantified was 400 bacteria/100 cells. As these figures were estimated, no standard deviation or SEM were calculated.

A5687 showed significantly higher levels of adhesion to co-cultures using one way analysis of variance than any other isolate tested ($P<0.001$ except 81/80 where $P<0.05$). Comparison of adherence observed in Caco-2 cells with Caco-2/HT29 co-culture assays is illustrated in figure 3.9.

Analysis of variance showed that there was no significant difference in the levels of adhesion observed for RA87, Wesley, P43/6/78^T and 28/94 in Caco-2 or Caco-2/HT29 cultures. Significantly higher levels of adhesion were observed in co-culture assays for 81/80 ($P<0.05$), FT6 ($P<0.01$) and A5687 ($P<0.001$).

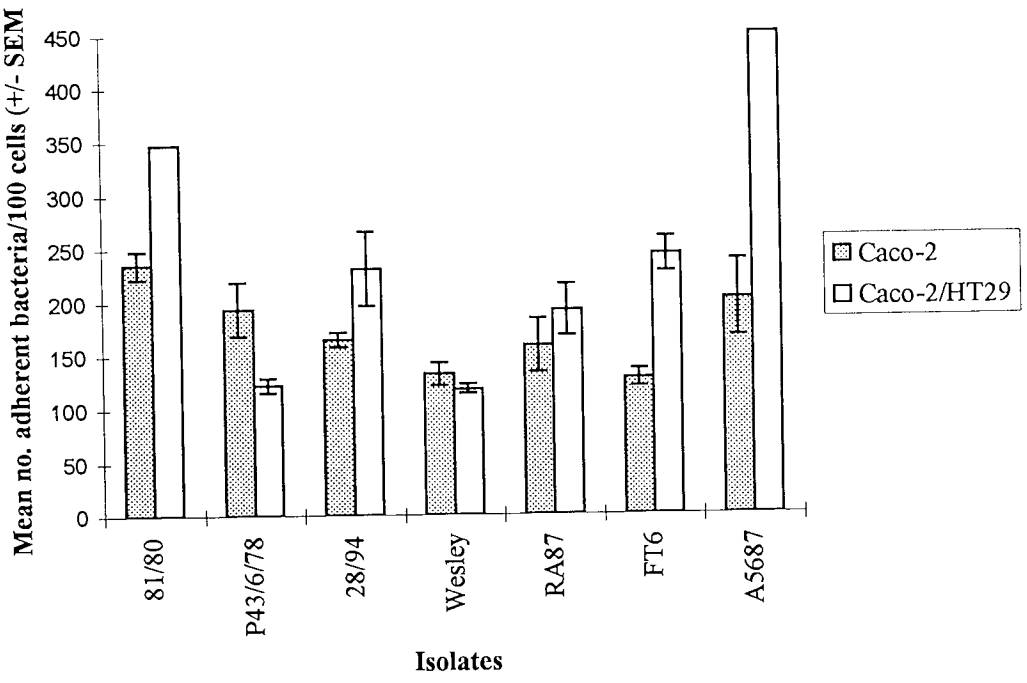


Figure 3.9: Comparison of adherence of test isolates to Caco-2 and Caco-2/HT29 adhesion assays. The mean values are shown with error bars representing the standard error of the mean (SEM).

3.3.3 Invasion assays

3.3.3.1 Scanning electron microscopy

After incubation of Caco-2 cells with intestinal spirochaetes for 6 hours, few spirochaetes were observed attached to the cell surface.

3.3.3.2 Quantitation of penetration of Caco-2 model by immunofluorescence

All isolates examined penetrated the Caco-2 cell layer (Appendix 14) after approximately 2 hours incubation. Occasionally, invasion was observed after one hour of incubation.

Figure 3.10 shows immunofluorescent spirochaetes in the basal media of inserts.

3.3.3.3 Evaluation of intracellular bacteria by acridine orange staining

Acridine orange is a vital stain which binds between DNA molecules. Viable cells are stained green by acridine orange and non-viable cells stained red. Intestinal spirochaetes were stained by acridine orange as described by Miliotis (1991) (Figure 3.11). After incubation with intestinal spirochaetes, no evidence of intracellular bacteria was observed (Figure 3.12).

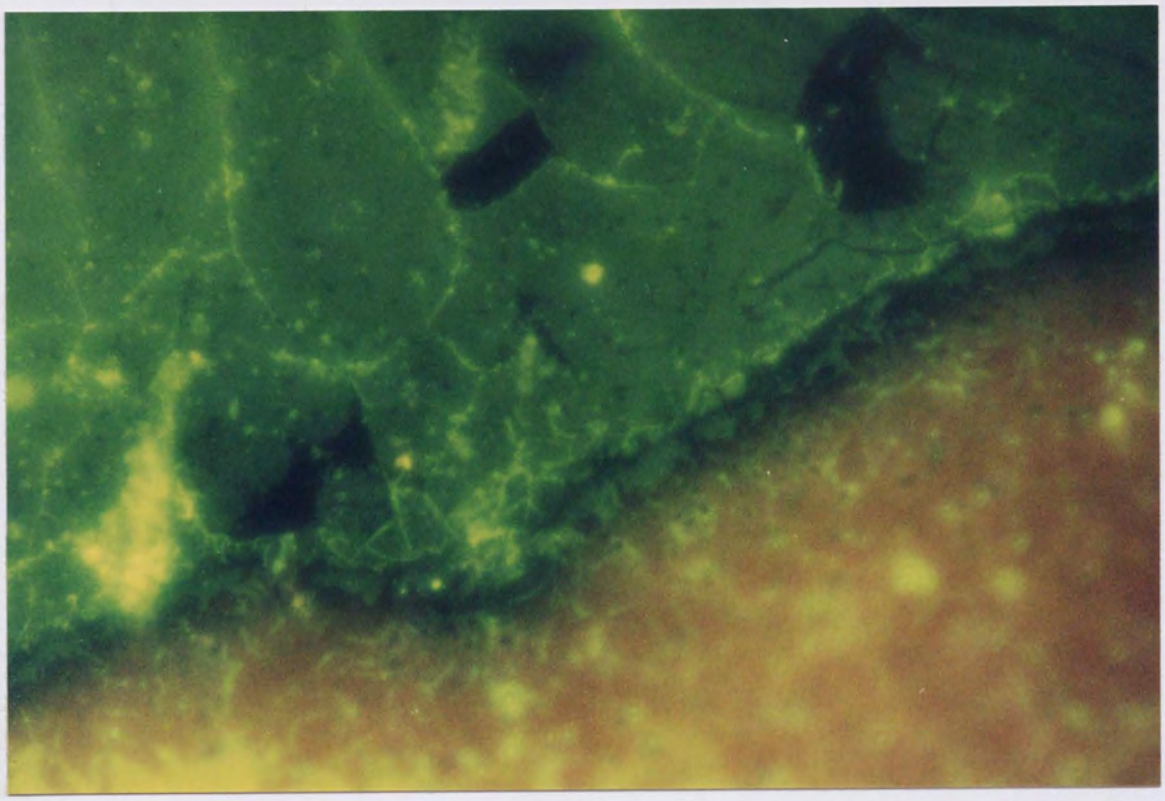


Figure 3.10: Intestinal spirochaetes (isolate 382/91) observed by immunofluorescence from samples of basal media taken after incubation with Caco-2 cell layer for 6 hours. Magnification x400.

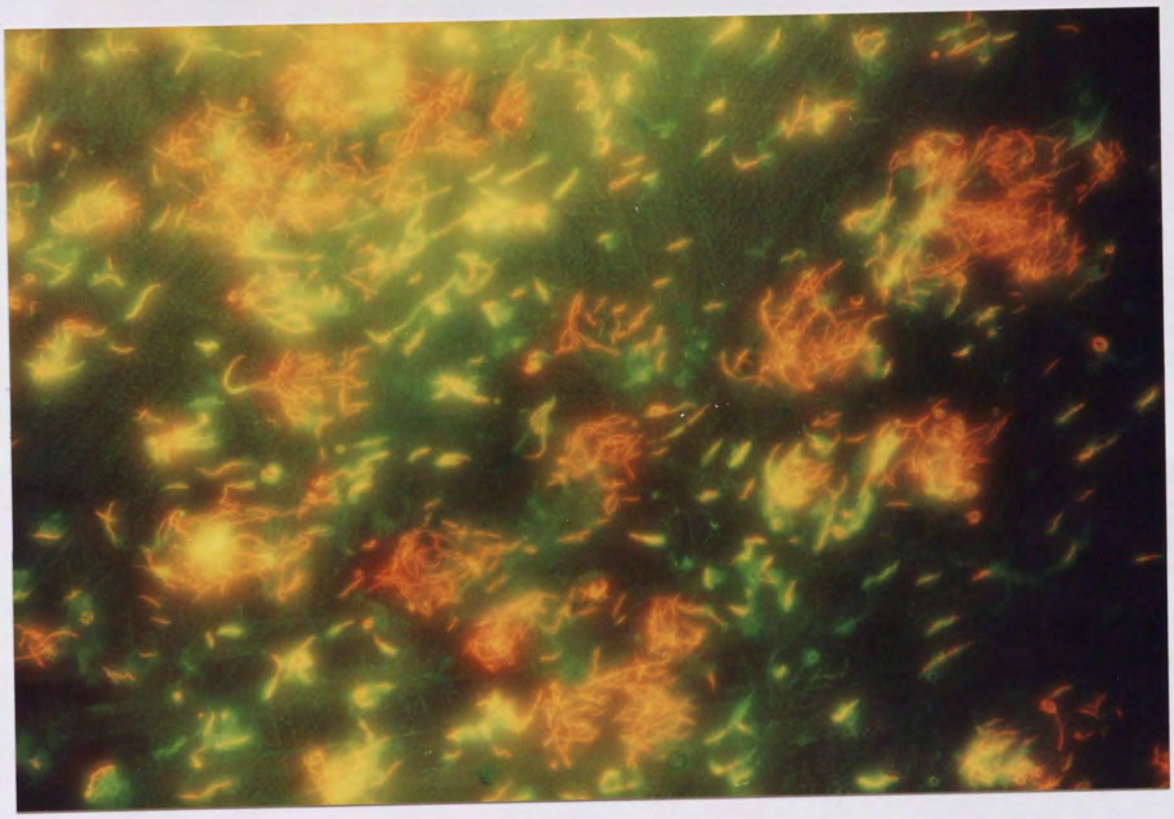
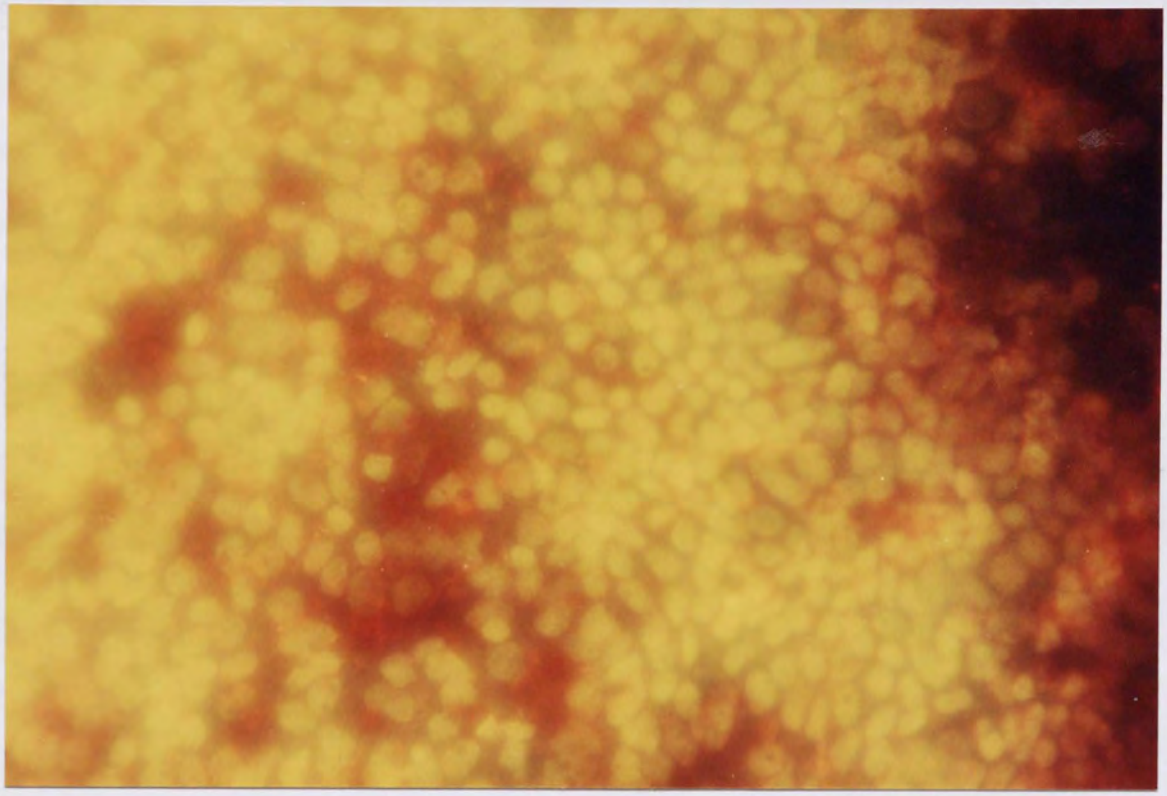


Figure 3.11: Human intestinal spirochaetes from a plate culture stained with acridine orange. Viable organisms were stained green, non-viable organisms appeared red. Magnification x400.

(a)



(b)

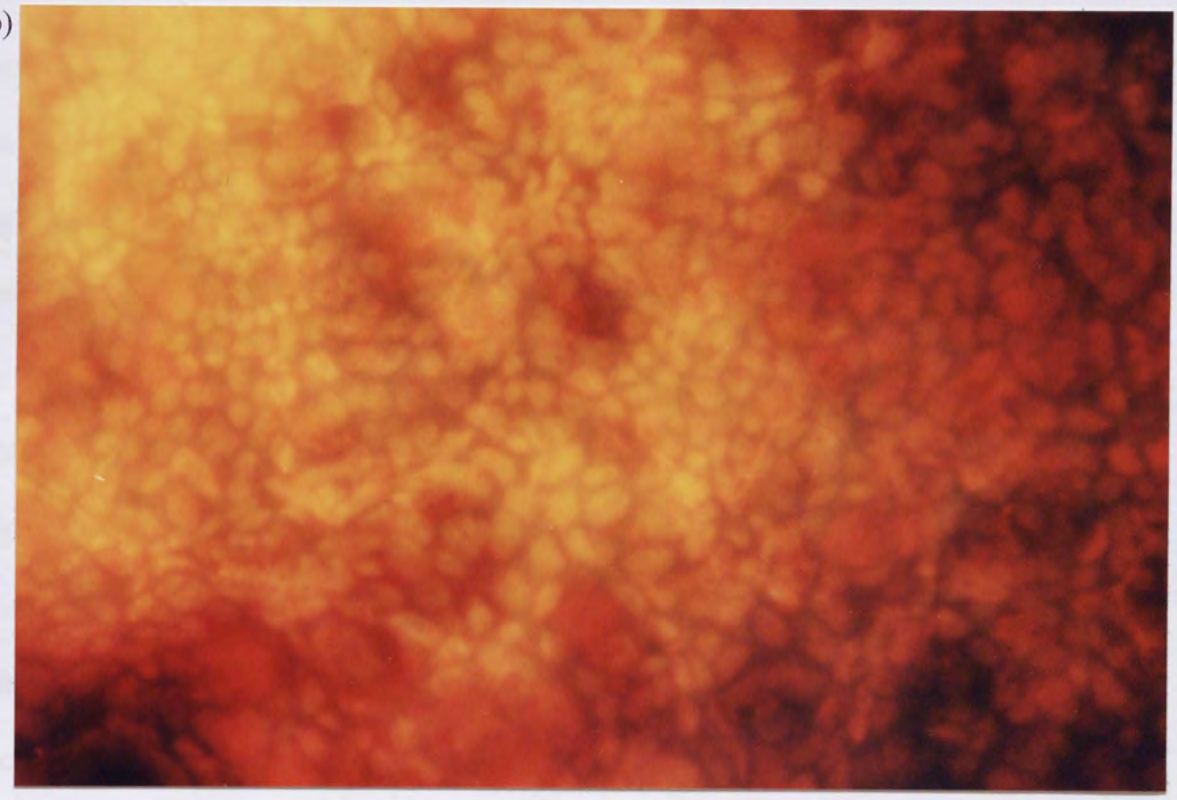


Figure 3.12: Caco-2 culture stained with acridine orange after incubation for 6 hours in the absence (a) or presence (b) of intestinal spirochaetes. Magnification x400.

3.4 Discussion

The adherence of isolate HRM16 in different growth phases showed no significant difference between exponential and stationary phase culture. Examination of individual experiments showed that bacteria at an inoculum of 10^8 spirochaetes (from either growth phase) adhered at significantly higher levels than either an inoculum of 10^6 or 10^7 spirochaetes. Spirochaetes are most motile in mid-log phase (Kennedy and Yancey, 1996) and so an inoculum of 10^8 exponential phase bacteria was selected. Growth phase has been demonstrated to be an important factor in both numbers of adhesive bacteria and adhesive pattern exhibited to cells. In *C. jejuni*, increased motility through a viscous environment (resembling mucus) significantly increased the invasion of Caco-2 cells (and therefore adhesion) by bacteria (Szymanski *et al.*, 1995). Growth phase affected the adherence of *A. caviae* to Caco-2 cells with most adhesion occurring during the early exponential phase of growth and the different growth phases showed differing adherence patterns, exponential phase cultures produced an aggregative pattern whilst stationary cultures gave a more diffuse pattern (Thornley *et al.* 1996).

The levels of adherence of isolates in the present study were low by comparison to the adhesion observed in previous studies of *S. innocens* and *S. hyodysenteriae* (Bowden *et al.*, 1989): in this study maximum adherence was approximately 4 spirochaetes per cell compared to 20 spirochaetes per cell (*S. hyodysenteriae*) and 10 spirochaetes per cell (*S. innocens*). This may be due to the use of a centrifugation step and media containing FBS, which they reported may affect adherence (Bowden *et al.*, 1989) or due to differences between the two different cell lines used: Caco-2 cells in this study and HenLe cells in study of the Bowden *et al.* (1989).

The Caco-2 cell model appeared to be a possible model for infection as the end-on adhesion of spirochaetes to cells (Figure 3.5) reflects the situation *in vivo* (Muniappa *et al.*, 1996; Muniappa and Duhamel, 1997a; Rodgers *et al.*, 1986). Considerable variation in the level of adhesion was observed amongst *S. pilosicoli* isolates within cluster 1 from Figure 2.5 such as isolates from group 1b some of which showed high levels of adherence (128/90) whilst others within the same group showed significantly lower levels of adhesion (PE90). The bacteraemic isolates showed varying levels of adhesion as may be expected from a genotypically heterogeneous group of organisms (section 2.3.1.3). These varying levels of adhesion did not suggest that the bacteraemic isolates had a particular capacity to adhere to and thus invade the host intestinal epithelium. The exception to this observation was isolate 382/91 which was so genotypically diverse from other isolates in the study that it could not be included in the analysis of PFGE data (section 2.3.1.1). This isolate showed significantly higher levels of adhesion than any other isolate tested suggesting that it may be better adapted to colonise the host epithelium and invade the host epithelial barrier.

The comparison of genotypically similar isolates from different animals showed that there was no significant difference in the levels of adhesion observed. This suggested that it may be possible for the human intestinal mucosa to be colonised by isolates from animals such as dogs supporting the hypothesis that cross-species transmission may occur. P43/6/78^T (which is porcine in origin) also showed levels of adhesion similar to human isolates further suggesting the feasibility of cross-species transmission. As cross-species transmission between pigs and humans has not been suggested previously, it is possible that the barrier to infection of humans with porcine isolates resides not with the ability of the spirochaete to adhere to epithelial cells but the nature of the barrier presented by the host. The adhesion of P43/6/78^T was

significantly higher than the adhesion of other porcine isolates (PWS/A^T the reference isolate of *S. intermedia* and JWPM which is an isolate of *S. hyodysenteriae*). The low levels of adherence in *S. hyodysenteriae* and *S. intermedia* suggested that these species may be less well adapted than *S. pilosicoli* to human epithelial cells or it is possible that they bind to different cell surface receptors and have differing host specificity. It may also indicate that *S. pilosicoli* which has been isolated from a number of different animals may be more adaptable.

The co-culture of Caco-2 and HT29 cells was a realistic infection model as it yielded similar levels of adhesion to those observed with Caco-2 cells although some isolates produced higher levels of adhesion with the co-culture model than with Caco-2 cells. During infection, biopsy samples have shown intestinal spirochaetes adhering to crypt (undifferentiated) cells as well as at villus tips (Surawicz *et al.*, 1987; Kostman *et al.*, 1995). The results suggested that intestinal spirochaetes were able to adhere to co-cultures at similar or higher levels than Caco-2 cells. It is possible that HT29 cells provide a larger number or different receptors with which spirochaetes can bind. Both of the models examined lack a mucus barrier, which would be required for a model to be representative of the intestinal epithelium. This function could be provided in a model containing differentiated HT29 (goblet) cells.

All isolates of intestinal spirochaetes tested were able to penetrate the Caco-2 model suggesting that they may be able to penetrate the human intestinal epithelium. Variation observed in the evaluation of penetration after one hour may be caused by insufficient formation of tight junctions in a small number of samples and for future experiments it may be advisable to use a TEER (Trans-epithelial electrical resistance) meter. This could be used to evaluate whether the cell layer tight junctions are in tact prior to and during the experiment. Use of a TEER meter on untreated cells would

indicate whether the penetration of the cell layer was caused by bacteria or due to incubation conditions. Staining of the Caco-2 layer after penetration did not reveal the presence of intracellular bacteria suggesting that penetration is via the paracellular route.

Based on the *in vitro* evidence of adhesion and invasion demonstrated by this and a number of other studies, it may now be possible to put forward a tentative hypothesis for *S. pilosicoli* infection *in vivo*. Spirochaetes in the lumen of the gastrointestinal tract are chemotactically attracted to mucin and other factors (Kennedy and Yancey, 1996). Motility enables spirochaetes to move to the mucosal surface, embed into the mucus covering of the mucosal surface and adhere to epithelial cells. Protease enzymes enable the break down of mucin which enhances the chemotaxis of other intestinal spirochaetes and affects cellular tight junctions or cell membranes allowing spirochaetes to penetrate the mucosal barrier (Muniappa and Duhamel, 1997).

The levels of adhesion observed with intestinal spirochaetes can be compared with a number of other studies. The study of *Bifidobacteria* species used Caco-2 cells which were 15 days post confluence and in a similar way to this study, observed considerable variation in adhesion both within and between species (Crociani *et al.*, 1995). Studies of *L. acidophilus* used Caco-2 cells at 15 days post confluence and although adhesion was found to be 18-155 bacteria/100 Caco-2 cells (Bernet *et al.*, 1994), this was within the range observed with intestinal spirochaetes. Studies of a range of *Lactobacillus* species that used Caco-2 cells at 21 days post-seeding showed adherence of up to 250 bacteria/100 cells which was similar to the level of adhesion observed in this study. Enterotoxigenic *E. coli* showed adhesion to Caco-2 cells (15 days post-confluence) at 1.8-2.39 bacteria/cell which did not cause the production of

lesions (Darfeuille-Michaud *et al.*, 1990). If the mean adhesion/100 cells is used to calculate adhesion of intestinal spirochaetes per cell, many of the isolates studied would be included in a similar range as that observed in enterotoxigenic *E. coli*.

In vivo studies using one day old chicks has found end-on adhesion of intestinal spirochaetes to epithelial cells using isolates from dogs and humans (Muniappa and Duhamel, 1997a) and a similar arrangement of spirochaetes was also observed in rectal biopsy samples (Rodgers *et al.*, 1986). The pattern of adhesion of spirochaetes to both models used reflected the situation *in vivo*, and so may be suitable for characterising the molecular basis for attachment to host epithelial cells.

In conclusion, intestinal spirochaetes show similar levels of adhesion to intestinal epithelial cell lines as other gastrointestinal bacteria. The adhesion model lacks a mucosal barrier. Data has suggested that the presence of a mucus layer may be significant as intestinal spirochaetes adhere to porcine gastric mucin (refer to section 5.3.2.5 of this thesis). Future work could investigate the addition of porcine gastric mucin to cells prior to exposure with intestinal spirochaetes and the development of a differentiated Caco-2/HT29 model.

Chapter 4: Immunoscreening of *S. pilosicoli* random genomic library

4.1 Introduction

4.1.1 Use of Lambda phage in construction of bacterial genomic libraries

Lambda phage have been used to generate bacterial genomic DNA libraries of a number of Gram-positive and Gram-negative organisms. The lambda ZAP II (Stratagene) system has been used to create a library of *Staphylococcus epidermidis* which was screened using a monoclonal antibody that was specific for an iron-regulated 32kDa lipoprotein. Using this system, an iron regulated operon of three genes was identified. The lipoprotein showed homology both to known adhesins and ABC transporters (for metal ions) (Cockayne *et al.*, 1998). Intestinal bacterial pathogens such as *C. difficile* have been studied using the lambda ZAP system. Polyclonal serum raised against heat-shocked *C. difficile* (bacterial adhesion to Caco-2 cells increased after heat shock) was used to screen a library. The clones selected expressed either a 40kDa or 27kDa protein and adhesion to cell lines was compared to heat shocked *C. difficile* (Karjalainen *et al.*, 1994).

Lambda ZAP II has also been used in the identification of major surface proteins of oral spirochaetes such as *T. denticola*. Polyclonal serum was raised in rabbit against whole cells but reacted with only one protein in immunoblots. A 53kDa protein was purified and observed to bind fibronectin, laminin and fibrinogen in a modified western blot adhesion assay (Haapasalo *et al.*, 1992). A library of *Treponema pallidum* subsp. *pallidum cfpA* gene (major cytoplasmic filament protein) was constructed in lambda ZAP II using DNA primers designed from the amino acid sequence (You *et al.*, 1996).

After screening, the protein identified contained 678 residues and had a molecular mass of 78.5kDa.

4.1.2 Aims

The epitope with which the monoclonal antibody BJL/AC1 reacts is likely to be surface exposed in *S. pilosicoli*, suggesting that during an infection, the host may form an immune response. The aim of this work was to generate a random genomic library of *S. pilosicoli* and screen it with BJL/AC1 to identify the nucleotide sequence for the protein with which the antibody reacts. Comparison of the sequence with other sequences whose function is known was used to identify the likely function of the protein.

Polyclonal serum was produced against whole intestinal spirochaetes in rabbit. This serum was used to screen the genomic library to identify genes for proteins to which an immune response had been raised. The gene sequences of three immunoreactive clones were established and compared to other known sequences to identify their likely function within the spirochaete.

4.2 Materials and Methods

4.2.1 Generation of a random genomic library

4.2.1.1 Extraction of DNA

Extraction of genomic spirochaete DNA was performed using a QIAamp tissue kit (Qiagen). A pure bacterial broth culture was centrifuged at 7500rpm (Beckman J2-20; JA-25.50 rotor) for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 100µl of ATL buffer (Qiagen) which was supplemented with 20µl of Proteinase K (90mg/ml final concentration) then vortexed. Cells were incubated at 55°C for 1-3 hours until lysis was complete, vortexing the sample every 20-30 minutes. To ensure that the DNA preparation was free of RNA, 20µl of RNase A (20mg/ml stock) was added, mixed, then incubated at 20-25°C for 2 minutes. AL buffer (Qiagen) (200µl) was added to the sample. After vortexing, the sample was incubated at 70°C for 10 minutes. 210µl of 100% ethanol was added to the sample and mixed thoroughly. The mixture was added to the spin column and centrifuged at 6500rpm (Eppendorf 5415C centrifuge) for 1 minute. 500µl of AW buffer (Qiagen) was then added and the spin column recentrifuged (6500rpm, Eppendorf 5415C centrifuge) for 1 minute. This step was repeated then followed by centrifugation for 2 minutes at 14000rpm to ensure that all AW buffer had been eluted. The DNA was then eluted twice by the addition of 200µl of AE buffer (Qiagen) or sterile double distilled water that was preheated to 70°C. The column was incubated at 20-25°C for 1 minute before centrifugation at 6500rpm for 1 minute. The DNA eluted was then pooled before quantitation.

4.2.1.2 Quantitation of DNA

The quantity of DNA in a preparation was estimated by mixing 2µl of Ethidium bromide (2µg/ml) with 2µl of sample on a piece of "parafilm". Alongside the sample, 2µl of ethidium bromide was mixed with 2µl of λ DNA diluted to concentrations ranging from 0.3-50µg/ml. The intensity of fluorescence observed under UV light (wavelength 254nm) was used to estimate the concentration of sample DNA compared to the λ DNA.

Accurate measurement of the DNA concentration of samples was performed by measuring the optical density of 10µl of the sample diluted into 1ml of sterile deionised water. The concentration was determined by measuring the optical density at 260nm based on an OD_{260} of 1.0 for 50µg/ml DNA and the purity of the DNA could be measured by calculating the ratio of the optical density at 260nm and 280nm. A pure sample of DNA had a 260:280 absorbance ratio of 1.8.

4.2.1.3 Digestion of genomic DNA and extraction of fragments

Partial digestion of the genomic DNA was conducted using the restriction endonuclease *Sau3AI* which has the cutting site 5'- GATC - 3'. Approximately 1µg of genomic DNA was restricted with 14 units of *Sau3AI* for 2 hours at 37°C. The digestion was then terminated by the addition of 0.5M EDTA pH 8.0. After the partial digest had been electrophoresed through 0.8% agarose for 45 minutes at 70V in 1x TBE, fragments in the region of 2-7kb were excised from the gel using the QIAquick gel extraction kit protocol (Qiagen). The area of gel containing the required fragments was excised using a sterile scalpel, and its weight determined. QX1 buffer (Qiagen) (3 gel volumes) was added to the gel (for a weight of agarose of 100mg, 1 gel volume was

equivalent to 100µl). The mixture was incubated at 50°C for 10 minutes, vortexing the mixture every 2-3 minutes until the gel slice had completely dissolved. To this mixture, 1 gel volume of 100% isopropanol was added and thoroughly mixed. This mixture was added to a spin column (Qiagen) then centifuged (14000rpm, Eppendorf 5415C centrifuge, 1 minute). QX1 buffer (0.5ml) was added to the column followed by centifuging (14000rpm, Eppendorf 5415C centrifuge, 1 minute) to ensure that all traces of agarose were removed. The column was washed by the addition of 0.75ml of PE buffer (Qiagen) and further centifugation (14000rpm, Eppendorf 5415C centrifuge, 1 minute). To ensure that all wash buffer had been eluted, the column was recentrifuged (14000rpm, Eppendorf 5415C centrifuge, 1 minute). DNA was eluted by adding 30µl of sterile water and incubating at 20-25°C for 2 minutes prior to centifugation (14000rpm, Eppendorf 5415C centrifuge, 1 minute).

4.2.1.4 Ethanol precipitation

In order to concentrate the DNA sample prior to ligation, an ethanol precipitation was performed. One tenth of a volume of 3M Sodium acetate pH 5.2 and two volumes of ethanol (100%) chilled to -20°C were added to the DNA sample. This was incubated at -20°C for 30 minutes to precipitate the DNA which was pelleted by centrifugation (13000rpm Eppendorf 5415C centrifuge, 30 minutes). The supernatant was removed using a vacuum aspirator. The DNA pellet was washed with 100µl of 70% ethanol prechilled to -20°C. The sample was further centrifuged at 13000rpm for 15 minutes to ensure the DNA pellet had not been dislodged during the addition of the ethanol. The ethanol was removed using an aspirator and the pellet allowed to air dry before resuspension in the appropriate volume of sterile deionised water to achieve the

required concentration. DNA was stored at -20°C prior to use.

4.2.1.5 Ligation of genomic DNA fragments into Lambda arms

A ligation reaction was set up as follows:

1µl λ phage arms (1µg)

6µl of DNA fragments

1µl of 10x ligase buffer

1µl of 10mM rATP(pH7.5)

1µl T4 DNA ligase (2 units)

The reaction was incubated at 12-14°C for 48 hours.

The ligation control reaction was incubated under the same conditions but contained:

1µl λ phage arms (1µg)

1.6µl pBub test insert (0.4µg)

0.5µl ligase buffer

0.5µl rATP pH 7.5

0.9µl sterile deionised water

0.5µl T4 DNA ligase

4.2.1.6 Packaging of product into lambda phage

An aliquot of freeze-thaw extract (Stratagene) was placed on dry ice while the sonic extract (Stratagene) was being thawed. The freeze-thaw extract was rapidly thawed until it was beginning to melt. DNA from the ligation reaction was added to the freeze-thaw extract (0.1-5.0µg of DNA in a volume of 1-5µl) and 15µl of sonic extract added. The mixture was stirred with a pipette tip to prevent the introduction of air

bubbles and centrifuged for 3-5 seconds (6500rpm, Eppendorf 5415C centrifuge) before incubating at 22°C for 2 hours. 500µl of SM buffer was added to the tube. Chloroform (20µl) was added and the contents of the tube mixed gently to lyse the cells. The tube was centrifuged (13000rpm, Eppendorf 5415C centrifuge, 30 seconds) to sediment cell debris and the supernatant transferred to a fresh tube. The packaged phage were stored at 4°C in preparation for titration.

The ligation control was packaged in the same way as the sample DNA. 5µl of the ligation control DNA was used.

In addition a packaging control consisting of wild type λ DNA was included to evaluate the packaging efficiency. Approximately 0.2µg (1µl) of wild type λcI857 *Sam*7 lambda control DNA was packaged as previously described (this section).

4.2.1.7 Maintenance of bacterial strains

Bacteria were maintained on appropriate antibiotic agar and each strain was sub-cultured weekly from a single colony. Strains XL1-MRF' and XLOLR were maintained on LB plates (10g NaCl, 10g tryptone, 5g yeast extract and 20g agar per litre, pH7.0) supplemented with 12.5µg/ml tetracycline. Strain VCS257 which was used only for titering packaging controls, was maintained on LB plates without any antibiotic supplement.

4.2.1.8 Bacterial culture

A single colony of bacteria was inoculated into the appropriate media and incubated for 16-18 hours at 30°C. Broth culture medium for *E. coli* strains XL1-MRF' and VCS257 was LB broth supplemented with 0.2% (w/v) maltose and 10 mM

magnesium sulphate whilst XLOLR was incubated in NZY broth (5g NaCl, 2g $MgSO_4.7H_2O$, 5g yeast extract, 10g casein hydrolysate and 15g agar per litre, pH7.5). 0.5ml of a culture with an OD_{600} of 0.5 was inoculated into a fresh broth culture and incubated at 37°C until the OD_{600} was 1.0. Bacteria were centifuged at 3000rpm (Beckman J2-21; rotor JA14) for 10 minutes then resuspended in 10 mM magnesium sulphate at an OD_{600} of approximately 2.0. Prior to use bacteria were stored at 4°C for up to 48 hours and were diluted to the required OD in 10mM magnesium sulphate.

4.2.1.9 Freezing bacteria

For storage, a 10ml culture of bacterial cells was grown from a single colony to an OD_{600} of 1.0-2.0. Freezing mixture (glycerol/50% (v/v) culture media) (4.5ml) was added to the culture and thoroughly mixed before the cells were aliquoted into 1ml samples and stored at -70°C.

4.2.1.10 Titration of packaged phage

1µl of the packaged λ phage and 1µl of a ten-fold dilution of packaged λ phage in SM buffer were added to 200µl of XL1-MRF' cells at an OD_{600} of 0.5. The bacteria and phage (from both the packaged sample and ligation control) were incubated at 37°C for 20 minutes. Before pouring onto dried NZY agar plates, 15µl of 0.5M IPTG, 50µl of 250mg/ml X-gal and 3ml of NZY top agar (48°C) were added. Plates were allowed to set for 10 minutes before incubation at 37°C for 16-18 hours. The numbers of clear plaques (phage contained an insert) and blue plaques (no insert incorporated) were counted to establish the number of recombinant plaques. For the packaging control, the bacteria used were VCS257, prepared in the same way as the XL1-MRF' cells for the

packaged sample and ligation control. 10µl of a 10^{-2} and 10^{-4} dilution of the packaging control phage were added to 200µl of VCS257 cells at an OD_{600} of 0.5 and incubated at 37°C for 20 minutes. Plates were prepared as described for the packaged sample and ligation control (section 4.2.1.10).

4.2.1.11 Genomic library amplification

XL1-MRF' were diluted to an OD_{600} of 0.5 in 10mM magnesium sulphate. 600µl of diluted bacteria were added to approximately 5.0 x 10^4 pfu and incubated at 37°C for 20 minutes. 6.5ml of melted NZY agar at 50°C was added to this, mixed then distributed evenly onto a dry 150mm NZY agar plate. The plates were incubated for 6-8 hours at 37°C. The plates were overlayed with 8-10ml of SM buffer and incubated at 4°C for 16-18 hours. The SM buffer was pooled and then the plate was rinsed with a further 2ml of SM buffer. Chloroform was added to a final concentration of 5% (v/v) and after thorough mixing, the sample was incubated at 20-25°C for 15 minutes. The supernatant was centrifuged at 1500rpm (Beckman JA2-20; JA14 rotor) for 10 minutes then recovered and transferred to a sterile glass universal. Chloroform and DMSO were added to a final concentration of 0.3% (v/v) and 7% (v/v) respectively. The library was stored at -70°C in 1ml aliquots. The amplified library was titered using 10^{-2}-10^{-8} dilutions as previously described (Section 4.2.1.10).

4.2.2 Identification of clones from the random genomic library of S. pilosicoli P43/6/78^T which reacted with the monoclonal antibody BJL/AC1

4.2.2.1 Immunoscreening of genomic library with the monoclonal antibody BJL/AC1

Screening was carried out using 150mm circular NYZ agar plates. Before use, plates were incubated at 37°C for 1 hour. Molten NZY top agar was maintained at a constant temperature (50°C). XL1-MRF' bacteria were diluted to an OD_{600} of 0.5 in 10mM magnesium sulphate. A total of 5.0 x 10^4 pfu from the amplified phage library stock was added to 600µl of diluted bacteria and incubated at 37°C for 20 minutes. NZY top agar (7.5ml) was mixed with the phage and evenly distributed on the surface of the pre-warmed plate. After allowing the top agar to solidify, the plate was incubated at 42°C for approximately 3.5 hours or until the plaques became visible. The agar was overlayed with a circular nitrocellulose membrane (Stratagene) which had been treated with 10mM IPTG 30 minutes prior to use, then incubated at 37°C for 3.5 hours. The membrane was removed and washed three times in TBS-Tween for 15 minutes with gentle agitation. Membranes were transferred to blocking solution for at least one hour at 20-25°C with gentle agitation or at 4°C for 16-18 hours, followed by transfer to fresh blocking solution containing the primary antibody at a dilution of 1:250. They were incubated in this antibody for at least three hours at 20-25°C with gentle agitation or at 4°C for 16-18 hours, then washed in TBS-Tween three times for five minutes each at 20-25°C with gentle agitation. Membranes were then incubated in fresh blocking solution containing goat anti-mouse alkaline phosphatase conjugated antibody (Stratagene) at a dilution of 1:5000 at 20-25°C for 1 hour with gentle agitation. As

previously described, membranes were washed in TBS-Tween and then once in TBS for five minutes with gentle agitation to remove any remaining Tween. Colour development was undertaken at 20-25°C with gentle agitation in the dark using 0.3mg/ml NBT and 0.15mg/ml BCIP in 100mM Tris-HCl pH 9.5, 100 mM NaCl and 5mM $MgCl_2$. After 30 minutes or when a positive colour reaction was observed, the membranes were rinsed in TBS and the colour development reaction terminated by washing the membranes in "Stop" solution (20mM Tris-HCl pH 2.9, 1mM EDTA). Membranes were air-dried and stored at room temperature in the dark.

The positions of the most intensely coloured putative clones were determined. The areas containing these clones were then removed by "coring" the area on the plate that correlated with the clone on the membrane. 500µl of SM buffer was added to each core and supplemented with 20µl of chloroform. The mixture was then vortexed briefly and incubated at 16-18 hours at 4°C to permit phage release. When "coring" from the primary screening plate many clones were included in the plug (up to 10^{6-7} pfu/cm^2). Secondary screening used these phage, which were plated at approximately 50-500 clones per plate. Screening was conducted as previously described. After membranes had been developed, plugs were cored from the plates to isolate single positive clones. If more than one plaque was cored, tertiary screening was performed. The phage cored from a single positive plaque were released as previously described and the insert excised into the phagemid vector.

4.2.2.2 Excision of a single clone

A culture of XL1-MRF' cells was grown to an OD_{600} of 1.0 (as described in section 4.2.1.8). 200µl of XL1-MRF' cells were combined with 250µl of phage stock

and 1μl of Exassist helper phage (Stratagene, stock concentration of 1.0 x 10^{10} pfu/ml) and incubated at 37°C for 20 minutes. NYZ broth (3ml) was added to the reaction and incubated for 3 -18 hours at 37°C with agitation. Simultaneous infection of *E. coli* with λ phage helper phage allowed the insert and flanking sequences to be replicated to form a single stranded circular DNA phagemid vector known as pBK-CMV. The reaction was incubated at 65°C for 20 minutes then centrifuged at 3000rpm (Eppendorf 5415C centrifuge) for 15 minutes. The *E. coli* were killed by the high temperature but packaged λ phagemid particles were resistant and these remained in the supernatant. The excised λ phagemid were stored at 4°C.

4.2.2.3 Plating of excised λ phagemids

E. coli XLOLR cells were grown as previously described in section 3.2.1.8 were diluted to an OD_{600} of 1.0. 200μl of cells, were then added to 100μl and 10μl respectively of the excised λ phagemid stock and incubated at 37°C for 15 minutes. To this, 300μl of NZY broth was added and incubated at 37°C for a further 45 minutes. 200μl of each mixture was spread onto LB-kanamycin agar plates (50μg/ml). Plates were incubated at 37°C for 16-18 hours. The phagemid contained a kanamycin resistance gene so that only XLOLR cells infected with the λ phagemid (double stranded DNA form) were able to grow. Single colonies were selected and streaked onto LB-kanamycin agar plates.

4.2.2.4 Extraction of DNA from positive clones

To extract DNA from clones which reacted with the monoclonal antibody BJL/AC1, a Clearcut™ Miniprep kit (Stratagene) was used according to the

manufacturer's instructions. In brief, a culture of NZY broth containing 50μg/ml kanamycin was loop inoculated with a single positive colony and incubated overnight at 30°C. An aliquot of 1.5ml was removed and centifuged at 14000rpm for 1 minute (Eppendorf 5415C centrifuge). After removing the supernatant, the cells were resuspended in approximately 100μl of Solution 1 (50mM Tris-HCl pH7.5, 10mM EDTA, 50μg/ml RNase A) to which approximately 125μl of Solution 2 (0.2M NaOH, 1% (w/v) SDS) was added. Finally approximately 125μl of solution 3 (Stratagene) was added and incubated at 4°C for 5 minutes. The mixture was centrifuged at 14000rpm (Eppendorf 5415C centrifuge) for 5 minutes and the pellet discarded. 15μl of resin was added to the supernatant, which was then loaded into the spin cup and centrifuged at 14000rpm (Eppendorf 5415C centrifuge) for 30 seconds. The DNA was washed twice in wash buffer (10mM Tris-HCl pH7.5, 100mM NaCl, 50% (v/v) ethanol), and centrifuged at 14000rpm for 30 seconds. Plasmid DNA was eluted in 100μl TE buffer (5mM Tris-HCl pH7.5, 0.1mM EDTA).

4.2.2.5 Determination of insert size

1μg of plasmid DNA extracted by the Clearcut™ Miniprep kit was digested with 1 unit of *BamHI* restriction endonuclease (Boehringer Mannheim) for 16-18 hours at 37°C. Alternatively 1μg of plasmid DNA was digested using 1 unit of *EcoRI* (Stratagene) and 1 unit of *PstI* (Stratagene) and incubated at 37°C for 16-18 hours. The fragments were electrophoresed through a gel (0.8% (w/v) agarose in 1x TBE). A kilobase ladder (New England Biolabs) and λ *EcoRI/HindIII* digest ladder (Sigma) were electrophoresed with the sample to enable size determination.

4.2.2.6 Preparation of DNA for sequencing

In order to prepare DNA suitable for automated sequencing, plasmid DNA was purified using the Promega Wizard® *Plus* Maxiprep kit. A 100-500ml culture of the appropriate clone was incubated at 37°C for 16-18 hours. The culture was centrifuged at 5000 rpm (Beckman J2-21; rotor JA14) for 10 minutes at 22-25°C, the supernatant discarded and the cells thoroughly resuspended in 15ml of cell resuspension solution (50mM Tris-HCl pH 7.5, 10mM EDTA, 100μg/ml RNase A). To this, 15ml of cell lysis solution (0.2M NaOH, 1% (w/v) SDS) was added and mixed gently by inversion. Lysis was complete when the solution became viscous and clear. 15ml of neutralization solution (1.32M potassium acetate, pH 4.8) was added and mixed by inversion. The mixture was centrifuged at 8000rpm (Beckman J2-21; rotor JA-25.50) for 15 minutes at 22-25°C and the cleared supernatant was filtered through Whatman No. 1 filter paper. Isopropanol (half the volume of the supernatant) was added and mixed by inversion. This mixture was centrifuged at 8000rpm (Beckman J2-21; rotor JA-25.50) for 15 minutes at 22-25°C. The supernatant was discarded and the DNA pellet resuspended in 2ml TE buffer. DNA purification resin (10ml) was added to the DNA, then mixed by inversion. The maxiprep column was attached to an aspirator and the DNA/resin mixture added. The vacuum pulled the mixture through the column which retained the DNA. Column wash solution (25ml) was added to the column allowing the vacuum to draw the solution through the column, washing the bound DNA and rinsing with 5ml of 80% ethanol. The vacuum was drawn on the column for one additional minute after the ethanol had passed through the column. After the vacuum was released, the column was placed in a 50ml screw cap tube and centrifuged at 2500 rpm in an MSE Chilspin

centrifuge with swing bucket rotor for 5 minutes. The column was then returned to the aspirator and the vacuum drawn for a further 5 minutes. To elute the DNA from the column, 1.5ml of preheated (65-70°C) water was added to the column and incubated for 1 minute before centrifuging in a 50ml screw cap tube (2500rpm, swing bucket rotor centrifuge, 5 minutes). To remove residual resin the DNA solution was first filtered through a 0.2μm syringe filter, then the eluate was centrifuged at 14000rpm (Eppendorf 5415C centrifuge) for 1 minute. The supernatant was transferred to a fresh tube and stored at -20°C.

4.2.2.7 DNA sequencing and database searching

DNA from Wizard maxipreps was supplied to Alta Biosciences (Birmingham) who sequenced clones by the Sanger dideoxy method using fluorescent labels. Open reading frames were identified and translated into amino acid sequences using DNA strider Version 1.2. Database searches were conducted from the nucleotide and derived amino-acid sequences using the GCG sequence analysis software (Program Manual for the Wisconsin package, version 8, 1994, Genetics Computer Group, 575 Science Drive, Madison, WI, USA) to identify similar sequences. Multiple alignments of these sequences was then made using the EGCG package (Program manual for EGCG version 8.1.0, March 1996, Peter Rice, The Sanger Center, Hinxton Hall, Cambridge, CB10 1RQ, UK). These alignments were used to generate dendrograms using PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein, 1993).

Chou-Fasman plots and Kyte-Doolittle hydropathy plots were generated from the derived amino acid sequence of the clone using the Protylze Structure Prediction Version 3.0 software (Scientific Educational software).

4.2.3 Immunoscreening of P43/6/78^T random genomic library with polyclonal serum

4.2.3.1 Generation of polyclonal serum to *S. pilosicoli*

Polyclonal serum to *S. pilosicoli* P43/6/78^T was raised using a New Zealand rabbit. P43/6/78^T antigen for dosing the rabbit was prepared as follows. Cultures of P43/6/78^T (3 x 20ml) were centrifuged at 3000rpm (Beckman J2-21; rotor JA-25.50) for 10 minutes. The pellet was resuspended in 1ml of saline then centrifuged (13000rpm, Eppendorf 5415C centrifuge, 5 minutes) and the pellet resuspended to a final concentration of 40mg/ml in saline. The bacteria were killed by exposure to UV light (wavelength 254nm) at a distance of 10cm for five minutes and aliquoted into 12 batches of 0.25ml. To one aliquot, an equal volume of Freunds complete adjuvant was added. Freunds incomplete adjuvant was added in equal volumes to 10 aliquots. The last aliquot was stored for use as a bacterial sample for testing sera. All samples were stored at -20°C prior to use. The rabbit was dosed by subcutaneous injection and on the first week inoculated with the Freunds complete adjuvant sample followed on consecutive weeks by dosing with aliquots containing the Freunds incomplete adjuvant. Serum samples were taken by ear vein bleed prior to dosing to ensure that the rabbit did not have any prior exposure to the antigen and also after 5 of the samples supplemented with Freunds incomplete adjuvant had been administered, to ensure that an immune response was being generated. Two weeks after the final doses had been administered, the serum was harvested by cardiac puncture. After collection, the whole blood was incubated at 4°C for 16-18 hours to permit clotting. The supernatant was centrifuged at 2000rpm (Beckman J2-20; JA14 rotor) for 10 minutes followed by centrifugation at 13000rpm for 5 minutes and the resulting serum was pooled prior to storage at -20°C.

4.2.3.2 Immune absorption of polyclonal serum with *E. coli* lysate

E. coli phage lysate (Stratagene), was diluted 1:10 (v/v) in TBS-Tween. Nitrocellulose membranes were immersed in the diluted lysate and incubated at 37°C for 30 minutes with gentle agitation. The membranes were removed from the lysate and allowed to air dry prior to washing in TBS three times for five minutes each. Excess moisture was removed and the membranes immersed in blocking solution for at least 30 minutes at 37°C. The membranes were then washed three times in TBS-Tween for five minutes. Polyclonal serum (section 4.2.3.1) was diluted fivefold in TBS-Tween and added to the first treated membrane and incubated at 37°C for 10 minutes. The procedure was repeated sequentially with each of the remaining membranes. The resulting antibody was then collected and stored at -20°C for future use.

4.2.3.3 Identification of clones which reacted with polyclonal serum.

The *S. pilosicoli* P43/6/78^T library was screened using the absorbed polyclonal serum (as described in section 4.2.2.1-3). The polyclonal serum was used at a dilution of 1:10 in TBS-Tween from the *E. coli* absorbed stock (a final dilution of 1:50). Goat anti-rabbit alkaline phosphatase conjugated antibody was used as described in section 4.2.2.1.

4.2.3.4 Extraction of DNA from positive clones

The phagemid DNA from positive clones was purified using the Wizard® *Plus* SV Miniprep kit. A single colony of a positive clone was incubated at 37°C for 16-18 hours in LB broth supplemented with 50μg/ml kanamycin. This culture (1-5ml) was

centrifuged (13000rpm, Eppendorf 5415C centrifuge, 5 minutes) and the supernatant was removed. The pellet was resuspended in 250μl cell resuspension solution (50mM Tris-HCl, pH7.5, 10mM EDTA, 100 μg/ml RNase A). To this, 250μl cell lysis solution (0.2M NaOH, 1% SDS) was added and mixed by inversion. The mixture was incubated until the suspension had become clear and viscous. Alkaline protease (10μl) was added to the lysate and incubated at 22-25°C for 5 minutes. To inhibit further action of the alkaline protease, 350μl of neutralization solution (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid) was added. This was mixed by inversion and the precipitate pelleted by centrifugation (14000rpm, Eppendorf 5415C centrifuge, 10 minutes). The cleared lysate (supernatant) was transferred to the spin column and centrifuged (14000rpm, Eppendorf 5415C centrifuge, 1 minute). The DNA retained on the column was washed by the addition of 750μl of column wash solution (60mM potassium acetate, 10mM Tris-HCl pH7.5, 50% (v/v) ethanol) and centrifugation (14000rpm, Eppendorf 5415C centrifuge, 1 minute). The DNA was washed with a further 250μl of column wash solution and after centrifugation (14000rpm, Eppendorf 5415C centrifuge, 1 minute) the column was recentrifuged (14000rpm, Eppendorf 5415C centrifuge, 2 minutes) to ensure that all of the wash solution had passed through the column. DNA was eluted by the addition of 100μl of nuclease-free water and centrifugation (14000rpm, Eppendorf 5415C centrifuge, 1 minute).

4.2.3.5 Determination of insert size, sequencing and database searches.

The size of insert in the clones, was determined as described in section 4.2.2.5. DNA was prepared for sequencing as described in section 4.2.2.6. Sequencing, database

searches and hydropathy plots were carried out as described in section 4.2.2.7.

4.2.3.6 Affinity purification of serum

After the elution of single clones which reacted with the polyclonal serum, affinity purification (Burnie and Clark, 1989) was used to isolate antibodies with which each clone reacted. From a culture of XL1-MRF', 200µl of cells diluted to an OD_{600} of 0.5 in 10mM $MgSO_4$ were added to 250µl of phage and incubated at 37°C for 20 minutes. NZY top agar (3ml) was added and after mixing was poured onto a plate with a 92mm diameter. The plates were incubated at 37°C until confluent lysis was apparent (usually 16-18 hours). A nitrocellulose membrane which had been pretreated with 10mM IPTG was incubated on each plate for 6 hours at 37°C. Membranes were washed three times in TBS-tween for 15 minutes then blocked in TBS/1% BSA for 1 hour before incubation in polyclonal serum for 16-18 hours at 4°C. This was followed by washing the membranes with TBS for 5 minutes and washing with 0.15M NaCl/0.05% (v/v) Tween 20. The antibodies were eluted from membranes by incubation with 0.15M NaCl, 0.05% (v/v) Tween 20 and 0.2M glycine HCl pH2.8 for 30 minutes. The reaction was stopped by the addition of 0.04g of Tris. The antibody solution was then diluted 1:1 in TBST/1% (w/v) BSA and used to probe western blots of whole cell extracts.

4.2.3.7 Western blotting with whole polyclonal serum and affinity purified serum

SDS-PAGE gels were used to separate proteins and the gels subsequently used for immunoblotting as described in sections 2.2.6.1 and 2.2.6.2. The polyclonal serum was applied to the membrane at a dilution of 1:50 in TBS-Tween and developed as described in section 2.2.6.2. SDS-PAGE gels which were subsequently immunoblotted

for development with the affinity purified serum (used without dilution after preparation as described in section 4.2.3.6) were electrophoresed with proteins from P43/6/78^{T}, a whole cell preparation of the clone from which the serum was purified and the *E. coli* strains used. Immunoblots were then developed as described in section 4.2.2.1.

4.3 Results

4.3.1 Generation of random genomic library

4.3.1.1 Calculation for determining the number of plaques required for a representative library

The calculation for determining the number of recombinant plaques required to produce a representative genomic library is (Sambrook *et al.*, 1989):

$$N = \frac{\ln (1 - P)}{\ln (1 - a/b)}$$

Where, N = Number of plaques

P = Probability of the presence of a gene

a = average fragment size

b = total genome size

If the probability of a gene being present is 0.99, the genome size was approximately 3Mb as determined by PFGE (section 2.3.1.1) and the average fragment size was approximately 4.5 kb,

$$N = \frac{\ln (1 - 0.99)}{\ln (1 - 4.5/3000)} = 3068 \text{ plaques}$$

To account for incorporation in either direction = 2 x N

On the assumption that there are three origins of replication = 3 x 2N = 1.8×10^4 plaques

For intestinal spirochaetes, the number of plaques required to have a 99% probability of incorporating any gene is 1.8×10^4 plaques.

4.3.1.2 Titration and amplification of P43/6/78^T genomic library

After ligation and packaging of P43/6/78^T fragments titration of the phage, showed that there were 6.1 x 10^4 pfu/ml which contained inserts. The number of packaged phage, therefore exceeded the requirement for a representative library.

After amplification of the library, titration showed that there were 1.74 x 10^7 pfu/ml recombinant phage.

4.3.2 Immunoscreening of P43/6/78^T library with the monoclonal antibody BJL/AC1

4.3.2.1 Sequencing of positive clones

After screening with BJL/AC1, the positive clone SR1 was selected for sequencing. SR1 contained an insert of approximately 2.6 kbp which was digested by *EcoRI* and *PstI* (section 4.2.2.5) into fragments of 551bp, 686bp, and 1394bp (Figure 4.1). The sequence of the insert showing the position of the open reading frame (ORF) is shown in Figure 1 (Appendix 1).

Sequencing of the clone revealed a single ORF of 825bp. This was translated into a 275 amino acid sequence using the Protylze Structure Prediction software.

4.3.2.2 Sequence similarities

Comparison of the DNA sequence from clone SR1 with GCG sequence analysis software showed that it had 54.1% identity to the *Thermatoga maritima* pyruvate oxidoreductase (POR) alpha subunit within a 796bp region.

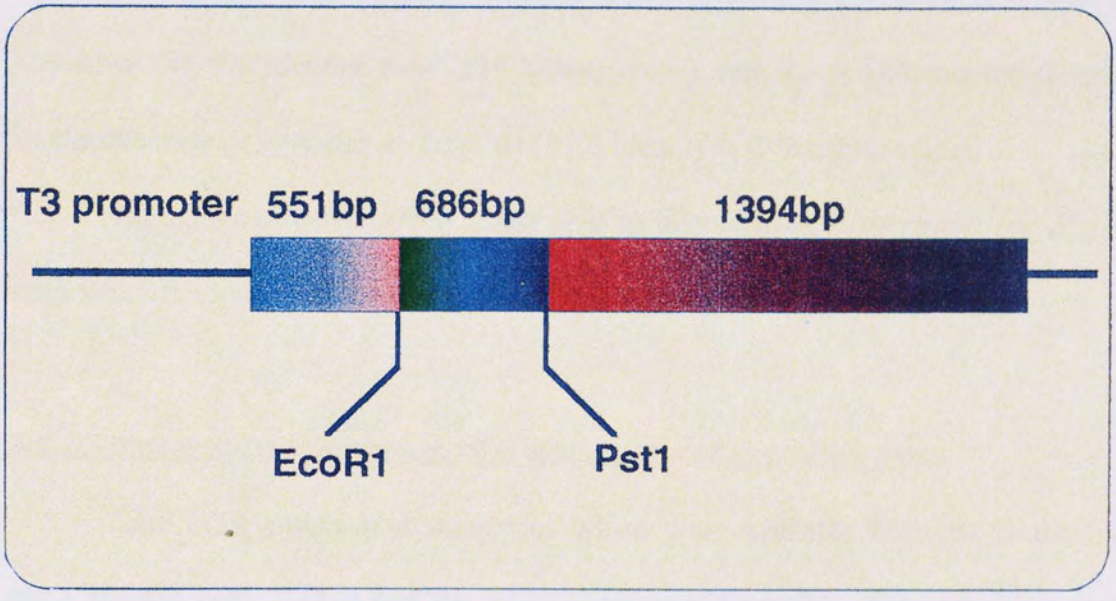


Figure 4.1 Diagrammatic representation of the insert in clone SR1 showing the restriction sites for digestion with *EcoRI* and *PstI*.

The amino acid sequence derived from the ORF was most closely related to the *H. pylori* pyruvate-ferredoxin oxidoreductase alpha subunit, showing 38.8% identity over 250 amino acids. It also showed significant homology to pyruvate-flavodoxin oxidoreductases in other intestinal organisms such as *E. coli* (28.7% identity in 258 amino acids) and *Giardia intestinalis* (25.1% identity over 251 amino acids). The ORF showed similarities to the PORs of extreme thermophiles such as the alpha subunit of *T. maritima* (38.6% identity over 254 amino acids) and the 2-ketovalerate-ferrodoxin oxidoreductase of *Pyrococcus furiosus* (34% identity in 262 amino acids).

Alignment of the deduced amino acid sequences from *T. maritima* and *H. pylori* with which SR1 is most closely related is shown in figure 4.2.

4.3.2.3 Phylogenetic relationships of sequences related to positive clone

Nine POR amino acid sequences which were available from GCG databases, were aligned with SR1 using the EGCG package ClustalW. This was exported to PHYLIP which generated a phylogenetic tree (Figure 4.3). The tree contained two branches which were separated at a distance of 3.5 Euclids. The first cluster contained SR1, *P. furiosus* (GenBank accession number X85250), *H. pylori* (AF015952) and *T. maritima* (X85250). The second cluster contained the remaining 6 PORs: *G. intestinalis* (L27221), *K. pneumoniae* (X01007), *E. coli* (Ae015952), *Rhodospirillum rubrum* (Q53046), *Synechocystis* sp. PCC 6803 (D64005) and *Anabaena* sp. PCC 7120 (L14925).

The RPF motif (figure 4.2) was common to all POR amino acid sequences examined.

```
          1                                                    50
pylori    FKDNGYIDGE FVLVESEHAA MSACVGAAAA GGRVSTATSS QGLALMVEVL
 therm    FVADGVVRTE MIPVESEHSA MSAVVGAAAA GARAMTATSA NGLALMHEIV
   sr1    MKANGRHTVC MIPGDGEHGA AGICYGATTA GGRVFNATSA NGLLFAMEQL
          :  :*       ::  :.**.*  .   **::* *.*. .***: :** :  * :

          51                                                   100
pylori    YQASGMRLPI VLNLVNRALA APLNIHGDHS DMYLSRDSGW ISLCTCNPQE
 therm    YIAASYRLPI VMPVVNRALS GPINIHCDHS DAMAERDSGW IQLFAETNQE
   sr1    PVQAGTRFPM VLNVVNRTVS GPLDIKCDQS DIMMALNTGW IIIMAHTTQM
             :. *:*: *: :***::: .*::*: *:* *     ::** * : : . *

          101                                                  150
pylori    AYDFTLMAFK IAEHQKVRVP TIVNQDGFLC SHTVQNVRPL -SDAVAYQFV
 therm    AYDFTILAVR LAEHEDVRLP VMVNLDGFIL SHGVEPVEFY -PDELVKKFV
   sr1    VYDFNIFALK IAE--KAKLP IIVSSDGFFT SHQKKKIHLF KNDKDVQDFL
          .***.::*.: ::*  ..::*  :*. ***:  **  : :.     *  . .*:

          151                                                  200
pylori    GEYQT-KHSL LDFDKPVSYG AQAEEEWHYE HKAQLHHAIM SASSVIEEVF
 therm    GELKP-MYPL LDTEHPVTWG PLDLYDYYFE HKRQQIEAME NVKKVFPEIA
   sr1    GKYTPEVTSV EPTKNPVTIG PYMNEDELTG SKLQLSQALE DSRAIIAEVF
          *:  . . : :  .:**: * .    :      * *  .*:  .   :: *:

          201                                                  250
pylori    NDFAKLTGRQ YHLTKTFQLE DAEIAIFALG TTYESAIVAA KEMRKK..GI
 therm    KEFEETFGRK YWFVEPYRME DAEHVMVALG STNSTIKYVV DELREE..GY
   sr1    EEFASLSGRK YSPIETHNME GAEVALMLCG SAYETGTLAV DEMRKANPNL
          ::* .  **: *   :...:* .** .:.  * :: .:   .. .*:*:   .

          251                                                  300
pylori    KAGVATIHSL RPFPYEKLGQ DLKNLKALAI LDKS------ ----------
 therm    KVGSLKIWMF RPFPKEQLQE LLNGRKSVVV LDRAVSFGAE APLYEAVKSA
   sr1    KIGAFAITQI RPFPGR---- --KNYKNYLL MLK------- ----------
          * *   *    **** .       :. *   : : :
```

Figure 4.2 Deduced amino acid sequence of ORF by comparison to most closely related sequences. Multiple sequence alignments of PORs from *H. pylori* **(pylori)** and *T. maritima* **(therm)** with the clone SR1 were generated using the EGCG program ClustalW (Gap opening penalty 10; gap extension penalty 0.05). "*" denotes a fully conserved residue; ":" denotes that one of the following "strong" groups is conserved STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY and FYW; "." denotes that a "weaker" group is conserved such as CSA, ATV, SAG, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM and HFY. The RPF motif which was common to POR sequences is marked in bold type and underlined.

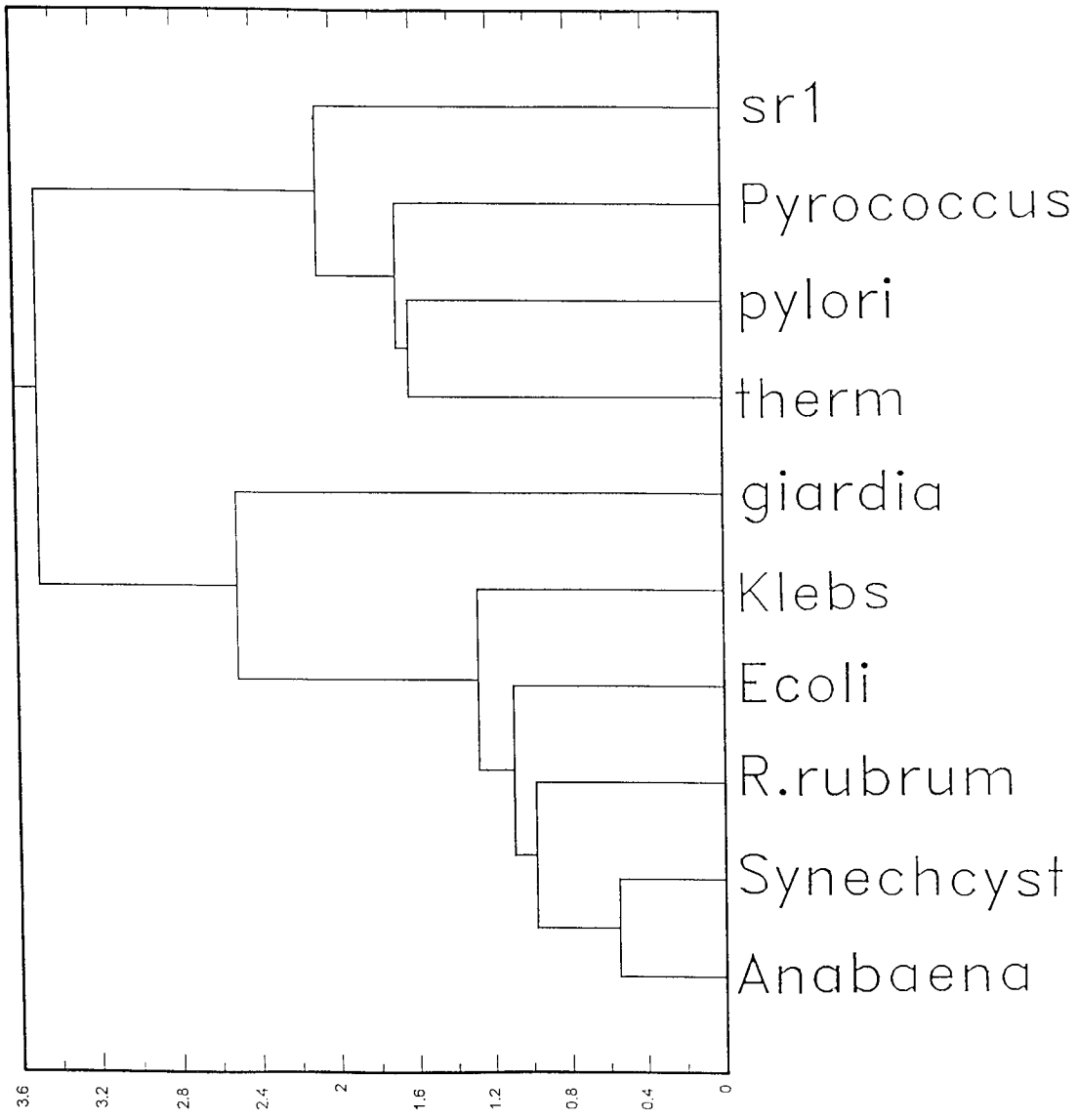


Figure 4.3: Phylogenetic tree of bacterial, protozoan and algal PORs. The tree was constructed using maximal parsimony as implemented by the PROTPARS program of PHYLIP. The tree was rooted by *G. intestinalis* (giardia). Other species indicated on the tree are *P. furiosus* (Pyrococcus), SR1, *H. pylori* (pylori), *T. maritima* (therm), *K. pneumoniae* (Klebs), *E. coli* (Ecoli), *R. rubrum* (R.rubrum), *Synechocystis* sp. PCC 6803 (Synechcyst) and *Anabaena* sp. PCC 7120 (Anabaena). The distance is shown in Euclids.

4.3.2.4 Hydropathy plots of derived amino acid sequences

Chou-Fasman plots of clone SR1 showed regions of α-helix, β-pleated sheet and turns (Figure 4.4). The residues between 180-200 showed the largest and most probable membrane spanning domain. A number of regions were found to contain hydrophobic residues by generation of a Kyte-Doolittle hydropathy plot (Figure 4.4). Using this method, the region most likely to be membrane spanning was residues 170-190.

4.3.3 Immunoscreening of P43/6/78^T library with polyclonal serum

4.3.3.1 Sequencing of immunoreactive clones

Six clones which reacted with polyclonal serum (as described in section 4.2.3) were selected for further study. Of these, two clones (2a and 5a) showed identical restriction fragment profiles to SR1. The T3 promoter end of clone 5a was sequenced and shown to contain the same insert as SR1. Three clones (1a, 3a and 6a) were selected for sequencing. Clone 6a contained an insert of approximately 1kb (Appendix 2) which was restricted into fragments of 678bp and 313bp by digestion with *EcoRI* and *PstI*. Clones 1a and 3a contained inserts which were 3.1kb (Appendix 3) and 2.6kb (Appendix 4) respectively.

The ORF of clone 6a was found by reading from the T7 promoter end. A ribosome binding site was identified and the coding regions contained 747bp which was translated into 249 amino acids. A termination codon was not found within the insert.

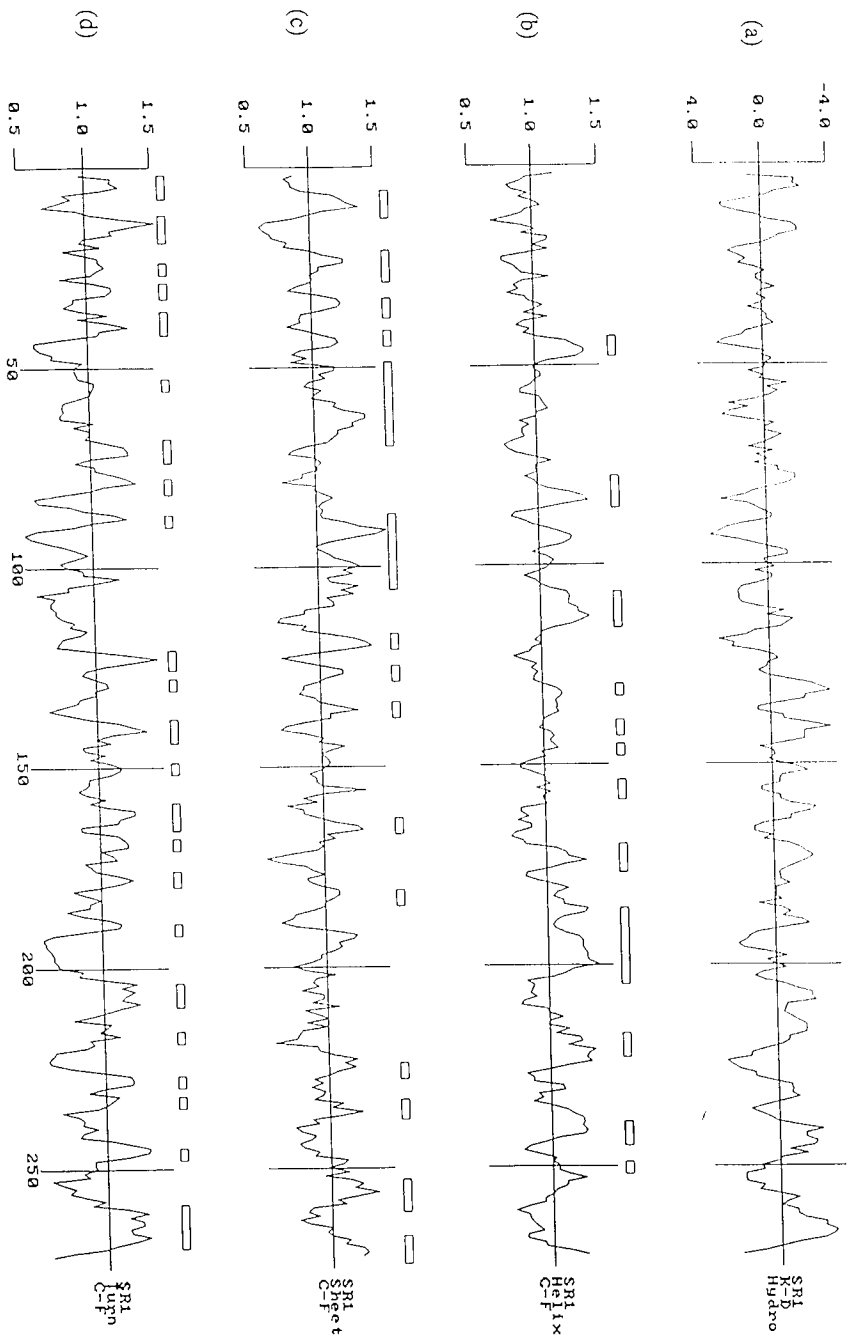


Figure 4.4: Kyte-Doolittle hydropathy plot (a) and Chou-Fasman plots (b-d) of α-helix (b), β-pleated sheet (c) and turns (d) which are represented by open boxes.

(Appendix 3). The first open reading frame was incomplete as it was at the beginning of the insert and consisted of 441bp. The second open reading frame consisted of 333bp, the third was 654bp and the fourth open reading frame was 648bp. The final open reading frame was located at the T7 end of the insert and was not complete as a termination codon was not found within the insert. From this open reading frame, 249bp were encoded by clone 1a.

Clone 3a contained one complete gene and two partial open reading frames. The sequences read from the T7 promoter end. The open reading frame at the T3 promoter end, contained a sequence of 513bp, but the termination codon was not contained within the insert. The second open reading frame consisted of a sequence of 975bp. A partial open reading frame was found at the T7 promoter end of the insert and this contained a 618bp region where the start codon was not contained within the insert.

4.3.3.2 Similarities with sequences in databases

Database searches using the nucleotide sequence of clone 6a showed high levels of homology within short coding regions. The largest fragments with the highest levels of identity to clone 6a1 were a putative basic protein in *C. pasteurianum* (54.3% identity in 282bp) and an unidentified region of *Bacillus subtilis* genomic DNA (52.2% identity in 324bp).

Comparison of the derived amino acid sequence of 6a with known sequences revealed it to have significant homology with arylsulphatase genes (*atsA*) from organisms such as *Pseudomonas aeruginosa* (31.9% identity in 229 amino acids), *Klebsiella aerogenes* (31.4% identity in 229 amino acids) and *E. coli* (32.8% identity in 131 amino acids). Multiple sequence alignments of arylsulphatase genes are illustrated in Figure 4.5.

The multiple genes within clone 1a showed high levels of DNA and amino acid homology to ribosomal proteins. The first (partial) open reading frame was similar to an elongation factor (tuf) in *S. hyodysenteriae* (92.1% identity in 178 amino acids), *T. pallidum* (65.2% identity in 181 amino acids) as well as non-spirochaetal bacteria such as *C. jejuni* (65.2% identity in 181 amino acids), *S. typhimurium* (70.2% identity in 181 amino acids), *H. pylori* (68.7% identity in 182 amino acids), *N. gonorrhoeae* (68.5% identity in 181 amino acids) and *T. maritima* (66.8% in 184 amino acids). The second open reading frame was found to have a significant level of homology to the ribosomal protein S10 (*rpsJ*) from a number of organisms including *S. hyodysenteriae* (99% identity in 101 amino acids), *B. burgdorferi* (60.4% in 101 amino acids), *T. pallidum* (59.4% identity in 101 amino acids), *T. maritima* (56.4% in 101 amino acids), *H. pylori* (56.7% identity in 97 amino acids) and *R. prowazekii* (52.6% in 101 amino acids). Sequences for the ribosomal protein L3 (*rplC*) showed a significant level of homology to the third open reading frame. These included *B. burgdorferi* (41.6% identity in 209 amino acids), *T. pallidum* (40.5% identity in 210 amino acids), and *T. maritima* (49.3% identity in 217 amino acids). The fourth open reading frame was similar to L4 (*rplD*) in organisms such as *B. burgdorferi* (44.5% identity in 211 amino acids), *T. pallidum* (37.8% identity in 209 amino acids), and *T. maritima* (44.6% identity in 186 amino acids). The fifth (partial) open reading frame, showed significant levels of homology to L2 (*rplB*) from organisms such as *B. burgdorferi* (57.6% identity in 85 amino acids), *T. pallidum* (48.2% identity in 83 amino acids), *H. pylori* (50.6% identity in 83 amino acids) and *T. maritima* (53.6% identity in 84 amino acids). Multiple sequence

```
                                                                      60
          1
Klebs     ---------MNKKAMAAAVSMILAGGAHAAQQERPNVIVIIADDMGYSDISPFGGEIPTP
Pseudomonas----------------------------SKRPNFLVIVADDLGFSDIGAFGGEIATP
6a        MRKNNNLTKGFCIGSSVLMAASAVSCSSETKSEKPNIVYIVLDDMGFGDLGCYGSSISTP
                                        .::**.: *: **:*:.*:. :*..*.**

                                                                     120
          61
Klebs     NLQAMAEQGMRMSQYYTSPMSAPARSMLLTGNSNQQAGMGGMWWYDS--TIGKEGYELRL
PseudomonasNLDALAIAGLRLTDFHTASTCSPTRSMLLTGTDHHIAGIGTMAEALTPELEGKPGYEGHL
6a        NIDALANNGIRYVNYFTSPLSSPSRASLLTGCEANKVGMGAVSQVDFG--DLAPNMSGRI
          *::*:*  *:*  ::.*:. .:*:*: **** . : .*:* :           . . ::

                                                                     180
          121
Klebs     TDRVTTMAERFKDAGYNTLMAGKWHLGFVPGATPKDRGFNHAFAFMGGGTSHFNDAIPLG
PseudomonasNERVVALPELLREAGYQTLMAGKWHLGLKPEQTPHARGFERSFSLLPGAANHYGFEPPYD
6a        KPEHAPITHTLKANGYNTYAIGKWHLGPYDEFTP--DGDKYHWPSGKGFDKNYNFIAGQA
          . . ..:.. ::  **:*   ******     **   * :  :.   *  .::.

                                                                     240
          181
Klebs     T-----VEAFHTYYTRDGER---VSLPDDFYSSEAYARQMNSWIKATPKEQPVFAWLAFT
PseudomonasESTPRILKGTPALYVED-ERYL-DTLPEGFYSSDAFGDKLLQYLKERDQSRPFFAYLPFS
6a        N------QFQPGGIIEGDEFIVPDTSDPNYHLSRDIVDRTLKYIDES-KNKPFFAYVAFG
                 :       .. *     :   .:: *     :  .::.   :.:*.**::.*

                                                                     300
          241
Klebs     APHDPLQAPDEWIKRFKGQYEQGYAEVYRQ-RIARLKALGIIHDDTP-LPHLELDKEWEA
PseudomonasAPHWPLQAPREIVEKYRGRYDAG-PEALRQERLARLKELGLVEADVEAHPVLALTREWEA
6a        AMHGPFNVAKKYIDKYKGKF-----------------------------------------
          * * *::.. : :.:::*::
```

Figure 4.5 Multiple sequence alignments of the derived amino acid sequence from clone 6a with known sequences from *Klebsiella aerogenes* (**Klebs**; Swissprot accession number P20713) and *Pseudomonas aeruginosa* (**Pseudomonas**; Swissprot accession number P51691) generated using the EGCG program ClustalW (Gap opening penalty 10; gap extension penalty 0.05). The symbols used ("*" ":" and ".") were as previously described in figure 4.2.

alignments of spirochaetal sequences are represented in Figure 4.6.

The first open reading frame in clone 3a was partially encoded within the insert, and the derived amino acid sequence showed a significant level of homology to ATP-binding proteins resembling ABC transporter proteins from a number of organisms. These included *Mycobacterium leprae* (48.8% identity in 168 amino acids), *Haemophilus influenzae* (43.2% in 169 amino acids) and *E. coli* (39.1% in 169 amino acids). The second open reading frame showed a significant level of homology to mannose-6-phosphate isomerase (*pmi*) genes in other organisms such as *Bacillus subtilis* (34.2% in 313 amino acids), *B. burgdorferi* (25% in 332 amino acids) and *S. typhimurium* (31.1% in 119 amino acids). The termination codon was not found within the insert for the final open reading frame. This partial gene appeared to encode a sorbitol dehydrogenase (L-iditol 2-dehydrogenase) protein. Homology was observed with *E. coli* (30.5% identity in 200 amino acids), *H. influenzae* (28.9% identity in 197 amino acids) and *B. subtilis* (29.4% identity in 187 amino acids). Alignment of amino acid sequences from clone 3a with known protein sequences are shown in Figure 4.7.

4.3.3.3 Hydropathy plots of putative proteins

Chou-Fasman plots showing the probable regions of α-helix, β-sheet and turns and Kyte-Doolittle hydropathy plots were constructed for the genes encoded by clones 1a, 3a and 6a.

Comparison of clone 1a deduced amino acid sequence with ribosomal protein amino acid sequences (Figure 4.6)

ORF1 - hypothetical elongation factor (tuf)

```
                                                                      60
         1
Borrelia TKCVKELLESMDNYFDLPERDIDKPFLLAVEDVFSISGRGTVATGRIERGIIKVGQEVEI
TreponemaAACIEELLAAMDSYFEDPVRDDARPFLLSIEDVYTISGRGTVVTGRIECGVISLNEEVEI
Gene 1a1 ---------------------------MSIEDVYSIPGRGTVVTGRIERGQIKKGDEVEI
SerpulinaCKCILDLLNALDTYIPDPVREVDKDFLMSIEDVYSIPGRGTVVTGRIERGKIEKGNEVEI
                                     :::***::*.*****.***** * *. .:****

                                                                     120
         61
Borrelia VGIKETRKTTVTGVEMFQKILEQGQAGDNVGLLLRGVDKKDIERGQVLSAPGTITPHKKF
TreponemaVGIKPTKKTVVTGIEMFNKLLDQGIAGDNVGLLLRGVDKKEVERGQVLSKPGSIKPHTKF
Gene 1a1 VGLRETKKTTCTGVEMFKKEV-VGIAGYNVGCLLRGIERKEVERGQVLAKPGTITPHKKF
SerpulinaVGIRPTQKTTCTGVEMFKKEV-VGIAGYNVGCLLRGIERKAVERGQVLAKPGTITPHKKF
         **:: *:**. **:***:* :  * ** *** ****:::* :******: **:*.**.**

                                                                     180
         121
Borrelia KASIYCLTKEEGGRHKPFFPGYRPQFFFRTTDVTGVVAL-EGKEMVMPGDNVDIIVELIS
TreponemaEAQIYVLSKEEGGRHSPFFQGYRPQFYFRTTDITGTISLPEGVDMVKPGDNTKIIGELIH
Gene 1a1 EAEVYILKKEEGGRHSGFVSGYRPQMYFRTTDVTGVINLPEGSPMIMPGDNANLL-----
SerpulinaEAEVYILKKEEGGRHSGFVSGYRPQMYFRTTDVTGVINLQGDAQMIMPGDNANLTIELIT
         :*.:* *.*******. *. *****::*****:**.: *  .  *: ****..:
```

ORF2 - hypothetical S10 ribosomal protein

```
                                                                      60
         1
Gene 1a2 -MKEQKIRVKLKAFDIELIDQSAQSIVASVKKTGARVSGPIPLPTSIRKVTVIRSPHVNI
Serpulina-MKEQKIRVKLKAFDIELIDQSAQSIVASVKKTGARVSGPIPLPTSIRKVTVIRSPHVNI
Borrelia MIAKDKIRVRLFSFDVKILDQSAESIVKAVQKAKAQIKGPIPLPTKIKKYTVLRSPHVNK
Treponema-MARERIRVKLCGFDVELVDQSSRAIVHAVQKAGAEVLGPIPLPTRMHKFTVLRSPHVNK
          : .::***:* .**::::***:.:** :*:*: *.: ******* ::* **:******

         61
Gene 1a2 KSREQFEMRVYKRLIDIFDVTPQTTESLKKLALPAGVDVQLK-
SerpulinaKSREQFEMRIYKRLIDIFDVTPQTTESLKKLALPAGVDVQLK-
Borrelia KSREQFEMRTHKRLIDILEPTSALMDSLMKLELPAGVEVDIK-
TreponemaKSREQFEMRTHKRLIDIIEPSQEVMNALMGLELSAGVDVRIKQ
         ********* :******:: :    ::*  * *.***:* :*
```

ORF3 - hypothetical ribosomal protein L3

```
                                                                      60
         1
Borrelia MLGLIGKKVGMTQIFQKNGIVVPVTVIEFQPNYIIGKKTVDRDGYSALIAGSVDLKSSKV
TreponemaMVGLIGQKVGMTQIFDARGCVTPVTVIRVEHNVVVGLKDVERFGYSAVILGTGCMKKSRI
Gene 1a3 MVGIIGRKLGMTTVFDETGNAIAVTVVEAGPCTVMQVRDNEKDGYNAIQLGYGAVKEKHL
         *:*:**:*:*** :*:  * . .***:.     ::  :  :: **.*:  *   :*.::

                                                                     120
         61
Borrelia SKPIKGQYKSLKDIEPKRYVIELKGLDG--YDAGDEIKVDVFKSVKYVDVTGTTKGKGFQ
TreponemaSKPYAGQFA--ERIPPVRVMREFRGFTLD-VSVGQVLDVRVLESVRYLDVCALSKGKGFQ
Gene 1a3 KKPQIGQFKK-ANLEPKKYLKEFRLDDSSAYTVGQELKVDIFQAGDFIDVSSLSKGRGFA
         .**  **:     : * : : *::        .*: :.* ::::  ::** . :**:**

                                                                     180
         121
Borrelia GAMKRHNFSGGPSSHGSKFHRHLGGTGQATTPARTFKGTKMAGRMAGNQQTIQNLEVVLI
TreponemaGVVKRWGFSGGRSSHGSKFHREAGSTGQCTSPGRTFKNVKMPGRMGAERVTVQNLRIERI
Gene 1a3 GVMKRHNYDGGPMSHGSNFRRRAGSIGCNSYPARVWKGKGMPGHMGNTLTTIQNLKVVEI
         *.:** .:.**  ****:*:*. *. *  : *.*.:*.  *.*:*.    *:***.:  *
         181
Borrelia DEEKRALLVKGPVPGAKGSFVVVKKSKK----------
TreponemaDVGLGVVMVRGAVPGRNKATVFLRTAVKRER--------
Gene 1a3 RPEDNLIMIKGSIPGAINGIVKITQAAKKKNKKKNSMTK
               ::::*.:**   . * :  : *
```

ORF4 - hypothetical L4 ribosomal protein

```
                                                                      60
         1
Borrelia MERKVFSKDGKEIGTINLDDRVFNIEISHGSIYNAIKNELSNLRVGTSSTKTRSEVRGSS
TreponemaMEKTVYSVEGVALRSVELDESVFGLSVNRGVIYYAINSELSNKRLGTACTKGRSEVHGSN
Gene 1a4 MEVVILNENGDSVGNLEVVDEIFKSEVNN-LLYEAIKNELANRRQGTHSTKTRAEVSGGG
         **  : . :*  : .::: : :*  .:..  :* **:.**:* * ** .** *:** *..

                                                                     120
         61
Borrelia KKPWKHKGTGRARVGTKRNPVWIGGGIALGPKPRDYSYRLPKKVKKLAFKSVLSLRAADE
TreponemaTKPYKQKGTGRARRGDKKSPLLVGGGTIFGPKPRDFHYALPKKVKRLAMKSLLSLKAQG-
Gene 1a4 KKPWRQKGTGRARAGSTRSPIWVGGGKTHTPKPRDYSYRLPKKMKRKALLSVLSLKYGN-
         .**:::******* * .:.*: :***    *****: * ****:*: *: *:***:  .

                                                                     180
         121
Borrelia NSFKVIENFSVESGKTKDLALIIKNFASFNG-KVVILLGNDDQM------IKRAGKNIRD
TreponemaDALTVIEDFTVESGKTRDLIQVLRHFAQRE--RTVFILQNDDAL------LKRAGRNIPT
Gene 1a4 NVLKVFEDFTFDAPKTKRMASFISKVKEPNSRKVAFVVGKDESLGDNYNKLLLSLRNIKD
         : :.*:*:*:.:: **: :  .: :. . :  :..::: :*: :      :  : :**

         181
Borrelia LKILSFDKLRVVDLFYAKNLIALESAVNKLNEFYIK---------
TreponemaLSFLSYNRLRAHDLFYGRKVLVLETAVHKIADFYRSKDAAQDGTY
Gene 1a4 LKLVNADSMSIHPLYYADEVYFTKTALSKLNARIKG---------
         *.::. : :    *:*. ::   ::*: *:
```

ORF5 - hypothetical L2 ribosomal protein

```
          1                                                          60
Borrelia  MGIKTYKPKTSSLRYKTTLSLDDLSKGNDPLKSKTKGKKFKSGRDSPGRISIRRRGGGHK
Treponema MALKMYRPMTAGLRGRVDLCRAELT-ARTPEKSLTRGKPAKAGRGAGGRISVRHRGGGHK
Gene 1a5  MAIKKFKPTTPSLRYRTVVDFSDIT-TNEPCKSLVCGKKRISGRGSNGRITMRRRGGGHK
          *.:* ::* *..** :. :   :::  . * ** . **   :**.: ***::*:******

          61
Borrelia  RKYRLIDFNRRDKFSIPARVASIEY
Treponema RRYRDIDFKR-DLHDIPGTVKTIEY
Gene 1a5  KLFRLVDFRR-DKHDIEAKVVSIEY
          : :* :**.* * ..* . * :***
```

Figure 4.6: Amino acid sequences of the genes in clone 1a shown as multiple sequence alignments with known sequences from spirochaetes *B. burgdorferi* (**Borrelia**; Genbank accession number U78193), *T. pallidum* (**Treponema**; Genbank accession number AE001202/AE000520) and *S. hyodysenteriae* (**Serpulina**; Genbank accession number U51635). Alignments were generated using the EGCG program ClustalW (Gap opening penalty 10; gap extension penalty 0.05). The symbols used ("*" ":" and ".") were as previously described in figure 4.2.

Comparison of clone 3a derived amino acid sequence with ABC transporter, pmi and sorbitol dehydrogenase amino acid sequences (Figure 4.7)

ORF1 - hypothetical ABC transporter

```
          1                                                          60
Synechocy-------MSEPVQPIIEFRGVSQSFGRKVILDDVDLKIYPGEAVGVIGPSGTGKSTILRI
Gene 3a1 ------------MNIIELKDVYKSFGSQKVLNGVNLIVNRGETLAVIGNSGCGKSVLIKH
M.leprae MAAIGGDGRMPMGVAIEVKGLTKSFGSSRIWEDVTLDIPAGEVSVLLGPSGTGKSVFLKS
Influenz ----------MNQNLIEVKNLTFKRGDRVIYDNLNLQVKKGKITAIMGPSGIGKTTLLKL
                    **.:.:  . *   : :.: * :  *:   ::* ** **:.:::

          61                                                         120
SynechocyVAGLLTPDSGEVIVHG-HRRQRS-IEEGEKALGVGLVFQQSALFDSLTVAENVGFTLYRD
Gene 3a1 LIGLLQPDSGSIIIDGNDINQISDNELTEVRKKFAMVFQGAALFDSLNVYENVSFGLRRI
M.leprae LIGLLRPERGSILIDGTDIIECSAKELYEIRTLFGVLFQDGALFGSMNLYDNTAFPLREH
Influenz IGGQLMPEQGEILFDGQDICRLSNRELYEVRKRMGMLFQSGALFTDISTFDNVAFPIREH
          : * * *: *.::..* .  . *  *  *    ..::** .*** .:.  :*..* : .

          121                                                        180
SynechocySDLRPR-EIRAIVEENLELVGLPGIGDRFPAELSGGMRKRVSLARAIVINPEQHQQYKNI
3a1       KKGMPESKIKVKVAEVLDMVGMPNIEHKMPSELSGGMKKRVGLARAIAMDPE-------I
M.leprae TKKKES-EIRDIVMEKLQLVGLGGDEKKFPGEISGGMRNVPGLARALVLDPQ-------I
Influenz THLPEN-LIRQIVLMKLEAVGLRGAAALMPSELSGGMARRAALARAIALDPD-------L
          .      *:  *   *: **: .    :*.*:**** .  .****:.::*:       :

          181
SynechocyLLYDEPTAGL
3a1       LLYDEPTTGL
M.leprae ILCDEPDSGL
Influenz IMFDEPFTGQ
                ::
```

ORF2 - hypothetical mannose-6-phosphate dehydrogenase

```
                                                                      60
          1
Borrelia  MNNEDNIFLMKNNIKEYDWGGINFIPNLLG-DKIDGKPKAEMWLG-AHKTFSSKILYKN-
Salmonel  MQ-K----LI-NSVQNYAWGSKTALTELYGIANPQQQPMAELWMG-AHPKSSSRITTANG
Bacillus  MTQSP--IFLTPVFKEKIWGG-TALRDRFG-YSIPSESTGECWAISAHPKGPSTVANGPY
3a2       MY----IFEFKEIGKESIWGG-NTLVKSYSKHFDKDKNIGESWEICDLKNDKNIVSNGEF
          *        :    ::  **. . : .  .      :  .* *      .  . :

                                                                     120
          61
Borrelia  EYVLLSDFLEDHK-ELLG---CN--DEFPFLLKVLSANKPLSIQIHPSKDIALKGYESEN
Salmonel  ETVSLRDAIEKNKTAMLGEAVANRFGELPFLFKVLCAAKPLSIQVHPNKRNSEIGFAKEN
Bacillus  KGKTLIELWEEHR-EVFG-GVEG--DRFPLLTKLLDVKEDTSIKVHP--DDY---YAGEN
3a2       KEKDISFLIEHLGEKLLGSNCKNQ-KDFPLLIKFIDAKDKLSIQVHP--DEE----YANK
          :   :    *.    ::*    .    :*:* *.: . .  **::**          ::

                                                                     180
          121
Borrelia  NKGIDINDPKRTYKDKNPKIELIYALSDFYALKGFLPLDEIKKIYEILELNFDFQSH---
Salmonel  AAGIPMDAAERNYKDPNHKPELVFALTPFLAMNAFREFSDIVSLLQPVAGAHSAIAHFLQ
Bacillus  EEGELGKTECWYIIDCKENAEIIYGHT---ARSKTELVTMINSGDWEGLLRRIKIKP---
3a2       KHNKHGKNEMWYIMEADENAKLLIGLKKG-IDKNNLKAAIENKENIEDMFNYFDIKK---
            .   .       : . : ::: . .     .         .

                                                                     240
          181
Borrelia  ---KDFVKTIFD--LQMYELEKIIEKILKNLDLIDNFR-G-YW--FNEIYNIYGIDVGLL
Salmonel  VPNAERLSQLFASLLNMQGEEK--SRALAVLKAALNSQQGEPWQTIRVISEYYPDDSGLF
Bacillus  ---GDFYYVPSG---TLHALCK----GALVLETQQNSD---------ATYRVYDYDR---
3a2       ---GDAFYIPNG---CIHAIMG----NSLIAEIQTPSD---------VTYRLYDWNR---
              :          :              .                  . *  :


                                                                     300
          241
Borrelia  VFLGMNILKLKPGEVVYTNSQEVHAYLKGDCIELMTNSDNVIRAGLTTKYIDKDEMLRVG
Salmonel  SPLLLNVVKLNPGEAMFLFAETPHAYLQGVALEVMANSDNVLRAGLTPKYIDIPELVANV
Bacillus  --LDSNGS---PRELHFAKA-----VN----AATVPHVD-----GYIDESTESRKGITIK
3a2       --VDKNGN---SRELHIEDS-----------FNVIKDIDAFQLKSKKENLLKENNIEINN
            :  *     . *     :              : . *     .   :  .  :

                                                                     360
          301
Borrelia  QFEE---GKLSFLNPDFQDNFSVFRLPNTNLKLIQKKIN--ENICINRNSAMVLLVLNGC
Salmonel  KFEPKPAGEL-LTAPVKSGAELDFPIPVDDFAFSLHDLAL-QETSIGQHSAAILFCVEGE
Bacillus  TFVQ---GEY-------------------FSVYKWDING-EAEMAQDESFLICSVIEGS
3a2       IFSN----EY-------------------FTTEEYLIKDKYTSKTNNKTFEIILVMEGN
           *      :                    :      :          .:  :   ::*

          361
Borrelia  VSINKS---LNLKKGESIFIGKKAENLFIDGDGEAFIAG-FN--
Salmonel  AVLRKDEQRLVLKPGESAFIG--ADESPVNASGTGRLARVYNKL
Bacillus  GLLKYEDKTCPLKKGDHFILPAQMPDFTIKGTCTLIVSHI----
3a2       GTITSDENSIELEAGKTVLLPAYLGEYSIYSEKGIKLLRVTV--
            :  .     *: *.  ::     :  : .     :
```

ORF3 - hypothetical sorbitol dehydrogenase

```
          1                                                          60
E.coli    DPNQEIGLGHECAGTVVAVGS-RVRKFKPGDRVNIEPGVPCGHCRYCLEGKYNICPDVDF
Bacillus  VVEKPFILGHECAGEIAAVGS-SVDQFKVGDRVAVEPGVTCGRCEACKEGRYNLCPDVQF
Influenz  LVTYPRILGHEIVGRVIESGIGMSDGVRVGDRVIVDPYVCCGQCYPCSIGRTNCCESLKV
Gene 3a3  ----------------------------------NDYCNNCYYCNNAKKHLCNNMTA
                                               *..*  *  .: : * .:

          61                                                         120
E.coli    MATQPNYRGALTHYLCHPESFTYKLPDNMDTMEGALVEPAAVGMHAAMLADVKPGKKIII
Bacillus  LATPP-VDGAFVQYIKMRQDFVFLIPDSLSYEEAALIEPFSVGIHAAARTKLQPGSTIAI
Influenz  IGVHI--DGGMQEVIRHPAHLLTKVPDNLPIHQLPLAEPLTIALHALHRTTLKSGEHIVI
Gene 3a3  VGVSL--DGGFAEYVTVKENLVFKVADNVSYESAAMVEPISCCLHGIDLMDIKQGDTVMV
          :..     *.: . :     :   :.*.:   . .: ** :  :*.     :: *. : :

          121                                                        180
E.coli    LGAGCIGLMTLQACKCLGATEIAVVDVLEKRLAMAEQLGATVVINGAKEDTIARCQQFTE
Bacillus  MGMGPVGLMAVAAAKAFGAGTIIVTDLEPLRLEAAKKMGATHIINIREQDALEEIKTITN
Influenz  IGAGAIGLMAALAAVQYGAIPILV-DILEQRLEYAKSLGIEHIVNPHKEDDIKRIKEITS
Gene 3a3  VGAGNIGLMMIQLLKYKGAVNIIAVEPFEKRRERAKKYGANIVIDPIN-DNTEEILKNNN
          :* * :***        **  * . :    *   *:. *   :::  : *   .    ..

          181                                                        240
E.coli    DMGADIVFETAGSAVTVKQAPYLVMRGGKIMIVGTVPGDS----AINFLKINREVTIQTV
Bacillus  DRGVDVAWETAGNPAALQSALASVRRGGKLAIVG-LPSQNEIPLNVPFIADN-EIDIYGI
Influenz  GRMAEVVMEASGANISIKNTLHYASFAGRIALTG-WPKTET-PLPTNLITFK-ELNIYGS
Gene 3a3  IFNIDKVIDCAGKVQTAKYSIKYAGKGAEIMLFG-LTAPDDEVKIKPFEMFQKELTIKTS
              : . : :*   : : :   .  ...: : *  .  .       :   : *: *

          241
E.coli    FRYANRYPVTIEAISSGRFDVKSMVTHIYDYRDVQQAFEESVNNKRDIIKGVIKISD
Bacillus  FRYANTYPKGIEFLASGIVDTKHLVTDQYSLEQTQDAMERALQFKNECLKVMVYPNR
Influenz  RTSKGEFEEALDMLATNKINASHIITKCIKFEEIPSFISDLSDHPENYLKINAVF--
Gene 3a3  FCQSLCF-------------------------------------------------
                :
```

Figure 4.7: Amino acid sequences of the genes in clone 3a shown as multiple sequence alignments with known sequences. For gene 3a1, sequences were *M. leprae* (**M. leprae**; PIR accession number S31144), *H. influenzae* (**Influenz**; PIR accession number B64182) and *Synechocystis* sp. PCC6803 (**Synechocy**; PIR accession number S74597). For the second open reading frame, the sequence was aligned with *B. burdorferi* (**Borrelia**; PIR accession number F70150), *B. subtilis* (**Bacillus**; PIR accession number

A69680) and *S. typhimurium* (**Salmonel**; Swissprot accession number P25081). The third open reading frame was aligned with *H. influenzae* (**Influenz**; PIR accession number Q57517), *B. subtilis* (**Bacillus**; Swissprot accession number Q06004) and *E. coli* (**E.coli**; PIR accession number F64937). Alignments were generated using the EGCG program ClustalW (Gap opening penalty 10; gap extension penalty 0.05). The symbols used ("*" ":" and ".") were as previously described in figure 4.2. Common motifs are indicated by bold type and underlining.

The predicted structure of each protein has been summarized in table 4.1

Table 4.1: Regions of α-helix, β-sheet and turns as predicted by Chou-Fasman plots

Gene	Number of α-helix regions	Number of β-sheet regions	Number of turns	Figure number
1a1	6	8	11	Appendix 5
1a2	4	4	6	Appendix 6
1a3	9	11	14	Appendix 7
1a4	9	7	13	Appendix 8
1a5	3	3	6	Appendix 9
3a1	6	8	9	Appendix 10
3a2	14	16	23	Appendix 11
3a3	8	11	15	Appendix 12
6a1	13	9	20	Appendix 13

4.3.3.5 Western blotting of whole polyclonal serum and affinity purification of polyclonal serum

Western blotting of whole cell proteins with polyclonal serum illustrated the development of an immune response to *S. pilosicoli* isolate P43/6/78^T. Probing of affinity purified serum with whole cell preparations of P43/6/78^T produced reactivity with a number of different proteins in all clones tested. An example is illustrated in Figure 4.8.

Figure 4.8 Immunoreactivity of affinity purified serum (clone 6a). From the right, gels were loaded with samples of prestained marker (1), P43/6/78^{T} (2), three clones of 6a (excised into XLOLR) (3-5), XL1-MRF (6) and XLOLR (7). Molecular weight standards are shown on the right in kDa.

4.4 Discussion

Monoclonal antibody BJL/AC1 reacted with clone SR1 which contained a gene that bears homology to PORs from a number of organisms. The selection of six clones at random which reacted with the polyclonal serum yielded two that produced the same restriction fragment pattern (as described in section 4.2.2.5) and sequencing of the T3 promoter end of one of these clones showed an identical insert. This suggested that it may be a dominant immunogenic protein which would be consistent with its apparent presence in the outer envelope of *S. pilosicoli* as shown by immunogold labelling (Lee and Hampson, 1995). They also observed labelling within the cell and this was likely to be caused by the production of high levels of the enzyme as it is important to metabolic and anabolic pathways. The structure of the protein which was encoded by the insert of SR1, contained a hydrophobic region at between approximately 170-200 amino acids which is consistent with a membrane spanning region. Staphylococcal transferrin binding protein has been identified as the enzyme glyceraldehyde-3-phosphate dehydrogenase suggesting enzymes can be isolated from the cell wall of microorganisms (Modun and Williams, 1998). PORs have previously been identified as membrane bound enzymes such as in the protozoan *Entamoeba histolytica* which causes human amoebiasis (Rodríguez *et al.*, 1998). Organisms such as *E. histolytica* were observed to contain fermentation enzymes such as POR which more closely resembled those from bacteria such as *K. pneumoniae* (49%) and the cyanobacteria *Anabaena* sp. (44%) than the enzymes from other protozoans such as *Giardia lamblia* (Rosenthal *et al.*, 1997). This suggested that the genes were derived from bacteria by horizontal transfer and may imply that bacterial PORs are membrane bound. POR activity has been identified in a large number of bacteria such as, *E. coli* (Genbank accession number Ae015952), *Anabaena* species (Genbank accession number L14925), *K. pneumoniae* (Genbank

accession number X01007), *R. rubrum* (Lindblad *et al.*, 1996), *T. maritima* (Blamey and Adams, 1994), *Thiobacillus ferrooxidans* (Powles and Rawlings, 1997) *Clostridium* species (Moulis *et al.*, 1996), *Campylobacter* species (Mendz *et al.*, 1997; Daucher and Krieg, 1995), *Lactobacillus lactis* (Ramos *et al.*, 1994), *Desulphovibrio africanus* (Pieulle *et al.*, 1997), *H. pylori* (Hughes *et al.*, 1995) and mutants of *Methanosarcina barkeri* (Fusaro) (Bock and Schönheit, 1995). This suggests that *S. pilosicoli* may share a common metabolic pathway with many other bacteria for the metabolism of pyruvate to acetate.

Most PORs consist either of a number of subunits which total 120kDa in size or one or more subunits of 120kDa. Bacteria such as the hyper-thermophilic bacterium *T. maritima* that inhabits hot springs, are generally viewed as ancestral species which evolved when the temperature of Earth was much higher than its current level (Woese, 1987; Woese *et al.*, 1990). These bacteria produce a POR which consists of 4 sub-units (Blamey and Adams, 1994). The α-sub-unit is 43 kDa, β-sub-unit 34 kDa, γ-sub-unit 23 kDa and the δ-sub-unit 13 kDa. Most mesophilic bacteria produce a POR which is a single sub-unit of 120kDa or two identical 120kDa sub-units which are believed to be a fusion of the four sub-unit system used by the Archaea. An exception to this is *H. pylori*, a mesophilic spiral bacterium which colonises the stomach and was shown to exhibit a four sub-unit POR of 43 kDa (α), 34 kDa (β), 23 kDa (γ) and 13 kDa (δ) (Hughes *et al.*, 1995). Similarly, the 2-ketovalerate-ferredoxin oxidoreductase of *Pyrococcus furiosus* with which the ORF of SR1 shows 34% identity, consists of 4 subunits 47 kDa (α), 34 kDa (β), 23 kDa (γ) and 13kDa (δ) (Heider *et al.*, 1996). Since the epitope to which BJL/AC1 reacts was shown to be a 29 kDa protein, and PORs are known to be at least 120 kDa in size, it is possible that *S. pilosicoli* has a 4 subunit POR

similar to that of organisms such as *H. pylori* and *T. maritima*. The level of DNA homology exhibited between the *S. pilosicoli* and *T. maritima* POR was significant and suggested that these organisms may have a close evolutionary relationship. However, further studies would be needed to investigate the phylogenetic descent of these bacteria and to elucidate the evolution of the enzyme.

The consensus sequence for ferredoxin clusters (C- -C- -) was not observed in the derived amino acid sequence of SR1 suggesting that this function may be found in other subunits. Promoter activation allowing POR production (*nifJ* gene) has been observed in *Anabaena* species when in iron depleted media (Bauer and Haselkorn, 1995). It would be of interest for future studies to identify whether the POR in *S. pilosicoli* is surface expressed, and to investigate whether *in vivo* (where conditions are iron limited) POR is regulated by the iron concentration in the media and whether it is involved in iron scavenging as well as metabolic functions. A POR has yet to be isolated from *S. pilosicoli* although there is evidence that a number of species including *S. hyodysenteriae*, *S. pilosicoli* and *S. innocens* metabolise pyruvate to acetate (Trott *et al.*, 1996a).

Clone 6a appears to encode part of a protein which shows significant homology to the structural gene *atsA* in *K. aerogenes* (Murooka *et al.*, 1990) and *P. aeruginosa* (Swissprot accession number P51691) which produces an arylsulphatase enzyme that is involved in the hydrolysis of arylsulphate esters. In addition to these bacteria, an arysulphatase enzyme has been identified in marine bacteria such as *Alteromonas carrageenovora* (Barbeyron *et al.*, 1995) and in pathogenic gastrointestinal bacteria such as *S. typhimurium* (Henderson and Milazzo, 1979) and *C. jejuni* (Yao and Guerry, 1996). The arylsulphatase of *C. jejuni* was identified as a 69kDa protein which did not show similarities to other known arylsulphatases (Yao and Guerry, 1996). In addition,

arylsulphatase has been implicated in the pathogenicity of oral bacteria from the *Campylobacter-Wolinella* family (Wyss, 1989). A homolog of the gene which regulates arylsulphatase expression has been characterised in *Bacteroides thetaiotaomicron* (an anaerobic organism which colonizes the colon). No evidence of arylsulphatase production has been shown in *B. thetaiotaomicron* and results suggested that in this case, the gene regulated production of enzymes which were involved in the utilization pathways of mucopolysaccharides such as heparin and chondroitin sulphate (Cheng *et al.*, 1992). Future studies could investigate whether the arylsulphatase gene fulfills a similar function in intestinal spirochaetes which in the host may be presented with both the mucus layer of the intestinal epithelium but also extracellular matrix components such as heparin sulphate which is a component of the basal lamina.

Clone 1a contained a number of ribosomal and associated proteins which showed a higher degree of similarity than observed for the other genes identified. The elongation factor (the first gene in the cluster) is involved in the binding of amino acyl t-RNA to ribosomes which are translating polypeptides. A common pattern among a number of bacteria is the positioning of the *rpsJ* gene (s10 ribosomal protein) downstream of the *tuf* gene. This arrangement was observed in *N. gonorrhoeae*, *R. prowazekii*, *T. maritima, T. pallidum*, *B. burgdorferi* and *S. hyodysenteriae*. Although the sequence downstream of the *tuf* and *rpsJ* genes has not been identified for *N. gonorrhoeae* and *S. hyodysenteriae*, *T. pallidum* and *B .burgdorferi* showed a similar arrangement of genes to clone 1a. In all cases the gene cluster beginning with the elongation factor (*tuf*) gene was followed by the S10 ribosomal protein, L3 and L4 proteins. The main difference observed was that in the case of *T. pallidum* and *B. burgdorferi*, an additional ribosomal protein gene (L23) was located between the L4 and L2 ribosomal proteins.

Clone 3a encodes two metabolic enzymes and one transporter protein. Both phosphomannose- isomerase (*pmi*) and sorbitol dehydrogenase are metalloproteins. In both cases, the metal ion preferred is zinc. A similar gene arrangement was observed in *Rhodobacter sphaeroides* which encoded an ABC transporter upstream of sorbitol dehydrogenase and mannitol dehydrogenase. Stein *et al.* (1997) suggested that this represented an operon which consisted of all the proteins required for transportation and metabolism of a variety of polyols. The arrangement of the genes observed in insert of *S. pilosicoli* genomic DNA in clone 3a may likewise represent part of an operon involved in the transport and metabolism of substrates such as mannose, which has been shown to be a substrate for *S. pilosicoli*, *S. hyodysenteriae* and *S. innocens* (Stanton, 1997).

Affinity purification produced a profile which showed that the purifed antibody responded to epitopes which appeared to be shared amongst a number of different proteins (including proteins in the *E. coli* strains examined). Shared epitopes have been observed in unrelated antigens of *B. burgdorferi*. A monoclonal antibody to a 93kDa protein was also observed to react with three (and possibly five) different antigens including a potential glyceraldehyde-3-phosphate dehydrogenase (Anda *et al.*, 1994).

In conclusion, immunoscreening an *S. pilosicoli* genomic library with polyclonal and species specific monoclonal antibodies has enabled the identification of a number of genes or gene clusters. Future work should involve the isolation and characterisation of the proteins described and investigations into whether they are virulence determinants.

Chapter 5: Adhesion of intestinal spirochaetes to extracellular matrix proteins

5.1 Introduction

5.1.1 Adhesion of organisms to mucin and extracellular matrix proteins

Mucin is one of the primary barriers preventing adherence of pathogens to mucosal surfaces. *S. hyodysenteriae* and *Campylobacter* species have both been demonstrated to be chemotactically attracted to mucin and L-serine and L-fucose (which are components of mucin), thus aiding movement of the bacteria from the gut lumen towards the mucosal surface (Kennedy and Yancey, 1996; Hugdahl *et al.*, 1988). In some cases, adhesion to mucin may represent the first stage in host colonisation as in *Pseudomonas aeruginosa* which has been found to adhere to immobilised mucin using its flagellar cap protein (encoded by *fliD*) (Arora *et al.*, 1998). The adhesion of *H. pylori* to gastrointestinal epithelial cell was inhibited by the presence of mucin (Simon *et al.*, 1997).

The extracellular matrix (ECM) is found surrounding most cells and, many bacteria possess molecules which can bind its components in addition to specific receptors for host cells. Amongst the most commonly studied ECM proteins are collagen (types I-VI), fibronectin, laminin (or laminin fragments), fibrinogen and vitronectin. Gram-positive and Gram-negative bacteria have been shown to bind ECM proteins and one of the most well characterised of these is *Staphylococcus aureus* which has been shown to reversibly bind to collagen (type II) (Speziale *et al.*, 1986) and fibronectin (Switalski *et al.*, 1983). Although binding of fibronectin did not affect adhesion *in vivo* (Flock *et al.*, 1996), mutants with a low fibronectin binding

phenotype, showed a reduction in the number of bacteria adhering to heart valves (Kuypers *et al.*, 1989).

E. coli is one of the most well defined Gram-negative bacteria in respect to adherence to ECM proteins and has been found to bind collagen (types I-VI), fibronectin and laminin using a number of different adhesins (Patti *et al.*, 1994). *Yersinia enterocolitica* adheres at the submucosal layer of the epithelium and has an outer membrane protein named YadA. This protein was found to be multi-functional, binding laminin (Skernik *et al.*, 1994) as well as collagen (types I-VI) and fibronectin (Schulze-Koops *et al.*, 1993). YadA possessed a hydrophobic region at the amino end (22 amino acids) and at the carboxy end. Removal of the hydrophobic region at the amino end affected the ability of bacteria to bind collagen and autoagglutinate, whereas bacteria lacking the carboxy hydrophobic region, were affected in transport of the protein to the outer membrane (Tamm *et al.*, 1993). N-terminally truncated mutants of YadA (lacking the amino hydrophobic region) lost the ability to adhere to polymorphonuclear leukocytes and induce oxidative burst, in addition to being attenuated *in vivo* (Roggenkamp *et al.*, 1996). *H. pylori* has also been demonstrated to have a high affinity for collagen (type IV) and laminin (higher than the *Campylobacter* species tested) and low level affinity for collagen (types I and II), vitronectin and fibronectin (Trust *et al.*, 1991).

A number of spirochaetes have been reported to adhere to ECM proteins. *T. denticola* has been demonstrated to bind immobilised fibronectin and laminin in a tip-orientated way but did not adhere to collagen (type IV) (Dawson and Ellen, 1990). The adhesion of *T. denticola* to both soluble and insoluble fibronectin has been confirmed (Haapasalo *et al.*, 1991) and after binding, *T. denticola* was shown to digest the fibronectin (Ellen *et al.*, 1994). The major surface protein complex has been

identified as consisting of a 53kDa protein which binds fibronectin and laminin (Haapalso *et al.*, 1992). *Borrelia garinii* has been demonstrated to bind fibronectin specifically and this reaction was shown to be saturable (Kopp *et al.*, 1995).

5.1.2 ELISA assay for bacterial adhesion to extacellular matrix proteins

One of the main approaches in the study of bacterial adhesion to ECM proteins is to examine their binding to immobilised proteins. The binding of *T. denticola* to fibronectin coated coverslips (Dawson and Ellen, 1990) and *P. aeruginosa* to mucin (which was assayed by viable counts after removal of adherent bacteria using a detergent) (Arora *et al.*, 1998) have been studied using this approach. ELISA type assays for adhesion using two different methodologies have been developed. The immobilisation of bacteria on microtiter plates followed by incubation with ECM proteins and development with anti-ECM protein antibodies has been performed successfully with *H. pylori* (Trust *et al.*, 1991). An alternative method involved immobilisation of the ECM protein on microtiter plates which were then incubated with whole bacteria or outer membrane preparations followed by immunological detection (Sperandio *et al.*, 1995) or bacterial staining (van der Flier *et al.*, 1995).

5.1.3 Aims

The objective of this part of the project was to investigate potential adhesion of intestinal spirochaetes to extracellular molecules. This involved the probing of intestinal spirochaete DNA with oligonucleotides based on known adhesin sequences of spirochaetes and gastrointestinal bacteria. In addition, an ELISA type assay was developed to investigate the adhesion of intestinal spirochaetes to extracellular matrix proteins.

5.2 Materials and Methods

5.2.1 Probing of Southern blots with oligonucleotide probes

5.2.1.1 Southern blotting of pulsed field gel electrophoresis gels

After PFGE gels had been electrophoresed as described in section 2.2.4.3, stained with ethidium bromide and destained for 1 hour (section 2.2.4.3), the gels were exposed to UV light (wavelength 254nm) using a UV transilluminator (UVP Inc) for 3 minutes. Gels were then incubated in denaturing solution (0.5M NaOH; 0.5M NaCl) at 22-25°C for 1 hour in the dark with gentle agitation. Denaturing solution was replaced with neutralization solution (1.5M NaCl; 0.5M Tris-HCl pH7.5) for 1 hour at 22-25°C in dark conditions with gentle agitation. A blot was then set up as follows. A sponge was placed in a plastic box containing 15x SSC (20x SSC stock solution: 3M NaCl, 0.3M tri-sodium citrate pH7.0). Six pieces of Whatman 3MM paper and positively charged nylon membrane (Boehringer Mannheim) were trimmed to the size of the pulsed field gel and soaked in 15x SSC. Three of these were placed on the sponge. The pulsed field gel was then removed from the neutralization solution and placed on top of the Whatman paper and sponge. The nylon membrane was placed on top of the gel and the remaining pieces of Whatman 3MM paper were positioned on top of the membrane. A number of paper towels were then added to the top of the Whatman 3MM paper and finally, a heavy weight was placed on the top. The blot was incubated at 22-25°C for 16-18 hours (to allow transfer of DNA by capillary action) then dismantled and the DNA fixed to the membrane by exposing the membrane to UV light (wavelength 254nm). Membranes were stored at 22-25°C until required for probing.

5.2.1.2 Design of oligonucleotide probes

Sequences were selected from two genes. Two probes were synthesised from YadA in *Yersinia enterocolitica* (Genbank accession no. X133881) and one probe synthesised from the gene BB0347 (a fibronectin binding protein) from *Borrelia burgdorferi* (Fraser *et al.*, 1997; genbank accession no. AE001141). The sequences of the probes were:

YADA1 5' - GCG CTG GTT CAA TTG CAA CAG G - 3'

YADA2 5' - GTT AAT TCT GTT GCA ATT GGT C - 3'

BB0347 5' -AGT TTG GTT TTA GAG ATT TAC AAT AAA - 3'

Oligonucleotide probes were synthesised by PE-Applied Biosystems UK. They were supplied lyophilised and were resuspended in sufficient sterile deionised water to ensure that all oligonucleotides were at a concentration of 100pmol in = 9µl.

5.2.1.3 DIG-labelling of oligonucleotide probes

A DIG oligonucleotide tailing kit (Boehinger Mannheim) was used to set up the following reaction at 4°C:

4µl tailing buffer (0.125M Tris-HCl, 1.25mg/ml bovine serum albumin, pH6.6)

4µl $CoCl_2$ solution (25mM)

100pmol of oligonucleotide or control unlabelled oligonucleotide (5µl of 20pmol/µl 30'mer in water)

1µl DIG-dUTP solution (1mM DIG-dUTP in water)

1µl dATP solution (10mM dATP in Tris buffer pH7.5)

1µl terminal transferase (50 units/µl in 0.2M Potassium cacodylate, 1mM EDTA, 200mM KCl, 0.2mg/ml bovine serum albumin, pH6.5, and 50% glycerol)

The volume was made up to 20µl with sterile deionised water.

The reaction was incubated at 37°C for 15 minutes then terminated by the addition of 2µl of a termination mixture made by the addition of 1µl of glycogen (20mg/ml in water) to 200µl of 0.2M EDTA pH8.0. The labelled oligonucleotide was precipitated by the addition of 2.5µl of 4M LiCl and 75µl 100% ethanol prechilled to -20°C. The mixture was incubated at -70°C for 30 minutes or at -20°C for 2 hours prior to centrifugation (13000rpm, Eppendorf 5415C centrifuge, 15 minutes). The supernatant was removed and the pellet washed with 50µl of 70% ethanol chilled to -20°C. The pellet was then dried using a speedvac SVC1000 (Stratech Scientific) and dissolved in 10µl of sterile deionised water.

5.2.1.4 Evaluation of tailing efficiency

The labelled oligonucleotides were diluted to 2.5pmol/µl in dilution buffer DNA (Herring sperm DNA 50µg/ml, in 10mM Tris-HCl, 1mM EDTA, pH8.0) based on the assumption that all of the oligonucleotide was retained and labelled. A series of tenfold dilutions (50µl sample volume where possible) were made from the sample and control oligonucleotides together with the control-tailed DNA (2.5pmol/µl of tailed 30'mer, provided by manufacturer). 1µl of each dilution was spotted onto the test strips provided and allowed to air dry. The strips were then subjected to immunological detection (section 5.2.1.5).

5.2.1.5 Immunological detection

Membranes were equilibrated with maleic acid buffer (1M maleic acid, 0.15M NaCl pH 7.5) for 1-5 minutes at 22-25°C. The buffer was removed and replaced with blocking solution (10x stock blocking solution consists of 10% (w/v) blocking reagent in maleic acid buffer: diluted to 1x in 1/10 dilution of maleic acid buffer) with which the membrane was incubated for 30 minutes at 22-25°C with gentle agitation. This was replaced with blocking solution containing 75mU/ml anti-DIG-alkaline phosphatase conjugate antibody. Membranes were incubated in the conjugate solution for 30 minutes at 22-25°C with gentle agitation. They were then washed twice in maleic acid buffer for 15 minutes. For development, membranes were first equilibrated in detection buffer (1M Tris-HCl, 0.1M NaCl, 50mM $MgCl_2$, pH9.5) for 2-5 minutes. Colour development was achieved by incubation of the membranes using colour substrate solution (detection buffer supplemented with 337.5µg/ml NBT and 175µg/ml of X-phosphate solution) in dark conditions.

5.2.1.6 Hybridisation of Southern blot membranes

Membranes were prehybridised in hybridisation solution (5xSSC,1/10 dilution of 10x stock blocking solution, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS) for at least 1 hour at the hybridisation temperature. This was replaced by 2.5ml hybridisation solution supplemented with the tailed oligonucleotide and 10µl/ml of Poly-A solution. Oligonucleotide was used at 10pmol/ml of hybridisation solution. Membranes were incubated with the probe for 6 hours redistributing the hybridisation solution at regular intervals. The hybridisation temperature used was determined as 5-10°C lower than the T_m value of the probe. Membranes were then washed twice in 2xSSC, 0.1% (w/v) SDS for 5 minutes at the hybridisation temperature followed by 2

washes in 0.1xSSC, 0.1% (w/v) SDS for 5 minutes at the hybridisation temperature. They were then developed as described in section 5.2.1.5.

For each blot a control reaction was also performed. A dilution series of control DNA (0.25mg/ml supercoiled pUC18 DNA in 10mM Tris-HCl pH7.6, 1mM EDTA) was made in DNA dilution buffer between 50pg/μl and 0.25pg/μl and DNA was denatured by heating at 95°C for 10 minutes followed by cooling at 4°C. Positively charged nylon membrane (Boehringer Mannheim) was loaded with 1μl spots of the diluted control DNA. After air drying, the DNA was bound to the membrane by crosslinking with a UV transilluminator at a wavelength of 254nm for 3 minutes.

5.2.2. ELISA assay

5.2.2.1 Plate coating

Collagen (Collaborative Biomedical Products) was diluted to 50μg/ml in 0.02M acetic acid. Fibronectin (Collaborative Biomedical Products) and Laminin (Collaborative Biomedical Products) were diluted to 50μg/ml in DMEM. Stock solutions of mucin was diluted to 50mg/ml in coating buffer (50mM sodium carbonate adjusted to pH9.6 with 50mM sodium bicarbonate solution). Each solution (100μl) was put into the first row of an Immulon-2 96-well plate (Dynatech). Serial 2-fold dilutions were made in each subsequent row of the plate using coating buffer. The final row was left blank so that once developed, the optical density measured in the blank row was taken as the background. Plates were incubated in the coating mixture for 16-18 hours at 4°C and were then washed three times in TBS-Tween. Following incubation for 16-18 hours at

4°C in TBS-Tween (to block the unbound areas of the plate), plates were stored at -20°C prior to use.

5.2.2.2 Bacterial adhesion to plates

Pure spirochaete broth cultures were centrifuged at 3000rpm (Beckman J2-20; J-25.50 rotor) for 10 minutes, resuspended in 1ml PBS and recentrifuged (13000rpm, Eppendorf 5415C, 5 minutes). They were resuspended to $1x10^8$ spirochaetes/ml in PBS and 100µl added to the plates so that each isolate tested was represented at each dilution. The plates were then incubated at 37°C for 1 hour to allow adhesion of the spirochaetes. Plates were then washed three times in TBS-Tween to remove excess spirochaetes.

5.2.2.3 Development ELISA assay

Plates were incubated with polyclonal serum (section 4.2.3.1) which recognised bound spirochaetes at the optimal dilution (1/32) for 16-18 hours at 4°C. The optimal dilution was determined by coating ELISA plates with intestinal spirochaetes (as described in section 5.2.2.1) and varying the dilution of polyclonal serum used. To determine the level of polyclonal serum binding to plates, one column was developed which had not been treated with spirochaetes. This optical density was then subtracted from the values of the experimental wells. Plates were washed three times in TBS-Tween to remove excess antibody and binding of the polyclonal serum was visualised by the addition of a Protein A-Horseradish peroxidase conjugate (0.25µg/ml) for 3 hours at 4°C. Plates were then washed three times in TBS-Tween and excess removed by repeated blotting. The substrate buffer was freshly prepared: 10mg of tetramethylbenzidine was dissolved in 1ml of DMSO. 100ml of acetate/citrate buffer

(0.1M sodium acetate adjusted to pH 6.0 with citric acid) was added followed by 20µl of H_2O_2. 100µl of this substrate buffer was added to each well of the plate and incubated at 22-25°C for approximately 5 minutes or until a blue coloration developed in the wells. 100µl of 2M H_2SO_4 was then added to each well which changes the colour reaction from blue to yellow. The optical density was measured using an Anthos reader 2001 (Labtec) at 450nm.

5.3 Results

5.3.1 Southern blotting

The oligonucleotide probes described in section 5.2.1.2 were DIG-labelled and used in hybridisation reactions with southern blots of human intestinal spirochaetes. The labelled probes did not hybridise to the human intestinal spirochaete genomic DNA. A control reaction was performed with each sample. The probe for the control reaction hybridised to the supercoiled pUC18 DNA which was visualised after colour development.

5.3.2 ELISA assay

5.3.2.1 Optimisation of polyclonal antibody dilution

The optimum dilution of polyclonal serum (section 4.2.3.1) was determined to be 1/32 as can be seen in figure 5.1. Isolate PE90 was used to characterise the optimum dilution for polyclonal serum. PE90 was selected as a representative isolate of *S. pilosicoli* which was found within the largest cluster of isolates (1b) of Figure 2.4 (dendrogram of relatedness as determined by PFGE).

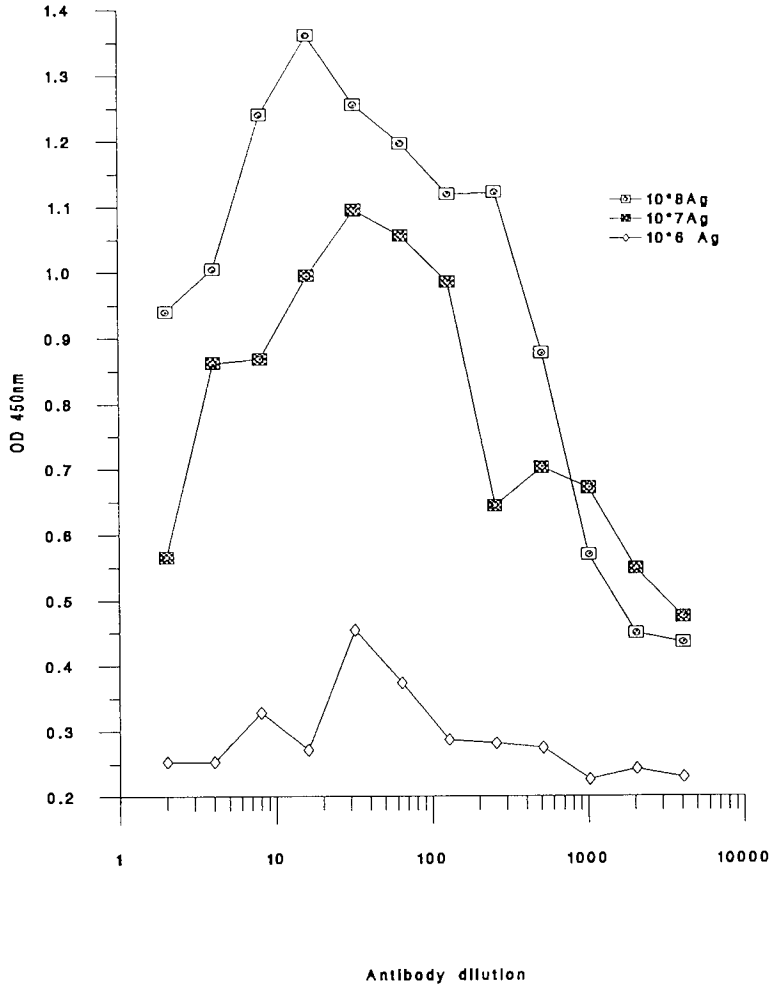


Figure 5.1: Graph illustrating the OD at 450nm of 2-fold antibody dilutions at different antigen (spirochaete) concentrations. 10*8/10*7/10*6 spirochaetes represents 10^8, 10^7 and 10^6spirochaetes. Optimal dilution was 1/16 (10^8 spirochaetes) and 1/32 at both 10^7 and 10^6 spirochaetes.

5.3.2.2 Adhesion of intestinal spirochaetes to immobilised collagen

The adherence of intestinal spirochaetes to collagen type 1 was tested at concentrations from 50μg/ml to 0.3μg/ml which included the 1μg/ml used to examine adherence by *V. cholerae* outer membrane preparations (Sperandio *et al.*, 1995). An example of the results observed can be seen in Figure 5.2. All isolates tested adhered to collagen type 1, although no trend was observed.

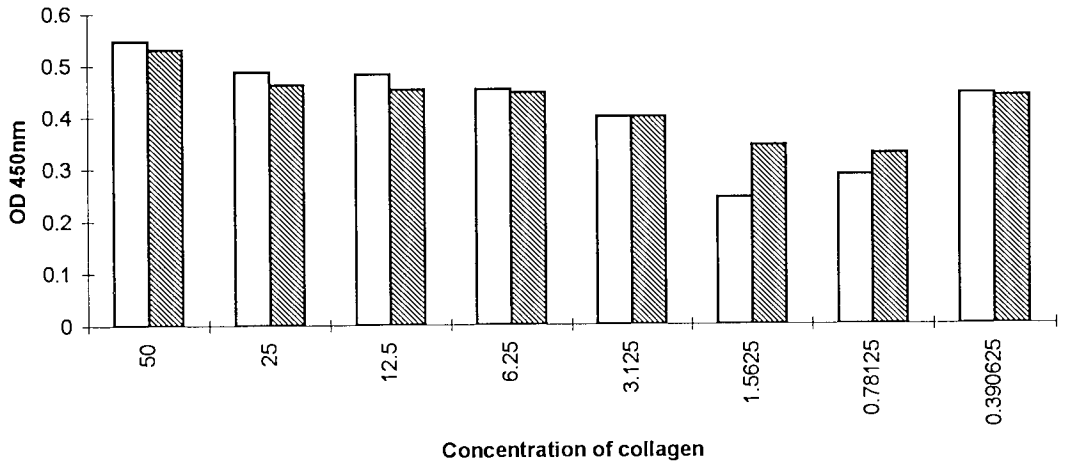


Figure 5.2: Adherence of intestinal spirochaete isolate 60 to immobilised collagen (in duplicate). The concentration of collagen is shown in μg/ml. Duplicate samples are shown for each concentration.

5.3.2.3 Adhesion of intestinal spirochaetes to immobilised fibronectin

All intestinal spirochaete isolates tested adhered to immobilised fibronectin. Binding to fibronectin was dependent on concentration and appeared to be saturable. An example of intestinal spirochaete binding to fibronectin is illustrated in Figure 5.3. Adhesion was tested at concentrations ranging between 50μg/ml and 2.4ng/ml.

Previous studies have observed that at 50μg/ml fibronectin forms a multi-layer (van der Flier *et al.*, 1995). The range of fibronectin concentrations used in this study were within the range used by van der Flier (1995) and Sperandio *et al.* (1μg/ml) (1995).

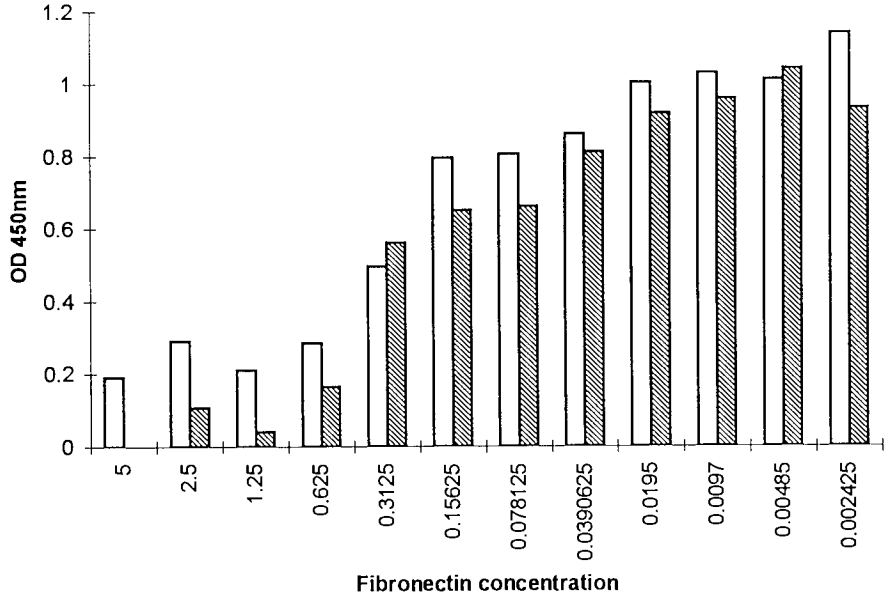


Figure 5.3: Adhesion of intestinal spirochaete isolate PE90 (in duplicate) to immobilised fibronectin. The fibronectin concentration is shown in μg/ml. Duplicate samples are shown for each concentration.

5.3.2.4 Adhesion of intestinal spirochaetes to immobilised laminin

Immobilised laminin was tested at concentrations ranging between 50μg/ml and 2.4ng/ml which encompassed 1μg/ml (the concentration used by Sperandio *et al.*, 1995). All isolates tested showed adhesion to laminin. An example of this can be seen in Figure 5.4.

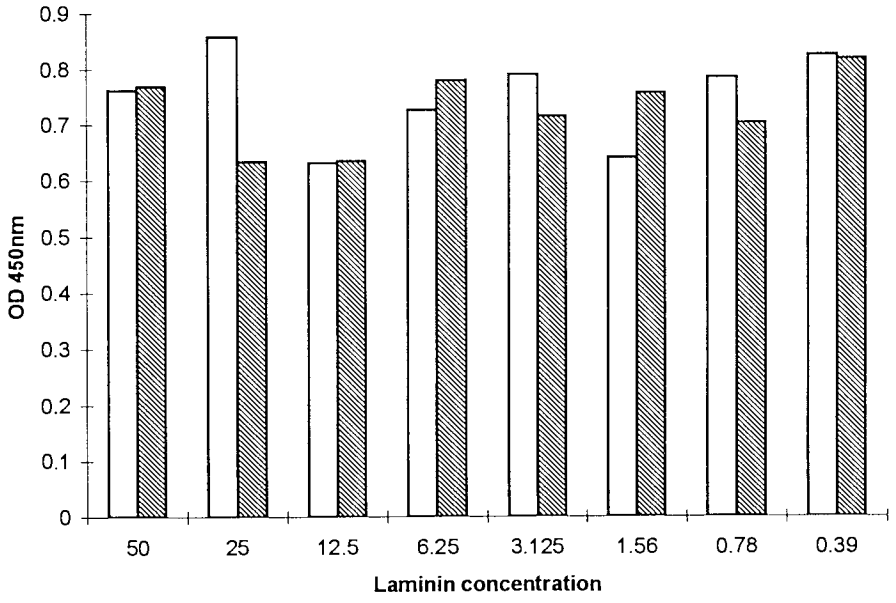


Figure 5.4: Adhesion of intestinal spirochaete isolate RA87 to immobilised laminin. Laminin concentration is shown in μg/ml. Duplicate samples are shown for each concentration.

Although intestinal spirochaetes appeared to bind to laminin, no trends were observed in the level of binding as the concentration of laminin was decreased.

5.3.2.5 Adhesion of intestinal spirochaetes to porcine gastric mucin

All isolates tested adhered to pig gastric mucin at concentrations of 5mg/ml to 0.97μg/ml. Figure 5.5 shows an example of the adherence of intesinal spirochaetes to mucin which was used at a range of concentrations which included the 50μg/ml of mucin used to study the adhesion of *P. aeruginosa* (Arora *et al.*, 1998).

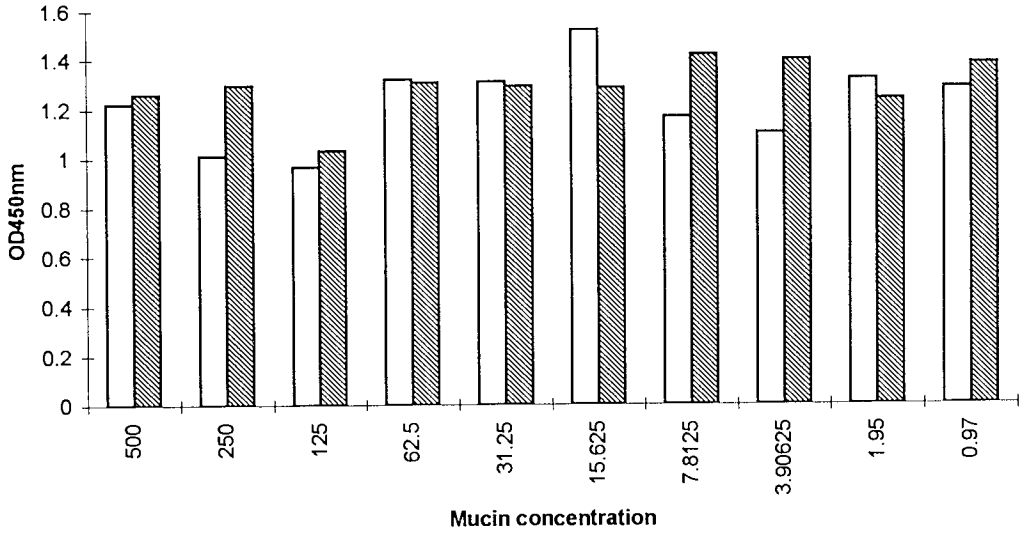


Figure 5.5: Adhesion of intestinal spirochaete isolate 128/90 to porcine gastric mucin (in duplicate). Concentration of mucin is shown in μg/ml. Duplicate samples are shown for each concentration.

Isolates showed high optical density (bacterial adhesion) to mucin at all concentrations used.

5.4 Discussion

The YadA and *B. burgdorferi* oligonucleotide probes did not adhere to genomic DNA from human intestinal spirochaetes. The YadA sequence used corresponded to the region of sequence which is known to be involved in collagen binding (Tamm *et al*, 1993; Roggenkamp *et al.*, 1996). The ELISA results suggested that intestinal spirochaetes may adhere to type 1 rat tail collagen, but lack of recognition of the YadA probe suggests that either intestinal spirochaetes do not adhere to the same epitope on the collagen molecule, or that the protein is encoded by a markedly different DNA sequence. No hybridisation of *S. pilosicoli* DNA to YadA sequences (or probes derived from other genes involved in the adhesion and invasion of enterobacteria) has been observed by Southern blotting under low stringency conditions (Hartland *et al.*, 1998).

The ELISA assay indicated that intestinal spirochaetes were able to adhere to fibronectin although adhesion increased as the concentration of fibronectin decreased. At a concentration of 50μg/ml, fibronectin forms a multiple layer and the interaction between the molecules results in conformational changes (van der Flier, 1995). The results obtained suggested that binding may occur at sites which are not as frequently exposed when the fibronectin is arranged as a multilayer.

Intestinal spirochaetes were found to bind laminin, in addition to collagen and fibronectin. All intestinal spirochaetes tested also adhered at high levels to porcine gastric mucin at each concentration tested. It is possible that this level of adhesion was observed because intestinal spirochaetes are chemotactically attracted to mucin. Chemotaxis to mucin and its components has been described for *S. hyodysenteriae* although chemotaxis was not observed in the one *S. pilosicoli* isolate tested (P43/6/78^{T}) (Kennedy and Yancey, 1996). The interaction of intestinal spirochaetes

with mucin may be the initial stage in colonisation of the epithelial surface, as described for *P. aeruginosa* (Arora *et al.*, 1998).

Whilst this study indicates that intestinal spirochaetes are able to adhere to ECM proteins, further work is required to investigate the nature of these interactions and its effect on infection. Future work could identify proteins which bind ECM components by Western blotting such as vitronectin binding of *Pneumocystis carinii* (Limper *et al.*, 1993) and laminin binding of *Leishmania donovani* (Ghosh *et al.*, 1996) as well as examine whether intestinal spirochaetes are able to digest the ECM proteins as described for a number of bacteria such as *Porphyromonas gingivalis* (Pike *et al.*, 1996) and *T. denticola* (Ellen *et al.*, 1994). Although adhesion of *S. pilosicoli* to gastrointestinal epithelial cells has been demonstrated (Chapter 3), adhesion to mucin may also be important for initial colonisation of the intestine as suggested for *Campylobacter* species (Lee *et al.*, 1986).

In conclusion, intestinal spirochaetes have been demonstrated to adhere to ECM proteins and mucin, supporting the hypothesis that mucin may be an important factor in the adhesion of intestinal spirochaetes to intestinal cells (Chapter 3). This is a characteristic which has been observed in other spirochaetes such as *T. denticola* and *B. garinii* which both adhere to fibronectin (Dawson and Ellen, 1990; Haapalso et al., 1992). Future experiments could use a modified western blotting technique (as described by Limper *et al.*, 1993 and Ghosh *et al.*, 1996) to identify bacterial proteins to which ECM proteins adhere. In addition, the cell culture models described (Chapter 3) could be treated with ECM proteins or mucin to investigate their effect on adhesion.

Chapter 6 : Concluding remarks

This study has enabled the identification of a large number of human and animal intestinal spirochaete isolates using species specific monoclonal antibodies. Generation of an expression gene library has enabled the identification of a number of proteins from *S. pilosicoli* by comparison with known DNA sequences which has given insight into both the biochemistry of intestinal spirochaetes and also the molecular arrangement of genes. A cell culture model was used for the study of adherence of intestinal spirochaetes to intestinal epithelial cells. *S. pilosicoli* has also been shown to be able to adhere to extracellular matrix proteins.

Characterisation of isolates by pulsed field gel electrophoresis has demonstrated that it was a suitable method for the specific differentiation of intestinal spirochaetes and shown a number of genotypic characteristics which have also been observed in typing methods such as multi-locus enzyme electrophoresis which are based on phenotype. These are the diversity exhibited by isolates within a single species and the possibility of cross-species transmission. This study has confirmed the observation made by MEE studies that certain bacteraemic isolates do not appear to be closely related suggesting that their invasive property may not be correlated to currently identified phenotypic or genotypic characteristics. The invasion assay used in this study confirmed that these isolates were not especially adapted for invasion by comparison with other *S. pilosicoli* isolates. Adherence of intestinal spirochaetes to the Caco-2 cells showed one isolate to be of significant interest. This isolate (designated 382/91) was obtained from the blood of a seriously ill patient in France. 382/91 was significantly different based on pulsed field gel electrophoresis (to the degree that it was omitted from the dendrograms generated) and adhered to the Caco-2 cells at significantly higher levels than any other isolate tested (including the other bacteraemic

isolates). During the invasion assays, no penetration of the epithelial layer was observed by the intracellular route suggesting that it was achieved by the paracellular route. This contrasts with the literature where there have been several reports of intracellular bacteria (Gebbers *et al.*, 1987; Rodgers *et al.*, 1986, Antonakopoulos *et al.*, 1982). The Caco-2 model does not produce a mucus barrier as observed *in vivo*. HT29 is a goblet cell line and further investigations could pursue invasion studies using co-culture of Caco-2 cells and differentiated HT29 cells to produce a differentiated cell layer with a covering of mucus. In addition preliminary work has suggested that intestinal spirochaetes may be suitable for use in the organ culture system used to investigate *S. typhimurium* adhesion to rabbit ileal tissue (Worton *et al.*, 1989; Amin *et al.*, 1994) .

The ability of intestinal spirochaetes to adhere to extracellular matrix proteins such as laminin, fibronectin and collagen which are found in the basal lamina or surrounding tissues has been demonstrated. It would be of interest to investigate whether intestinal spirochaetes are also able to digest these components as observed in other spirochaetes (such as *T. denticola*, Ellen *et al.*, 1994). Adherence to the extracellular matrix (including the basal lamina) and digestion of their components could enable intestinal spirochaetes to invade the host epithelium by the paracellular route.

The generation of a random genomic library of *S. pilosicoli* has facilitated the identification of a number of previously uncharacterised genes. These included genes for a pyruvate oxidoreductase which resembled the ancestral form found in bacteria such as *T. maritima* and *H. pylori*, an ABC transporter, sorbitol dehydrogenase, mannose-6-phosphatase isomerase and a cluster of ribosomal proteins. The molecular arrangement of the ribosomal protein cluster was similar to that observed in other

spirochaetes such as *B. burgdorferi* and *T. pallidum* as well as other organisms such as *T. maritima*. Identification of a cluster of genes encoding an ABC-transporter, sorbitol dehydrogenase and mannose-6-phosphate isomerase may indicate the presence of an operon which is involved in uptake and metabolism. Having generated a representative random genomic library it may be possible to use this library to investigate potential adhesins using the Caco-2 cell model. Previous work has shown that the *E. coli* strains used for screening lambda libraries are unable to adhere to Caco-2 cells which were then used to screen the library for factors involved in the adhesion of *C. difficile* (Karjalainen *et al.*, 1994). If positive clones can be identified using this method, it would also enable the examination of factors which enhance or inhibit adhesion (such as ECM proteins) and to identify whether more than one factor is involved in adherence.

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Appendix 1

1 - CGTCCCGAAA GTACAGTGGA ATGGGTGCTA ATAGTGCTGG TAAAATGCTT -50
GCAGGGCTTT CATGTCACCT TACCCACGAT TATCACGACC ATTTTACGAA

51 - GCAGAAGTAG GCGTCTTAAC ACAAGGTTTC TATGGTGCTG CATTCTCAAG -100
CGT CTTCATC CGCAGAATTG TGTTCCAAAG ATACCACGAC GTAAGAGTTC

101 - TTATGGTTCA GAAAAGAAAGGTTCTCCAGT AAAATCTTTT GTAAGGTTTT -150
AATACCAAGT CTTTTCTTTC CAAGAGGTCA TTTTAGAAAA CATTCCAAAA

151 - CAGAAACTGA AGTTAGAGTA AACTCTACTG TAGAAGAACC ACATGTAGTG -200
GTCTTTGACT TCA ATCTCAT TTGAGATGAC ATCTTCTTGG TGTACATCAC

201 - GCAGTGTTCC ACATGAATCT TCTAAAAAAC CCTCTTACAT TAGCTGGTGT -250
CGTCACAAGG TGTACTTAGA AGTTTTTTTG GGAGAATGTA ATCGACCACA

251 - TAAATAAGAT GCTATTGTTA TTTTTAACAC TAATAAAACA CCTGATGAGG -300
ATTTATTCTA CGATAACAAT AAAAATTGTG ATTATTTTGT GGACTACTCC

301 - CAAGAGATTT TGCTAAATTA CATGGCGGTA AAGTAGTTTG TATTGATGCA -350
GTTCTCTAAA ACGATTTAAT GTACCGCCAT TTCATCAAAC ATAACTACGT

351 - ATAAAAATCG CTAATGATTT AAAACTTCCT TCACAAGCTG CTAATACTAT -400
TATTTTTAGC GATTACTAAA TTTTGAAGGA AGTGTTCGAC GATTATGATA

401 - CATAATGGGT GCTATGGTTA ATCAGCTTGA TTTCATAGAT TCTGCTAAGT -450
GTATTACCCA CGATACCAAT TACTCGAACT AAAGTATCTA AGACGATTCA

451 - TTGAAGAGCA AATCAGAAAA CAATTCGGCG GAAGAAAAGCTGAATTAGTT -500
AACTTCTCGT TTAGTCTTTT GTTAAGCCGC CTTCTTTTCG ACTTAATCAA

501 - GAGCCAAATA TTGAAGCTTT CAGAAAAGGC GGAAGTGAAT CTGTTGTTAA -550
CTC GGTTTAT AACTTCGAAA GTC TTTTCCG CCTTCACTTA GACAACAATT

551 - AGAATTCAAA GCTGACGGAA AATATCCTTA TATTCCTTAT AAAAAACCAG -600
TCTTAAGTTT CGACTGCCTT TTATAGGAAT ATAAGGAATA TTTTTTGGTC

601 - AACCTGTATA TGGTAAAAAT AACCAATTAA CAGGCGGTTA TATCAATGCT -650

TTGGACATAT ACCATTTTTA TTGGTTAATT GTCCGCCAAT ATAGTTACGA

651 - GCTGGTAACT CTACTTTAAA AGATTTACAA GTTACTCGTA CTGGTAATAT -700
CGACCATTGA GATGAAATTT TCTAAATGTT CAATGAGCAT GACCATTATA

701 - ACCTGTATTC AATCCTGCTA ACTGTATTGA TTGTGCTAAC TGTGAAGTTG -750
TGGACATAAG TTAGGACGAT TGACATAACT AACACGATTG ACACTTCAAC

751 - TTTGTCCTGA CCTTTGTATC GTTTGGGAAA AAGGACCAGA CAGGAAAGAC -800
AAACAGGACT GGAAACATAG CAAACCCTTT TTCCTGGTCT GTCCTTTCTG

801 - CCTAATAAAA CTGCTATGAA TATGATGGGT ATAGATTATC AATACTGTAA -850
GGATTATTTT GACGATACTT ATACTACCCA TATCTAATAG TTATGACATT

851 - AGGTTGTTTA AAATGTGTTA GAGCTTGCCC TAAAGGACCT TATTCTGGTA -900
TCCAACAAAT TTTACACAAT CTCGAACGGG ATTTCCTGGA ATAAGACCAT

901 - AATTTGAAAA AGATCAGCAG GCTTTAAGAA TAGAAGTAGA AGCTAATTGT -950
TTAAACTTTT TCTAGTCGTC CGAAATTCTT ATCTTCATCT TCGATTAACA

951 - GATGTTGATA AACTTACATA CAGACGTTAT AAAAAATAAT AAGGAGCGTA-1000
CTACAACTAT TTGAATGTAT GTCTGCAATA TTTTTTATTA TTCCTCGCAT

1001 - TAAATGGCAC AAAAATATAA TCTTGCTGAG CAAGAAAACA TACTTGAAAG -1050
ATTTACCGTG TTTTTATATT AGAACGACTC GTTCTTTTGT ATGAACTTTC

1051 - TGGTAATGAA TTAGCAGCCA TTGCAGCCGC TCAAATCAAT TATCACGTTA -1100
ACCATTACTT AATCGTCGGT AACGTCGGCG AGTTTAGTTA ATAGTGCAAT
-35 -10 ⇓+1
1101 - **TGGGTT**ACTACCCAATCACT CCA**TCTACT**C AAATTGCT**GA GTAC**TTAGAT -1150
ACCCAATGAT GGGTTAGTGA GGTAGATGAG TTTAACGACT CATGAATCTA

1151 - GAA**ATG**AAAGCTAATGGCAG ACATACTGTT TGCATGATTC CTGGTGACGG -1200
CTTTACTTTC GATTACCGTC TGTATGACAA ACGTACTAAG GACCACTGCC

1201 - TGAACATGGT GCAGCTGGTA TCTGTTATGG TGCTACTACT GCAGGAGGAA-1250
ACTTGTACCA CGTCGACCAT AGACAATACC ACGATGATGA CGTCCTCCTT

1251 - GAGTATTCAA TGCTACTTCT GCTAACGGTT TACTTTTTGC TATGGAACAA -1300
CTCATAAGTT ACGAGAAGA CGATTGCCAA ATGAAAAACG ATACCTTGTT

1301 - TTACCTGTTC AAGCTGGTAC TAGATTCCCT ATGGTATTAA ACGTTGTAAA -1350
AATGGACAAG TTCGACCATG ATCTAAGGGA TACCATAATT TGCAACATTT

1351 - CAGAACTGTT TCTGGTCCAT TAGATATTAA ATGTGACCAA TCTGACATAA -1400
GTCTTGACAA AGACCAGGTA ATCTATAATT TACACTGGTT AGACTGTATT

1401 - TGATGGCTCT TAACACTGGT TGGATTATCA TTATGGCTCA TACTACTCAG -1450
ACTACCGAGA ATTGTGACCA ACCTAATAGT AATACCGAGT ATGATGCGTC

1451 - ATGGTTTATG ACTTCAATAT ATTTGCATTA AAAATTGCTG AGAAAGCTAA -1500
TACCAAATAC TGAAGTTATA TAAACGTAAT TTTTAACGAC TCTTTCGATT

1501 - ACTTCCTATC ATTGTTTCTT CTGACGGATT CTTTACTTCT CACCAAAAGA -1550
TGAAGGATAG TAACAAAGAA GTCTGCCTAA GAAATGAAGA GTGGTTTTCT

1551- AAAAAATCCA CCTTTTCAAA AATGATAAAG ATGTTCAAGA TTTCTTAGGT -1600
TTTTTTAGGT GGAAAAGTTT TTACTATTTC TACAAGTTCT AAAGAATCCA

1601 - AAATATACTC CTGAAGTTAC TTCTGTTGAG CCTACTAAGA ACCCTGTTAC -1650
TTTATATGAG GACTTCAATG AAGACAACTC GGATGATTCT TGGGACAATG

1651 - TATTGGGCCT TACATGAATG AAGACGAATT AACAGGTAGT AAATTACAAC -1700
ATAACCCGGA ATGTACTTAC TTCTGCTTAA TTGTCCATCA TTTAATGTTG

1701 - TTTCTCAAGC TTTAGAAGAT TCTAGAGCTA TTATTGCTGA AGTATTTGAA -1750
AAAGAGTTCG AAATCTTCTA AGATCTCGAT AATAACGACT TCATAAACTT

1751 - GAGTTTGCTT CTCTTTCTGG AAGAAAATAC TCTCCTATAG AAACTTCATA -1800
CTCAAACGAA GAGAAAGACC TTCTTTTATG AGAGGATATC TTTGAAGTAT

1801 - ACATGGAAGG AGCTGAAGTT GCTTTAATGC TTTGCGGTTC TGCTTATGAA -1850
TGTACCTTCC TCGACTTCAA CGAAATTACG AAACGCCAAG ACGAATACTT

1851 - ACTGGTACTT TGGCTGTTGA TGAAATGAGA AAAGCTAATC CCTAATCTTA -1900
TGACCATGAA ACCGACAACT ACTTTAGTCT TTTCGATTAG GGATTAGAAT

1901 - AAATTGGTGC TTTCGCTATT ACTCAGATTC GTCCTTTCCC TCAAAAAGAA -1950
TTTAACCACG AAAGCGATAA TGAGTCTAAG CAGGAAAGGGAGTTTTTCTT

1951 - TTACAAAAAT TACTTGCTAA TGTTAAAGTA GTTGTTGTAG GTGATAGACA -2000
AATGTTTTTA ATGAACGATT ACAATTTCAT CAACAACATC CACTATCTGT

2001 - AGATACTTAT TCTGGTATGG GCGGTAATAT GTCTACTGAG ATTAGAGCTG -2050
TCTATGAATA AGACCATACC CGCCATTATA CAGATGACTC TAATCTCGAC

2051 - CTCTTAAAAA TGACCCTAAC AACAAATCTT CTATTGTTAG CAGAGTTTAT -2100
GAGAATTTTT ACTGGGATTG TTGTTTAGAA GCTAACAATC GTCTCAAATA

2101 - GCTCTTGGCG GTACTGAGTT TACTCTTGAC AAAGCTAAAG AATTATTTGA -2150
CGAGAACCGC CATGACTCAA ATGAGAACTG TTTCGATTTC TTAATAAACT

2151- ATTAGGTTTA AAAGAATTAG CTAAAGCTGG TTTCTGTTGA AAAACACTCT -2200
TAATCCAAAT TTTCTTAATC GATTTCGACC AAAGACAACT TTTTGTCAGA

2201 - TATTTAGAAC AATATATGGG TGACCTAATG TTAAAATGAA ACCTATACAT -2250
ATAAATCTTG TTATATACCC ACTGGATTAC AATTTTACTT TGGATATGTA

2251 - GACCCTTTAA CTTTAGAGAG CCAAAAATCA GGCATTACTG TTACTATGAA -2300
CTGGGAAATT GAAATCTCTC GGTTTTTAGT CCGTAATGAC AATGATACTT

2301 - CGAACAAATTCATAAACTTG ATGTTAAAGT TCCCACTTCT TAGAGAATTA -2350
GCTTGTTTAA GTATTTGAAC TACAATTTCA AGGGTGAAGA ATCTCTTAAT

2351 - ACTGGTAAAG CTTATCGTTA TCGTTATGCT CAAGCTCATG GTGCTTGTAA -2400
TGACCATTTC GAATAGCAAT AGCAATGCGA GTTCGAGTAC CACGAACATT

2401 - CGGTTGTGGT ATATTCTCTG GTATCAATAC TTTCATGAAA GGTATAGAAG -2450
GCCAACACCA TATAAGAGAC CATAGTTATG AAAGTACTTT CCATATCTTC

2451 - GTTCTGTTGT ACTTTTGGTA CATACTGGTT GTTCTATGGT TGTTACTACT -2500
CAAGACAACA TGAAAACCAT GTATGACCAA CAAGATACCA ACAATGATGA

2501 - GGTTATCCTT ACAGCTCTTA TAGAACTACT TATGTTCACA ACTTGTTCCA -2550
CCAATAGGAA TGTCGAGAAT ATCTTGATGA ATACAAGTGT TGAACAAGGT

2551 - AAACGGTGCT GGTACTCTTT CTGGTATTGT AGAAATGTAT CATGAGAGAA -2600
TTTGCCACGA CCATGAGAAA GACCATAACA TCTTTACATA GTACTCTCTT

2601 - AAAGAAGAGGAGAAATTGAC GGACCAGAA
TTTCTTCTCC TCTTTAACTG CCTGGTCTT

Appendix 1: Nucleotide sequence of clone SR1. The open reading frame is indicated by underlining. Start codon and potential ribosome binding site -10 and -35 promoters are indicated by bold type.

Appendix 2

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1 -    CATTTTTTTG CGACCGTAGG GAGTGCCTGT GGTATTAACA CATATGAAGC -50
       GTAAAAAAAC GCTGGCATCC CTCACGGACA CCATAATTGT GTATACTTCG

51 -   CAGCTTTTCG GCGAATGGCT ATATTTTATC TTAAATTTAA TAAAGTATCT -100
       GTCGAAAAGC CGCTTACCGA TATAAAATAG AATTTAAATT ATTTCATAGA

101 -  TACATATAAG ATAATTTTAG TATTATTTCG TACTAATTTT ATATTTGCAC -150
       ATGTATATTC TATTAAAATC ATAATAAAGC ATGATTAAAA TATAAACGTG
                                                    -35
151 -  TTTTTGCGGC GGGAAAAAGT TGACTAAAAA ATGACAAACT TAAAAATTTT -200
       AAAAACGCCG CCCTTTTTCA ACTGATTTTT TACTGTTTGA ATTTTTAAAA
                      -10              ⇓+1RBS
201 -  CAGTATATAT AAAAAATACG AATTGCAAGGAGAATAAAAT AGTATATGAG -250
       GTCATATATA TTTTTTATGC TTAACGTTCC TCTTATTTTA TCATATACTC

251 -  AAAAAATAAT AATTTAACTA AAGGTTTTTG TATAGGGTCA TCTGTACTCA -300
       TTTTTTATTA TTAAATTGAT TTCCAAAAAC ATATCCCAGT AGACATGAGT

301 -  TGGCTGCATC TGCAGTGTCA TGCTCAAGTG AAACTAAATC AGAAAAGCCT -350
       ACCGACGTAG ACGTCACAGT ACGAGTTCAC TTTGATTTAG TCTTTTCGGA

351 -  AATATTGTTT ATATAGTATA GATGATATGG GATTCGGTGA TTTGGGTTGT -400
       TTATAACAAA TATATCATAT CTACTATACC CTAAGCCACT AAACCCAACA

401 -  TATGGTTCAA GCATATCCAC ACCAAATATT GATGCTTTAG CTAATAATGG -450
       ATACCAAGTT CGTATAGGTG TGGTTTATAA CTACGAAATC GATTATTACC

451 -  TATAAGGTAT GTTAATTATT TTACTTCTCC TTTAAGTTCT CCAAGCAGAG -500
       ATATTCCATA CAATTAATAA AATGAAGAGG AAATTCAAGA GGTTCGTCTC

501 -  CATCACTTCT TACAGGGTGC GAAGCTAATA AGGTTGGAAT GGGAGCGGTA -550
       GTAGTGAAGA ATGTCCCACG CTTCGATTAT TCCAACCTTA CCCTCGCCAT

551 -  AGTCAGGTTG ATTTCGGAGA TTTAGCACCT AATATGTCAG GAAGAATAAA -600
       TCAGTCCAAC TAAAGCCTCT AAATCGTGGA TTATACTGTC CTTCTTATTT

601 -  GCCCGAACAT GCTCCTATAA CTCATACTTT AAAAGCAAAT GGATATAATA -650
       CGGGCTTGTA CGAGGATATT GAGTATGAAA TTTTCGTTTA CCTATATTAT

651 -  CTTATGCTAT AGGTAAATGG CATTTAGGTC CTTATGATGA GTTTACACCA -700
       GAATACGATA TCCATTTACC GTAAATCCAG GAATACTACT CAAATGTGGT

701 -  GACGGAGATA AATATCATTG GCCTTCAGGA AAGGGGTTCG ATAAGAATTA -750
       CTGCCTCTAT TTATAGTAAC CGGAAGTCCT TTCCCCAAGC TATTCTTAAT

751 -  TAATTTTATA GCAGGACAGG CTAATCAATT TCAGCCGGGC GGTATTATAG -800
       ATTAAAATAT CGTCCTGTCC GATTAGTTAA AGTCGGCCCG CCATAATATC

801 -  AAGGTGATGA GTTTATAGTT CCTGATACTT CAGACCCTAA TTATCATTTG -850
       TTCCACTACT CAAATATCAA GGACTATGAA GTCTGGGATT AATAGTAAAC

851 -  AGTAGAGATA TAGTTGATAG AACATTAAAA TATATTGATG AATCTAAAAA -900
       TCATCTCTAT ATCAACTATC TTGTAATTTT ATATAACTAC TTAGATTTTT
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901 - TAAACCTTTC TTTGCTTATG TGGCATTCGG AGCTATGCAT GGACCTTTCA -950
ATTTGGAAAG AAACGAATAC ACCGTAAGCC TCGATACGTA CCTGGAAAGT

951 - ATGTTGCTAA AAAATATATA GATAAATATA AAGGCAAATT T
TACAACGATT TTTTATATAT CTATTTATAT TTCCGTTTAA A

Appendix 2: Nucleotide sequence of the clone 6a from the T7 promoter end. The gene sequence is indicated by underlining and the ribosomal binding site (RBS) and potential -10 and -35 promoters are indicated in bold type.

Appendix 3

Open reading frame (1a1)

1 - CAGTACGTGA AACAGATAAA GACTTCTTAA TGTCAATCGA AGACGTATAC -50
GTCATGCACT TTGTCTATTT CTGAAGAATT ACAGTTAGCT TCTGCATATG

51 - TCAATCCCTG GAAGAGGTAC AGTTGTTACA GGTAGAATAG AAAGAGGACA -100
AGTTAGGGAC CTTCTCCATG TCAACAATGT CCATCTTATC TTTCTCCTGT

101 - AATCAAAAAA GGTGACGAAG TAGAAATCGT TGGTTTAAGA GAAACTAAGA -150
TTAGTTTTTT CCACTGCTTC ATCTTTAGCA ACCAAATTCT CTTTGATTCT

151 - AAACTACTTG TACTGGTGTA GAAATGTTCA AGAAAGAAGT TGTTGGTATA -200
TTTGATGAAC ATGACCACAT CTTTACAAGT TCTTTCTTCA ACAACCATAT

201 - GCTGGTTATA ACGTTGGATG TCTTTTAAGA GGTATTGAAC GTAAAGAAGT -250
CGACCAATAT TGCAACCTAC AGAAAATTCT CCATAACTTG CATTTCTTCA

251 - AGAAAGAGGACAGGTATTAG CTAAACCAGG TACAATCACA CCTCATAAAA -300
TCTTTCTCCT GTCCATAATC GATTTGGTCC ATGTTAGTGT GGAGTATTTT

301 - AATTCGAAGC AGAAGTTTAT ATCTTGAAAA AAGAAGAAGGTGGAAGACAT -350
TTAAGCTTCG TCTTCAAATA TAGAACTTTT TTCTTCTTCC ACCTTCTGTA

351 - AGCGGTTTCG TAAGCGGTTA CAGACCACAA ATGTACTTCA GAACAACAGA -400
TCGCCAAAGC ATTCGCCAAT GTCTGGTGTT TACATGAAGT CTTGTTGTCT

401 - CGTAACAGGA GTTATCAACT TACCAGAAGG TTCTCCAATG ATAATGCCAG -450
GCATTGTCCT CAATAGTTGA ATGGTCTTCC AAGAGGTTAC TATTACGGTC

451 - GTGATAACGC TAACTTACTA TAGAGTTAAT CAGCCAAATC GCTATGGAAG -500
CACTATTGCG ATTGAATGAT ATCTCAATTA GTCGGTTTAG CGATACCTTC

500 - AGAAACAAAGATTCGCTATA CGTGAAGGTG GTAAGACAGT AGGTAACGGT -550
TCTTTGTTTC TAAGCGATAT GCACTTCCAC CATTCTGTCA TCCATTGCCA

551 - GTTGTAACAA AAATATTAGA ATAATTTAAG GAAAGAAATA AAGGGATATG -600
CAACATTGTT TTTATAATCT TATTAAATTC CTTTCTTTAT TTCCCTATAC

601 - TTTTAATATT AAATGTATCC CTTAATATAA AGAATAAAAG CGAGTAATTC -650
AAAATTATAA TTTACATAGG GAATTATATT TCTTATTTTC GCTCATTAAG

651 - AAACTATGAA AGAACAGAAAATAAGAGTTA AATTAAAAGC CTTTGATATA -700
TTTGATACTT TCTTGTCTTT TATTCTCAAT TTAATTTTCG GAAACTATAT

701 - GAATTAATTG ATCAATCAGC TCAGTCAATA GTTGCTAGTG TAAAGAAGAC -750
CTTAATTAAC TAGTTAGTCG AGTCAGTTAT CAACGATCAC ATTTCTTCTG

751 - AGGTGCAAGA GTATCAGGAC CTATACCACT ACCTACAAGC ATAAGAAAGG -800
TCCACGTTCT CATAGTCCTG GATATGGAGA TGGATGTTCG TATTCTTTCC

801 - TAACAGTAAT AAGAAGTCCG CATGTAAACA TTAAGTCAAG AGAGCAGTTC -850
ATTGTCATTA TTCTTCAGGC GTACATTTGT AATTCAGTTC TCTCGTCAAG

851 - GAGATGAGAGTTTACAAGAG ATTAATAGAT ATCTTTGATG TAACACCTCA -900
CTCTACTCTC AAATGTTCTC TAATTATCTA TAGAAACTAC ATTGTGGAGT

901 - AACAACTGAG TCTTTGAAGA AGTTGGCACT TCCAGCTGGC GTAGATGTAC -950
TTGTTGACTC AGAAACTTCT TCAACCGTGA AGGTCGACCG GATCTACATG

951 - AGTTGAAGTA AGAAAGGTTT TTTAAACCGT GTGAATAAAA CTTTACTACG -1000
TCAACTTCAT TCTTTCCAAA AAATTTGGCA CACTTATTTT GAAATGATGC

-35

1001 - TTTTGTTTGT ATGGTTATAA TTTTAATAGT TTGACTTAAA AA**TTGGTA**AA-1050
AAAACAAACA TACCAATATT AAAATTATCA AACTGAATTT TTAACCATTT

-10 ⇓+1**RBS** **Open reading frame 3 (1a3)**

1051 - AAAAGAAGTA G**TAAAAT**CAT TATAG**AGGA**T TAGCCG**ATGG** TAGGAATAAT -1100
TTTTCTTCAT CATTTTAGTA ATATCTCCTA ATCGGCTACC ATCCTTATTA

1101 - TGGCAGAAAA TTGGGTATGA CAACAGTTTT CGATGAAACT GGCAATGCTA -1150
ACCGTCTTTT AACCCATACT GTGTTCAAAA GTTACTTTGA CCGTTACGAT

1151 - TAGCAGTAAC AGTTGTAGAG GCTGGGCCAT GCACAGTTAT GCATTGGTAA -1200
ATCGTCATTG TCAACATCTC CGACCCGGTA CGTGACAATA CGTAACCATT

1201 - AAAAAGAAGT AGTAAAATCA TTATAGAGGA TTAGCCGATG GTAGGAATAA -1250
TTTTTCTTCA TCATTTTAGT AATATCTCCT AATCGGCTAC CATCCTTATT

1251 - TTGGCAGAAA ATTGGGTATG ACAACAGTTT TCGATGAAAC TGGCAATGCT -1300
AACCGTCTTT TAACCCATAC TGTTGTCAAA AGCTACTTTG ACCGTTACGA

1301 - ATAGCAGTAA CAGTTGTAGA GGCTGGGCCA TGCACAGTTA TGCAGGTTAG -1350
TATCGTCATT GTCAACATCT CCGACCCGGT ACGTGTCAAT ACGTCCAATC

1351 - AGATAATGAG AAAGATGGCT ATAATGCTAT TCAATTAGGT TATGGTGCTG -1400
TCTATTACTC TTTCTACCGA TATTACGATA AGTTAATCCA ATACCACGAC

1401 - TAAAAGAAAA GCATTTAAAA AAGCCACAAA TAGGGCAATT TAAAAAAGCA -1450
ATTTTCTTTT CGTAAATTTT TTCGGTGTTT ATCCCGTTAA ATTTTTTCGT

1451 - AATTTAGAGC CTAAGAAATA TTTAAAAGAA TTTAGGTTAG ATGATTCAAG -1500
TTAAATCTCG GATTCTTTAT AAATTTTGTT AAATCCAATC TACTAAGTTC

1501 - TGCTTATACA GTTGGTCAGG AACTTAAAGT AGATATATTT CAAGCGGGCG -1550
ACGAATATGT CAACCAGTCC TTGAATTTCA TCTATATAAA GTTCGCCCGC

1551 - ATTTTATAGA TGTTAGTTCT TTGAGCAAAG GTAGAGGATT TGCCGGCGTA -1600
TAAAATATCT ACAATCAAGA AACTCGTTTC CATCTCCTAA ACGGCCGCAT

1601 - ATGAAAAGAC ATAATTACGA TGGTGGTCCT ATGAGTCATG GTTCTAATTT -1650
TACTTTTCTG TATTAATGCT ACCACCAGGA TACTCAGTAC CAAGATTAAA

1651 - CAGAAGAAGA GCAGGTTCTA TAGGTTGTAA CAGTTACCCT GCAAGAGTAT -1700
GTCTTCTTCT CGTCCAAGAT ATCCAACATT GTCAATGGGA CGTTCTCATA

1701 - GGAAAGGCAA AGGTATGCCT GGGCATATGG GTAATCTTTA ACTACTATAC -1750
CCTTTCCGTT TCCATACGGA CCCGTATACC CATTAGAAAT TGATGATATG

1751 - AAAACTTAAA AGTAGTTGAA ATAAGACCAG AAGATAACTT GATTATGATT -1800
TTTTGAATTT TCATCAACTT TATTCTGGTC TTCTATTGAA CTAATACTAA

1801 - AAAGGTTCTA TACCTGGAGC TATAAACGGT ATAGTAAAAA TCACTCAAGC -1850
TTTCCAAGAT ATGGACCTCG ATATTTGCCA TATCATTTTT AGTGAGTTCG

⇓+1

1851 - AGCTAAGAAG AAAAATAAGA AGAAGAACTC AATGACTAAA TAATAAAAGC -1900
TCGATTCTTC TTTTTATTCT TCTTCTTGAG TTACTGATTT ATTATTTTCG

RBS Open reading frame 4 (1a4)

1901 - AGGATGATAA AATGGAAGTA GTAATACTAA ATGAAAATGG AGATAGCGTA -1950
TCCTACTATT TTACCTTCAT CATTATGATT TACTTTTACC TCTATCGCAT

1951 - GGTAATTTAG AGGTAGTTGA CGAGATATTC AAATCAGAAG TTAACAACAA -2000
CCATTAAATC TCCATCAACT GCTCTATAAG TTTAGTCTTC AATTGTTGTT

2001 - TCTACTTTAC GAAGCAATCA AAAATGAGTT GGCGAACAGA CGTCAAGGAA -2050
AGATGAAATG CTTCGTTAGT TTTTACTCAA CCGCTTGTCT GCAGTTCCTT

2051 - CTCACTCTAC TAAAACAAGA GCAGAAGTTT CAGGAGGCGG TAAAAAGCCT -2100
GACTGAGATG ATTTTGTTCT CGTCTTCAAA GTCCTCCGCC ATTTTTCGGA

2101 - TGGAGACAAA AAGGTACAGG TAGAGCAAGA GCAGGTTCTA CACGTTCACC -2150
ACCTCTGTTT TTCCATGTCC ATCTCGTTCT CGTCCAAGAT GTGCAAGTGG

2151 - AATTTGGGTA GGCGGTGGTA AAACACATAC TCCTAAGCCT AGAGATTATA -2200
TTAAACCCAT CCGCCACCAT TTTGTGTATG AGGATTCGGA TCTCTAATAT

2201 - GTTATAGATT GCCTAAAAAG ATGAAACGTA AGGCTCTATT GTCTGTTTTA -2250
CAATATCTAA CGGATTTTTC TACTTTGCAT TCCGAGATAA CAGACAAAAT

2251 - TCTTTGAAAT ATGGTAACAA TGTTCTTAAA GTTTTTGAGG ATTTCACTTT -2300
AGAAACTTTA TAGGATTGTT ACAAGAATTT CAAAAACTCC TAAAGTGAAA

2301 - TGATGCTCCA AAGACAAAAA GAATGGCAAG TTTTATAAGT AAGGTTAAAG -2350
ACTACGAGGT TTCTGTTTTT CTTACCGTTC AAAATATTCA TTCCAATTTC

2351 - AGCCAAATAG CAGAAAAGTA GCATTTGTAG TAGGTAAAGA TGAGTCATTA -2400
TCGGTTTATC GTCTTTTCAT CGTAAACATC ATCCATTTCT ACTCAGTAAT

2401 - GGTGATAATT ACAATAAGTT ATTATTATCT TTAAGAAACA TCAAAGATTT -2450
CCACTATTAA TGTTATTCAA TAATAATAGA AATTCTTTGT AGTTTCTAAA

2451 - AAAGCTTGTA AATGCAGACA GTATGTCTAT ACATCCTTTA TATTATGCTG -2500
TTTCGAACAT TTACGTCTGT CATACAGATA TGTAGGAAAT ATAATACGAC

2501 - ATGAAGTATA CTTTACTAAA ACAGCTTTAT CTAAATTAAA TGCTAGAATT -2550
TACTTCATAT GAAATGATTT TGTCGAAATA GATTTAATTT ACGATCTTAA

2551 - AAGGGATAAG AAATATGAGC ATGTATTCAC TTTTAATTGA GCCTATACTT -2600
TTCCCTATTC TTTATACTCG TACATAAGTG AAAATTAACT CGGATATGAA

2601 - ACAGAAAAGA GTAATATCCT TAGAACTGAG CCTAAAGGAA CAGAGAAGCG -2650
TGTCTTTTCT CATTATAGGA ATCTTGACTC GGATTTCCTT GTCTCTTCGC

2651 - TTATTATGTA TTTAGAGTAA GACAGGACGC TAATAAGCAA GAGTTGAAGA -2700
AATAATACAT AAATCTCATT CTGTCCAGCG ATTATTCGTT CTCAACTTCT

2701 - AAGCGGTTGT TTTTTTATTT AATGTACATC CGCTAGATTG TAAGATAATA -2750
TTCGCCAACA AAAAAATAAA TTACATGTAG GCGATCTAAC ATTCTATTAT

2751 - AATGTAAAGC CTAAGAAGAA AAATCGCAGA ATGAGCAGAC GCGGGTTATA -2800
TTACATTTCG GATTCTTCTT TTTAGCGTCT TACTCGTCTG CGCCCAATAT

2801 - CACGCAGTTA TAAAAAAGCG ATAATAGTTC TCGA**TGGCAA**AGAATCAATA -2850
GTGCGTCAAT ATTTTTTCGC TATTATCAAG AGCTACCGTT TCTTAGTTAT
-10 ⇓+1**RBS** **Open reading frame (1a5)**
2851 - GA**TATAGT**AAAATAAGTT**GA GGGA**TACCGA**TG**GCTATTAA GAAATTTAAA -2900
CTATATCATT TTATTCAACT CCCTATGGCT ACCGATAATT CTTTAAATTT

2901 - CCGACAACAC CAAGTTTACG TTATCGTACA GTAGTTGATT TTTCGGATAT -2950
GGCTGTTGTG GTTCAAATGC AATAGCATGT CATCAACTAA AAAGCCTATA

2951 - AACAACAAAT GAGCCTTGTA AATCATTAGT ATGCGGTAAA AAACGCATAA -3000
TTGTTGTTTA CTCGGAACAT TTAGTAATCA TACGCCATTT TTTGCGTATT

3001 - GCGGAAGAGGTTCAAACGGT CGTATCACTA TGCGTCGTCG CGGTGGTGGA -3050
CGCCTTCTCC AAGTTTGCCA GCATAGTGAT ACGCAGCAGC GCCACCACCT

3051 - CACAAGAAAT TATTTAGATT AGTAGATTTT AGAAGAGATA AGCATGATAT -3100
GTGTTCTTTA ATAAATCTAA TCATCTAAAA TCTTCTCTAT TCGTACTATA

3101 - AGAAGCTAAA GTAGTTTCTA TAGAGTAT
TCTTCGATTT CATCAAAGAT ATCTCATA

Appendix 3: Nucleotide sequence of clone 1a. The gene sequences (open reading frames) are indicated by underlining and the ribosomal binding sites (RBS), potential -10 and -35 promoters and start codons are indicated by bold type

Appendix 4

1 - TAGTCCAGTA GTAGGTTCAT CATATAATAA TATTTCAGGG TCCATTGCAA -50
ATCAGGTCAT CATCCAAGTA GTATATTATT ATAAAGTCCC AGGTAACGTT

51 - TAGCACGAGC AAGCCCAACA CGTTTTTTCA TTCCTCCAGA CAATTCTGAA -100
ATCGTGCTCG TTCGGGTTCT GCAAAAAAGT AAGGAGGTCT GTTAAGACTT

101 - GGCATTTTAT GTTCTATATT TGGCATGCCA ACCATATCCA ACACTTCAGC -150
CCGTAAAATA CAAGATATAA ACCGTACGGT TGGTATAGGT TGTGAAGTCG

151 - TACTTTTACC TTTATCTTG CTTTCCGGCA TACCCTTTTT TATTCTTCTA -200
ATGAAAATGG AAATAGAAC GAAAGGCCGT ATGGGAAAAA ATAAGAAGAT

201 - AGCCCAAAAC TTACATTCTC ATAAACATTC AAAGAGTCAA ATAAAGCAGC -250
TCGGGTTTTG AATGTAAGAG TATTTGTAAG TTTCTCAGTT TATTTCGTCG

251 - CCCCTGAAAA ACCATAGCAA ACTTTTTTCT AACTTCTGTA AGTTCATTAT -300
GGGGACTTTT TGGTATCGTT TGAAAAAAGA TTGAAGACAT TCAAGTAATA

301 - CAGATATCTG ATTTATATCA TTACCATCTA TTATAATACT TCCGGAATCT -350
GTCTATAGAC TAAATATAGT AATGGTAGAT AATATTATGA AGGCCTTAGA

351 - GGCTGTAATA AACCTATTAA GTGTTTTATA AGAACGCTTT TTCCACAGCC -400
CCGACATTAT TTGGATAATT CACAAAATAT TCTTGCGAAA AAGGTGTCGG

401 - GGAGTTACCT ATAACCGCTA ATGTTTCGCC TCTATTAACT ATGAGATTAA -450
CCTCAATGGA TATTGGCTAT TACAAAGCGG AGATAATTGA TACTCTAATT

451 - CACCATTCAA GACCTTTTGA CTGCCAAAAC TTTTATATAC ATCTTTTAAT -500
GTGGTAAGTT CTGGAAAACT GACGGTTTTG AAAATATATG TAGAAAATTA

501 - TCTATTATAT TCATAAAACG AAAATTTCCC TCAAACAGT AACTCTTAAT -550
AGATAATATA **AGTA**TTTTGC TTTTAA**AGGG** AGTTTGTCA TTGAGAATTA

Open reading frame 1 (3a1) **RBS**

551 - AATTTTATTC CTTTTTCACT ATATATACTA TATTCTCCAA GATATGCAGG -600
TTAAAATAAG GAAAAAGTGA TATATATGAT ATAAGAGGTT CTATACGTCC

601 - TAAAAGAACA GTTTTTCCAG CCTCTAATTC AATACTATTT TCATCACTTG -650
ATTTTCTTGT CAAAAAGGTC GGAGATTAAG TTATGATAAA AGTAGTGAAC

651 - TTATTGTTCC ATTTCCTTCC ATTACTAATA TTATTTCAAA TGTTTTATTA -700
AATAACAAGG TAAAGGAAGG TAATGATTAT AATAAAGTTT ACAAAATAAT

701 - TTAGTTTTAG AAGTGTATTT ATCTTTTATA AGATATTCTT CTGTTGTGAA -750
AATCAAAATC TTCACATAAA TAGAAAATAT TCTATAAGAA GACAACACTT

751 - ATATTCATTT GAAAATATAT TATTGATTTC TATATTATTT TCTTTTAATA -800
TATAAGTAAA CTTTTATATA ATAACTAAAG ATATAATAAA AGAAAATTAT

801 - AATTTTCTTT TTTTGATTTC AATTGAAATG CATCTATATC TTTAATAACA -850
TTAAAAGAAA AAAACTAAAG TTAACTTTAC GTAGATATAG AAATTATTGT

851 - TTAAAAGAGT CTTCTATATG AAGTTCTCTT GAATTACCAT TTTTATCTAC -900
AATTTTCTCA GAAGATATAC TTCAAGAGAA CTTAATGGTA AAAATAGATG

901 - TCTATTCCAA TCATAAAGTC TGTAGGTAAC ATCACTTGGA GTTTGTATTT -950
AGATAAGGTT AGTATTTCAG ACATCCATTG TAGTGAACCT CAAACATAAA

951 - CTGCTATTAA ACTATTACCC ATTATAGCAT GTATACAGCC ATTTGGTATA -1000

GACGATAATT TGATAATGGG TAATATCGTA CATATGTCGG TAAACCATAT

1001 - TAAAAAGCAT CTCCTTTTTT TATATCAAAA TAATTAAACA TATCTTCTAT -1050
ATTTTTCGTA GAGGAAAAAAATATAGTTTT ATTAATTTGT ATAGAAGATA

1051 - GTTTTCTTTA TTTTCTATAG CAGCTTTCAA TTTATTTTTA TCAATACCTT -1100
CAAAAGAAAT AAAAGATATC GTCGAAAGTT AAATAAAAAT AGTTATGGAA

1101 - TTTTTAACCC TATTAAAAGT TTAGCATTTT CATCTGCTTC CATAATATAC -1150
AAAAATTGGG ATAATTTTCA AATCGTAAAA GTAGACGAAG GTATTATATG

1151 - CACATTTCAT TTTTGCCATG TTTATTATGT TTTTTATTAG CATATTCTTC -1200
GTGTAAAGTA AAAACGGTAC AAATAATAGA AAAAATAATC GTATAAGAAG

1201 - ATCGGGGTGA ACTTGTATTG ATAATTTATC TTTCGCATCT ATAAATTTTA -1250
TAGCCCCACT TGAACATAAC TATTAAATAG AAAGCGTAGA TATTTAAAAT

1251 - TTAATAGAGG GAAATCTTTT TGATTTTTAC AATTACTTCC CAATAGTTTT -1300
AATTATCTCC CTTTAGAAAA ACTAAAAATG TTAATGAAGG GTTATCAAAA

1301 - TCGCCGAGAT GTTCTATTAA AAATGATATA TCTTTTTCTT TAAACTCTCC -1350
AGCGGCTCTA CAAGATAATT TTTACTATAT AGAAAAAGAAATTTGAGAGG

1351 - ATTAGAAACA ATGTTTTTAT CATTTTTTAA ATCACATATT TCCCAGCTTT -1400
TAATCTTTGT TACAAAAATA GTAAAAAATT TAGTGTATAA AGGGTCGAAA

1401 - CGCCGATATT TTTATCTTTA TCAAAATGTT AGAATAAGA TTTACTAAT -1450
GCGGCTATAA AAATAGAAAT AGTTTTACAA TCTTATTCT AAATGATTA

1451 - GTGTTGCCGC CCCAGATGGA CTCTTTACCA ATTTCTTTAA ATTCAAATAT -1500
CACAACGGCG GGGTCTACCT GAGAAATGGT TAAAGAAATT TAAGTTTATA

1501 - ATACATAGTT ACATTATATA AAATAAAAAA AATAATTCAA TACAATAAAA -1550
TATGTATCAA TGTAATATAT TTTATTTTTT TTATTAAGTT ATGTTATTTT

Open reading frame 2 (3a2)

1551 - TTTAATTATT TAAATACCGC ACGTTAAATT AAGCAATAAA TTATTAATAT -1600
AAATTAATAA ATTTATGGCG TGCAATTTAA TTCGTTATTT AATAATTATA

1601 - GTTTTAATAT AATTTTTATT ATAAACAATA GTTATTAAAG CGTGCGTTGA -1650
CAAAATTATA TTAAAAATAA TATTTGTTAT CAATAATTTC GCACGCAACT

1651 - ATGGATTTTT AATTTAAATA AAAACTTGGG TGGGGTGCTT TTTTATTTAG -1700
TACCTAAAAA TTAAATTTAT TTTTGAACCC ACCCCACGAA AAAATAAATC

1701 - TAGACAAAAA GATAATAAAA TTAGAATTTT AAAATGATAC AATAAAAAAT -1750
ATCTGTTTTT CTATTATTTT AATCTTAAAA TTTTACTATG TTATTTTTTA

1751 - AAAGGGTGGG GTATGTTATA AAAACAAAAAAATAATAAAAA GGAGATGCTT -1800
TTTCCCACCC CATACAATAT TTTTGTTTTT TATTATTTTT CCTCTACGAA

1801 - TTTTATATAC CAAATGTTAA TACCCCTCTT ATTAATAAAA ACTATTATAC -1850
AAAATATATG GTTTACAATT ATGGGGAGAA TAATTATTTT TGATAATATG

1851 - TATAATTATG CTTTAATTAA CACTTTGCCG TCTTTTGCAT ATAGCTTATT -1900
ATATTAATAC GAAATTAATT GTGAAACGGC AGAAAACGTA TATCGAATAA

1901 - AGTAAATACA TCATTAATAT TCTCTAAATC AACAATGTCA GTGATAATTT -1950
TCATTTATGT AGTAATTATA AGAGATTTAG TTGTTACAGT CACTATTAAA

1951 - CTGATACATC TATTACTCCT TTAGCAAGTA AATTAGCAGC CCTTTCAAAA -2000
GACTATGTAG ATAATGAGGA AATCGTTCAT TTAATCGTCG GGAAAGTTTT

2001 - GCATAAGGAT TGACAAAAAG AAGTTTTTAT CGTAAGCTCT TTTTGAAACA -2050
CGTATTCCTA ACTGTTTTTC TTCAAAAATA GCATTCGAGA AAAACTTTGT

2051 - TTTCAAAAGG TTTTATTTTT ACTTCATCAT CAGGAGCAGT AAGTCCAAAA -2100
AAAGTTTTCC AAAATAAAAA TGAAGTAGTA GTCCTCGTCA TTCAGGTTTT

2101 - AGCATTATTT CAGCACCCTT GCCTGCATAT TTAATAGAAT ATTTAGCAGT -2150
TCGTAATAAA GTCGTGGGAA CGGACGTATA AATTATCTTA TAAATCGTCA

2151 - CTGTACTTTT CCAGCACAAT CTATAACTTT ATCAATATTA AATATATTAT -2200
GACATGAAAA GGTCGTGTTA GATATTGAAA TAGTTATAAT TTATATAATA

2201 - TATTTTTTAG TATTTCTTCA GTATTATCAT TAATAGGGTC TATTACAATA -2250
ATAAAAAATC ATAAAGAAGT CATAATAGTA ATTATCCCAG ATAATGTTAT

2251 - TTAGCACCAT ATTTTTTAGC TCTTTCTCTT CTTTTTTCAA ACGGCTCTAC -2300
AATCGTGGTA TAAAAAATCG AGAAAGAGAAGAAAAAAGTT TGCCGAGATG

2301 - AGCAATTATA TTAACAGCCC CTTTATATTT TAAAAGTTGA ATCATCATAA -2350
TCGTTAATAT AATTGTCGGG GAAATATAAA ATTTTCAACT TAGTAGTATT

2351 - GCCCAATATT TCCAGCACCA ACTACCATCA CAGTATCACC CTGCTTAATA -2400
CGGGTTATAA AGGTCGTGGT TGATGGTAGT GTCATAGTGG GACGAATTAT

2401 - TCCATTAAAT CTATACCATG CAAGCAACAA GAAATAGGTT CAACCATAGC -2450
AGGTAATTTA GATATGGTAC GTTCGTTGTT CTTTATCCAA GTTGGTATCG

2451 - CGCACTCTCA TAAGAAACAT TATCAGCTAC TTTAAATACT AAGTTCTCTT -2500
GCGTGAGAGT ATTCTTTGTA ATAGTCGATG AAATTTATGA TTCAAGAGAA

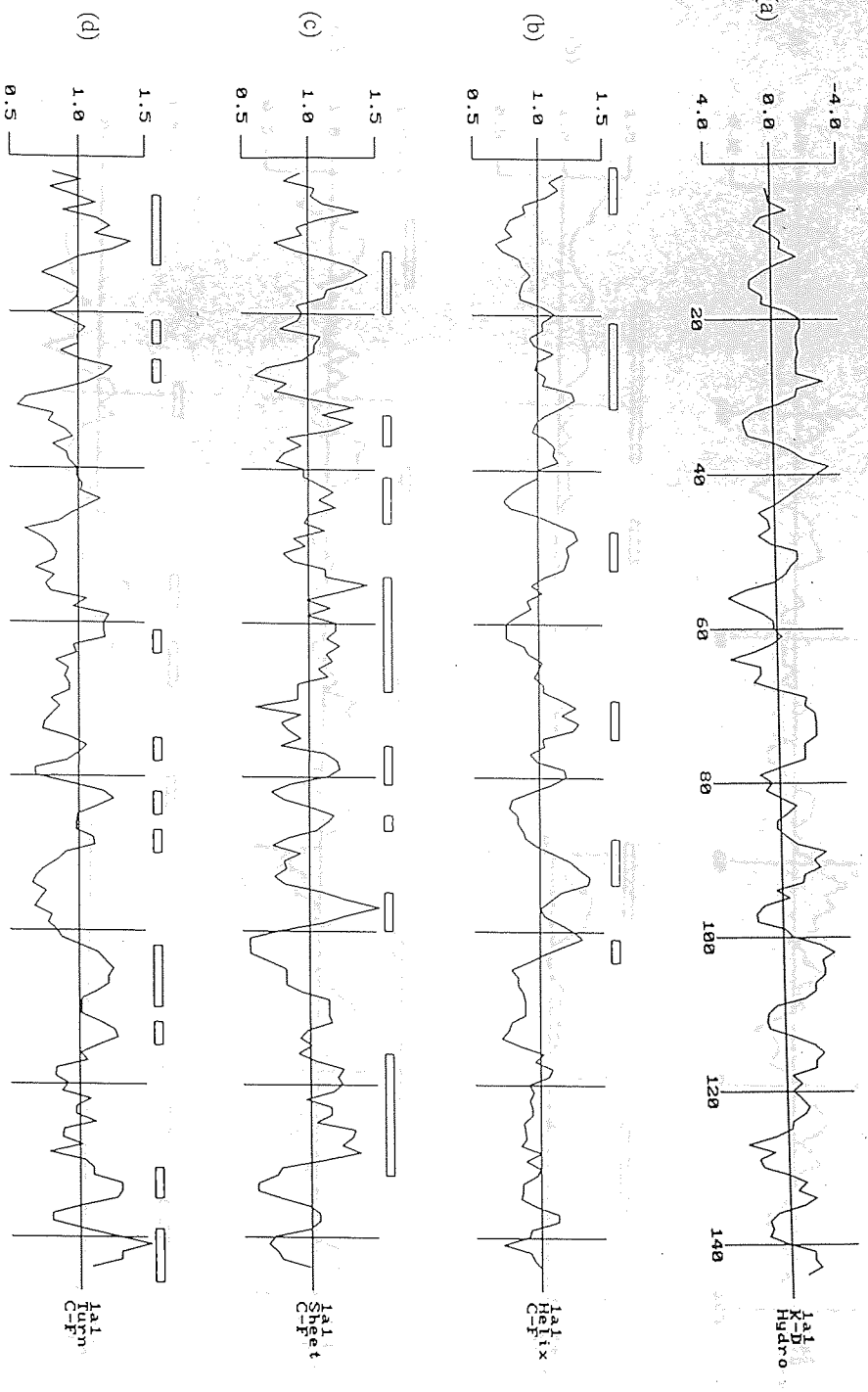
2501 - TAACTGTAAC ATACTCAGCA AAACCCCCGT CTAATGATAC TCCAACAGCA -2550
ATTGACATTG TATGAGTCGT TTTGGGGGCA GATTACTATG AGGTTGTCGT

2551 - GTCATATTGT TGCATAGGTG TTTTTTAGCA TTATTACAAT AGTAGCAATT -2600
CAGTATAACA ACGTATCCAC AAAAAATCGT AATAATGTTA TCATCGTTAA

2601 - ATTACAATAG TCATTAG
TAATGTTATC AGTAATC
Open reading frame 3 (3a3)

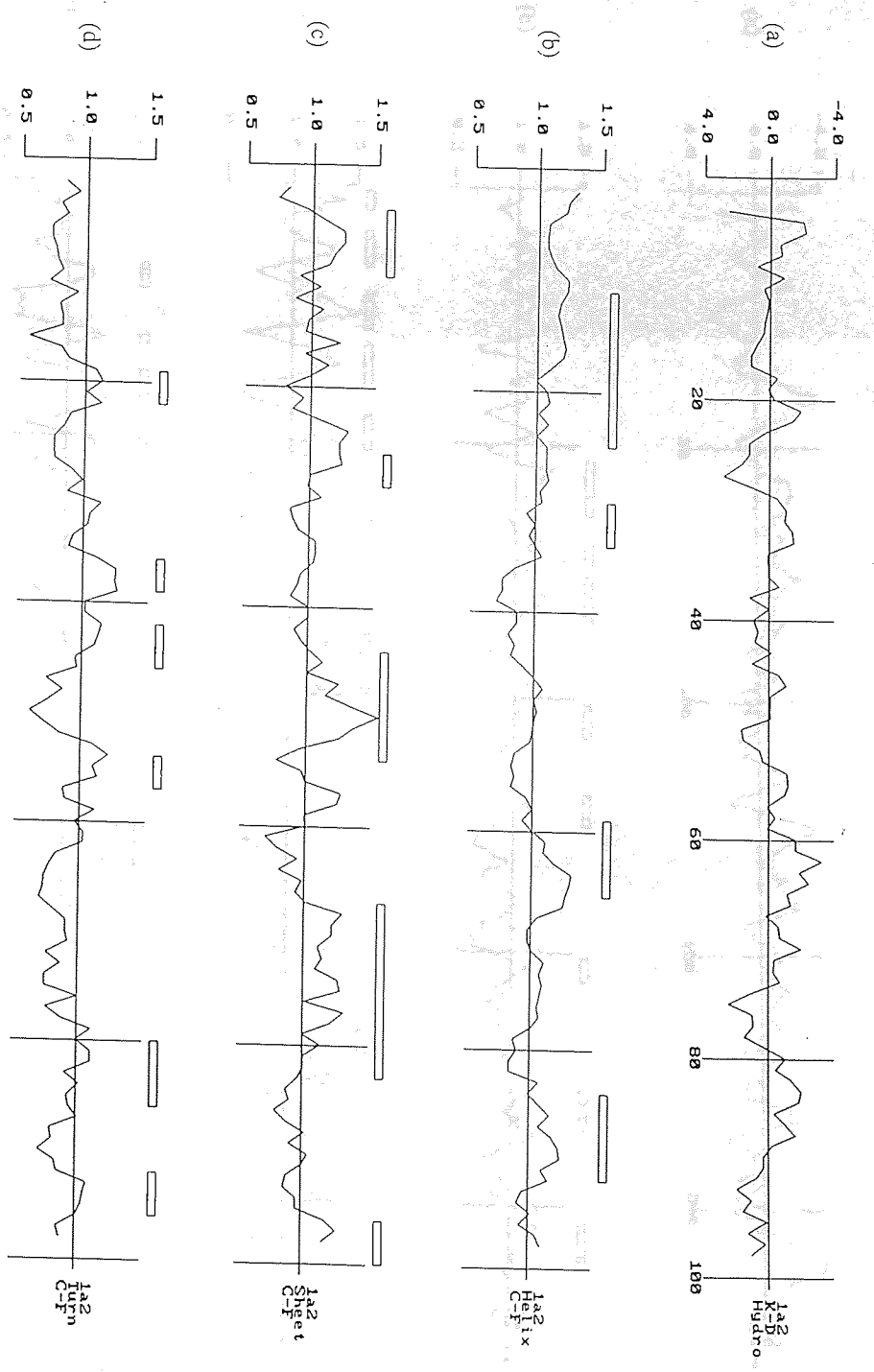
Appendix 4: Nucleotide sequence of clone 3a showing putative gene sequences underlined and start codons and ribosome binding sites in bold type.

Appendix 5



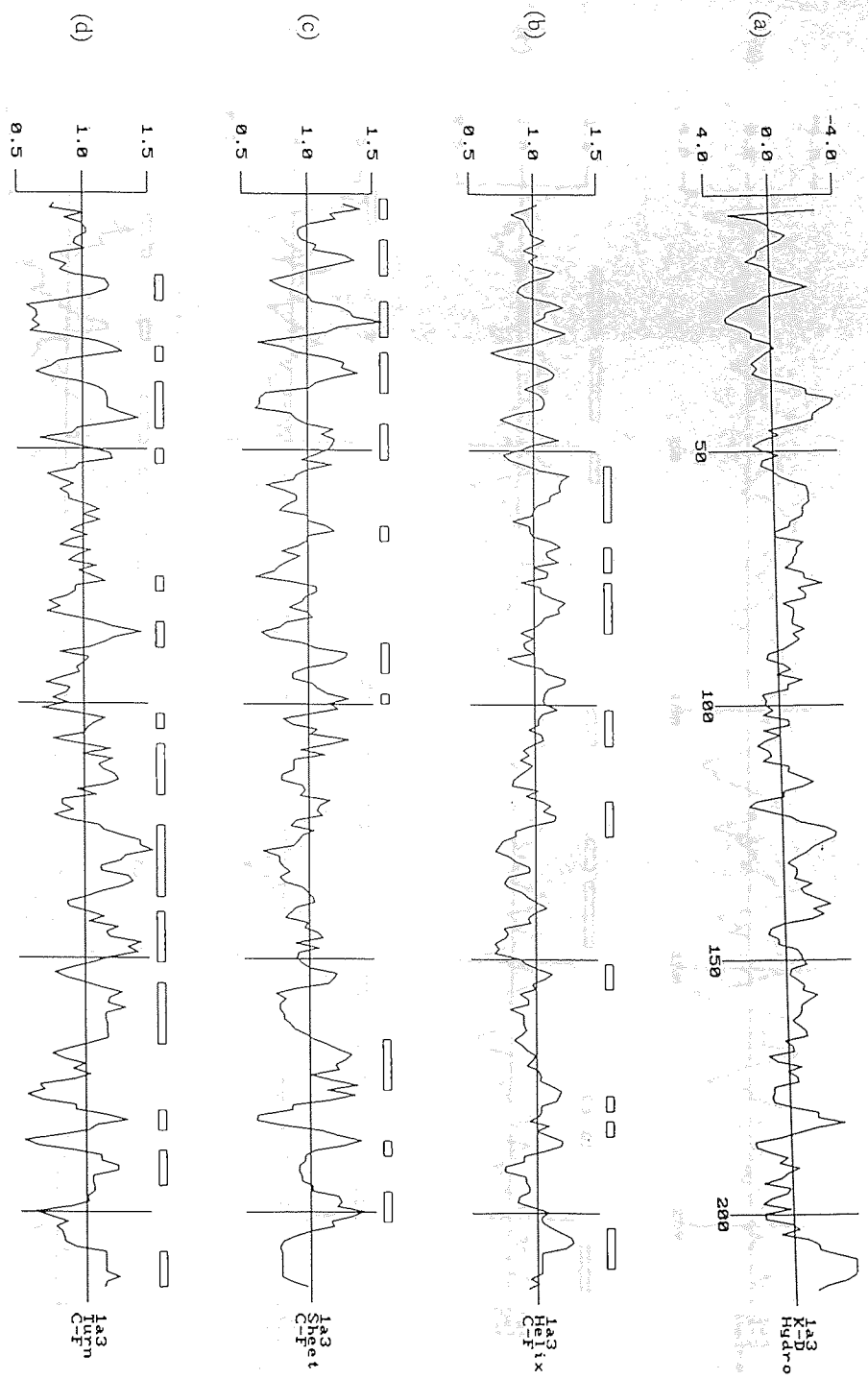
Appendix 5: Kyte-Doolittle hydropathy plot (a) and Chou-Fasman plots (b-d) of α-helix (b), β-pleated sheet (c) and turns (d) (which are represented by open boxes) for sequence 1a1.

Appendix 6



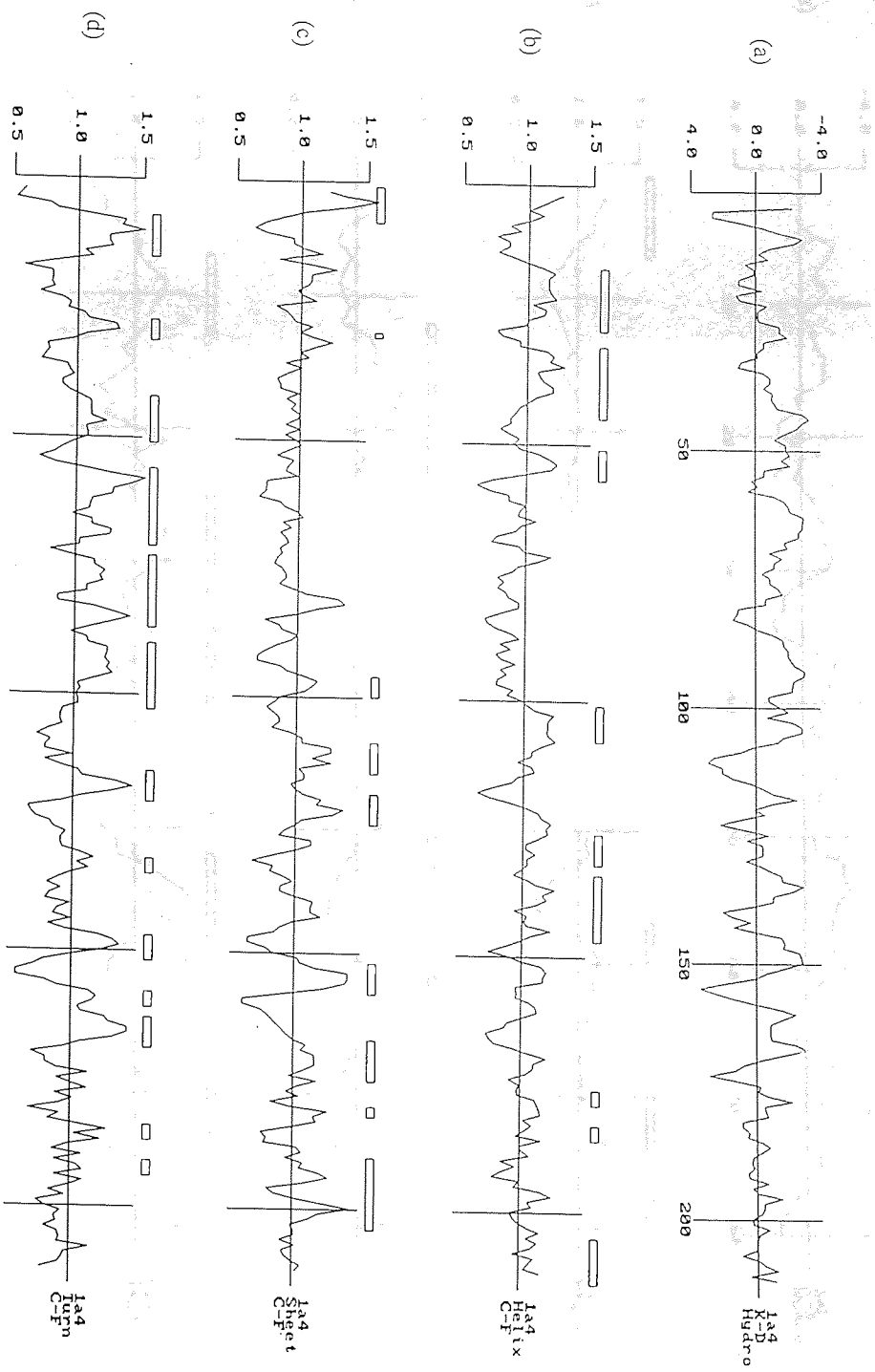
Appendix 6: Kyte-Doolittle hydropathy plot (a) and Chou-Fasman plots (b-d) of α-helix (b), β-pleated sheet (c) and turns (d) (which are represented by open boxes) for sequence 1a2.

Appendix 7



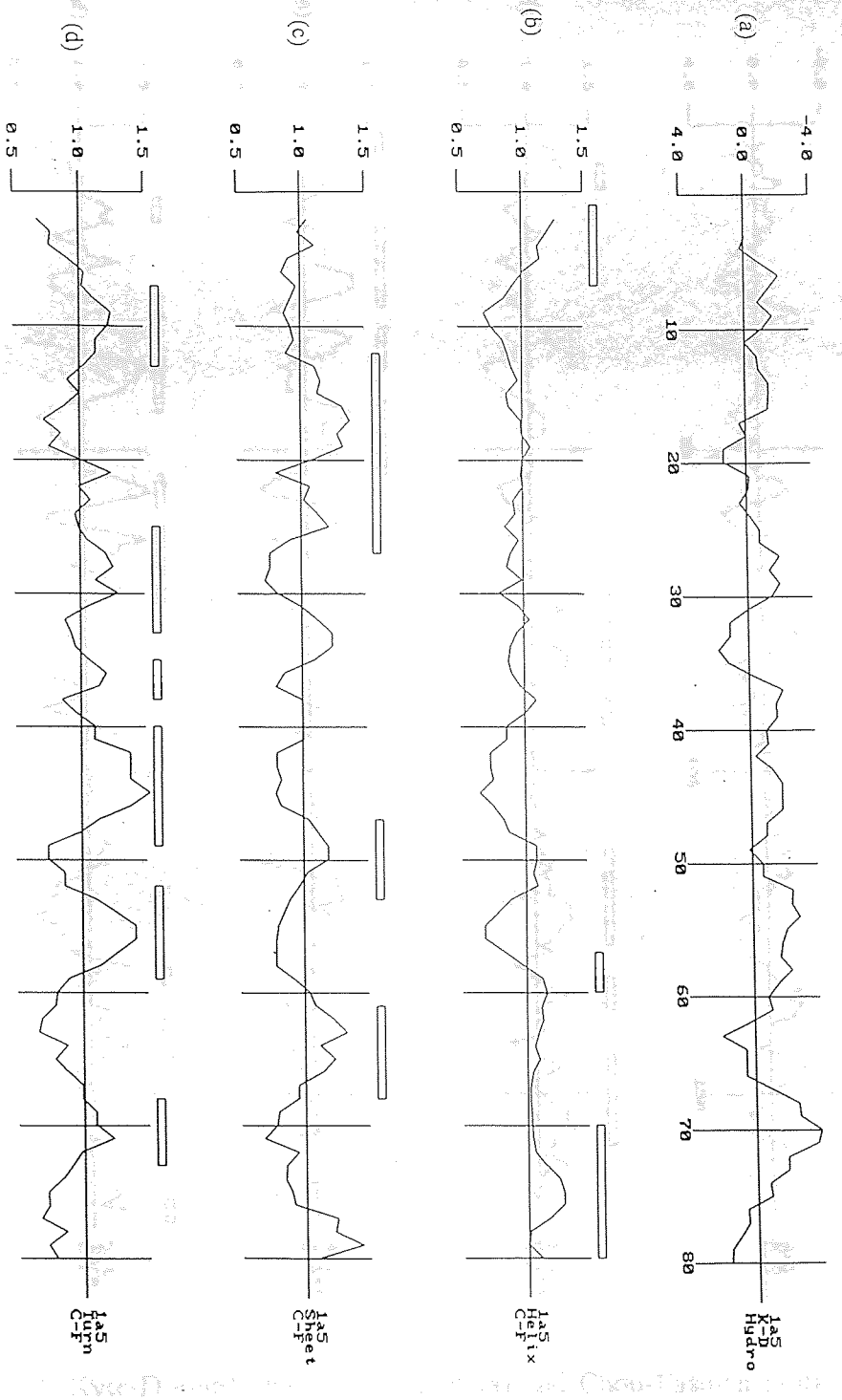
Appendix 7: Kyte-Doolittle hydropathy plot (a) and Chou-Fasman plots (b-d) of α-helix (b), β-pleated sheet (c) and turns (d) (which are represented by open boxes) of sequence 1a3.

Appendix 8



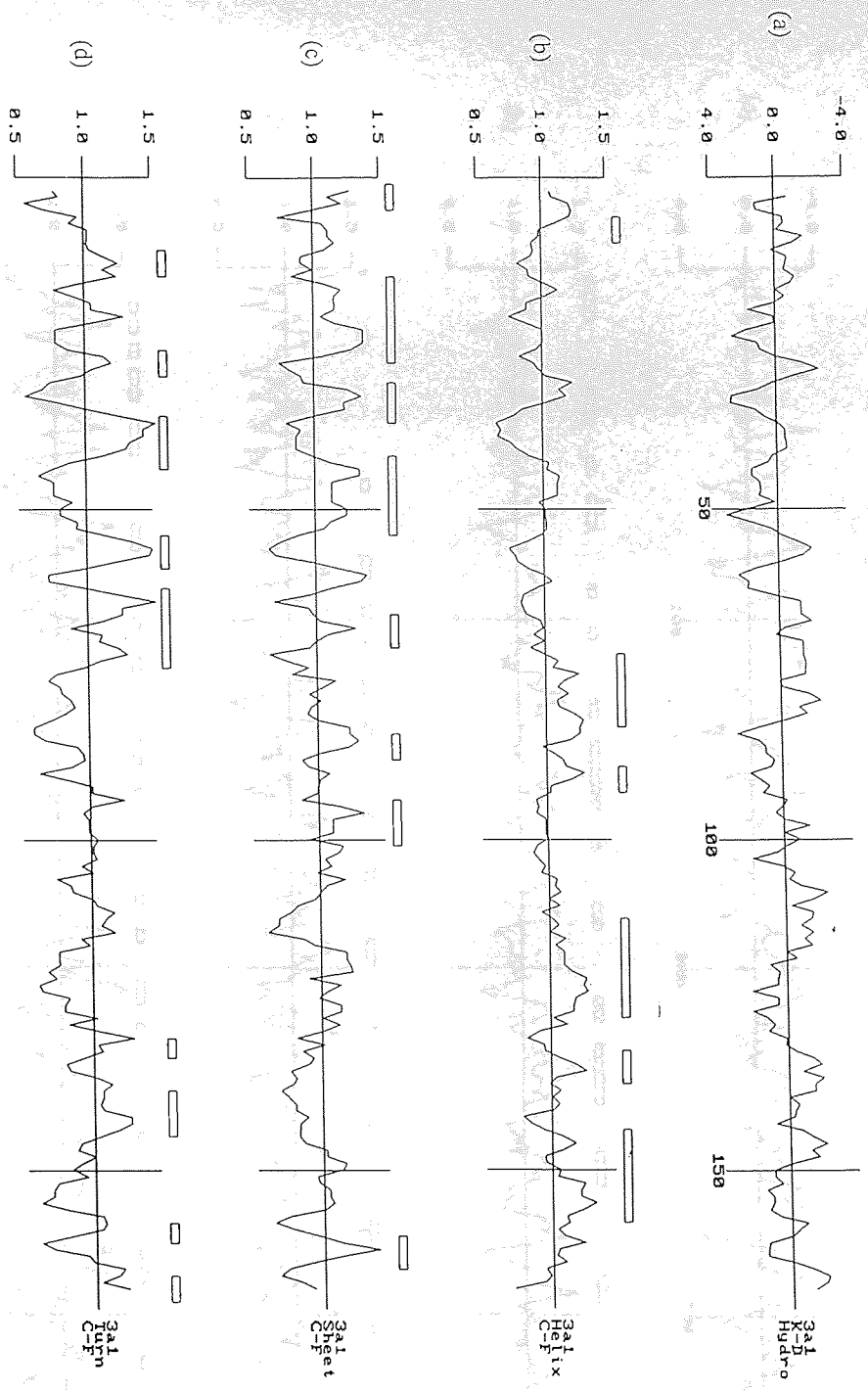
Appendix 8: Kyte-Doolittle hydropathy plot (a) and Chou-Fasman plots (b-d) of α-helix (b), β-pleated sheet (c) and turns (d) (which are represented by open boxes) of sequence 1a4.

Appendix 9



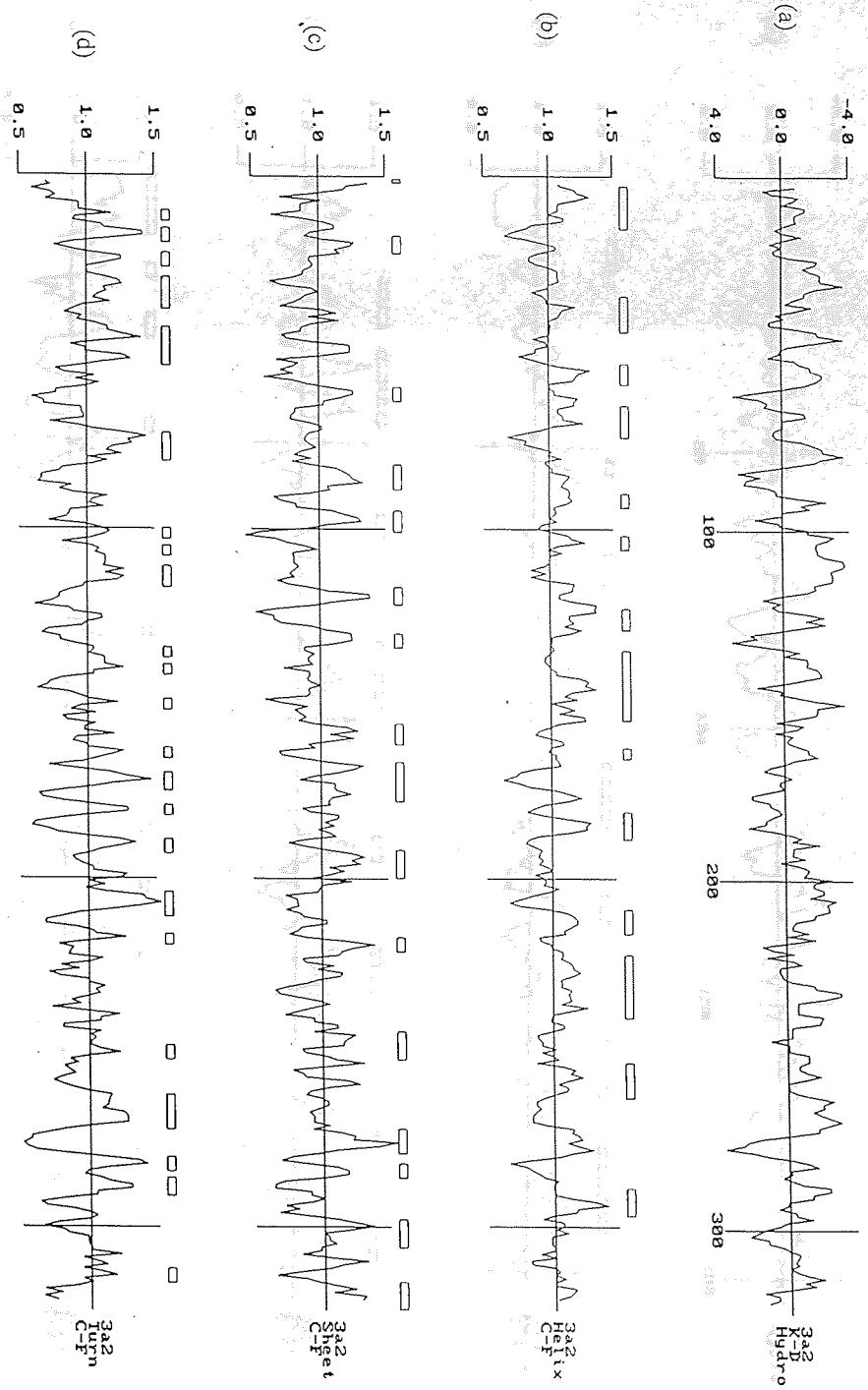
Appendix 9: Kyte-Doolittle hydropathy plot (a) and Chou-Fasman plots (b-d) of α-helix (b), β-pleated sheet (c) and turns (d) (which are represented by open boxes) of sequence 1a5.

Appendix 10



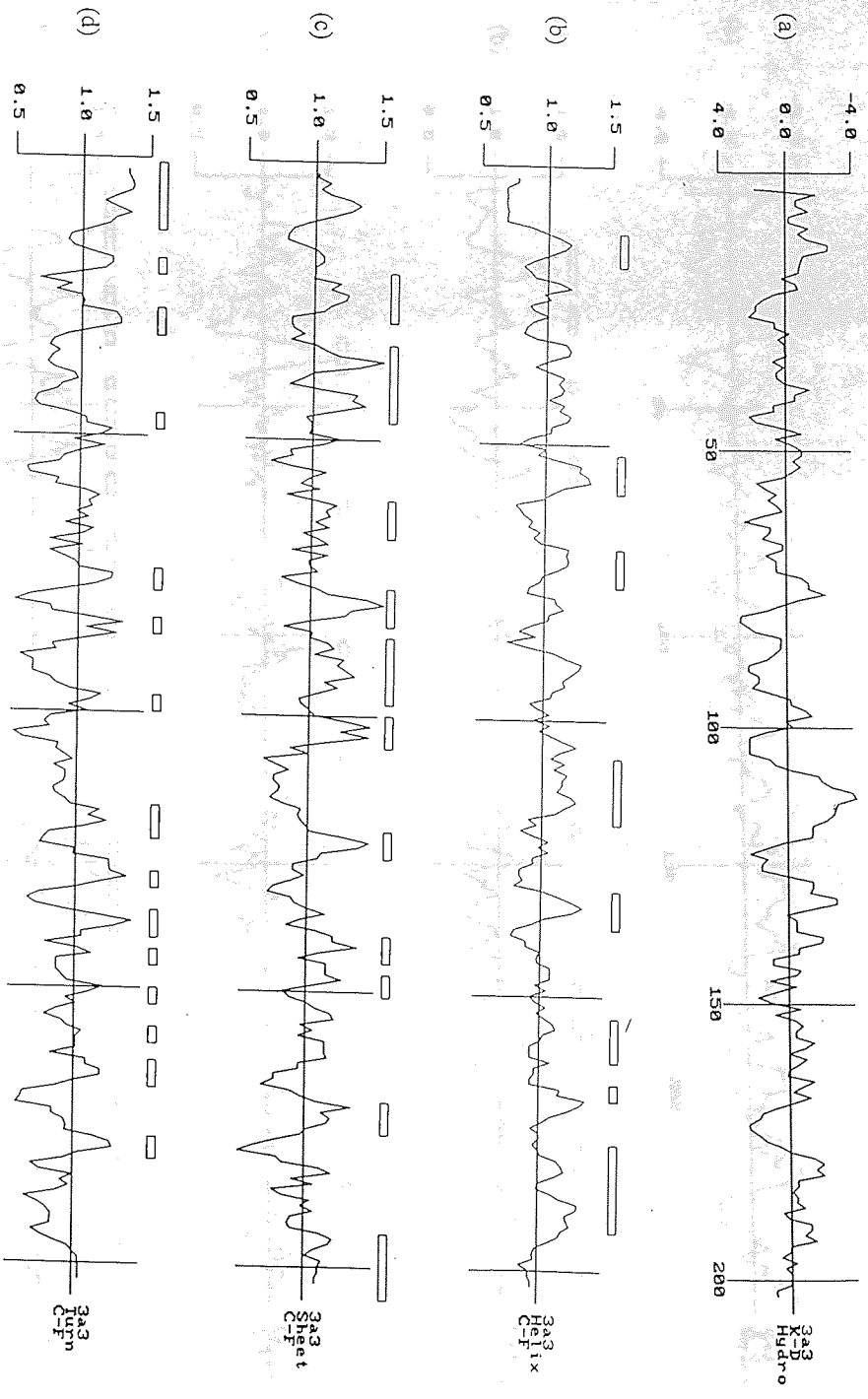
Appendix 10: Kyte-Doolittle hydropathy plot (a) and Chou-Fasman plots (b-d) of α-helix (b), β-pleated sheet (c) and turns (d) (which are represented by open boxes) of sequence 3a1.

Appendix 11



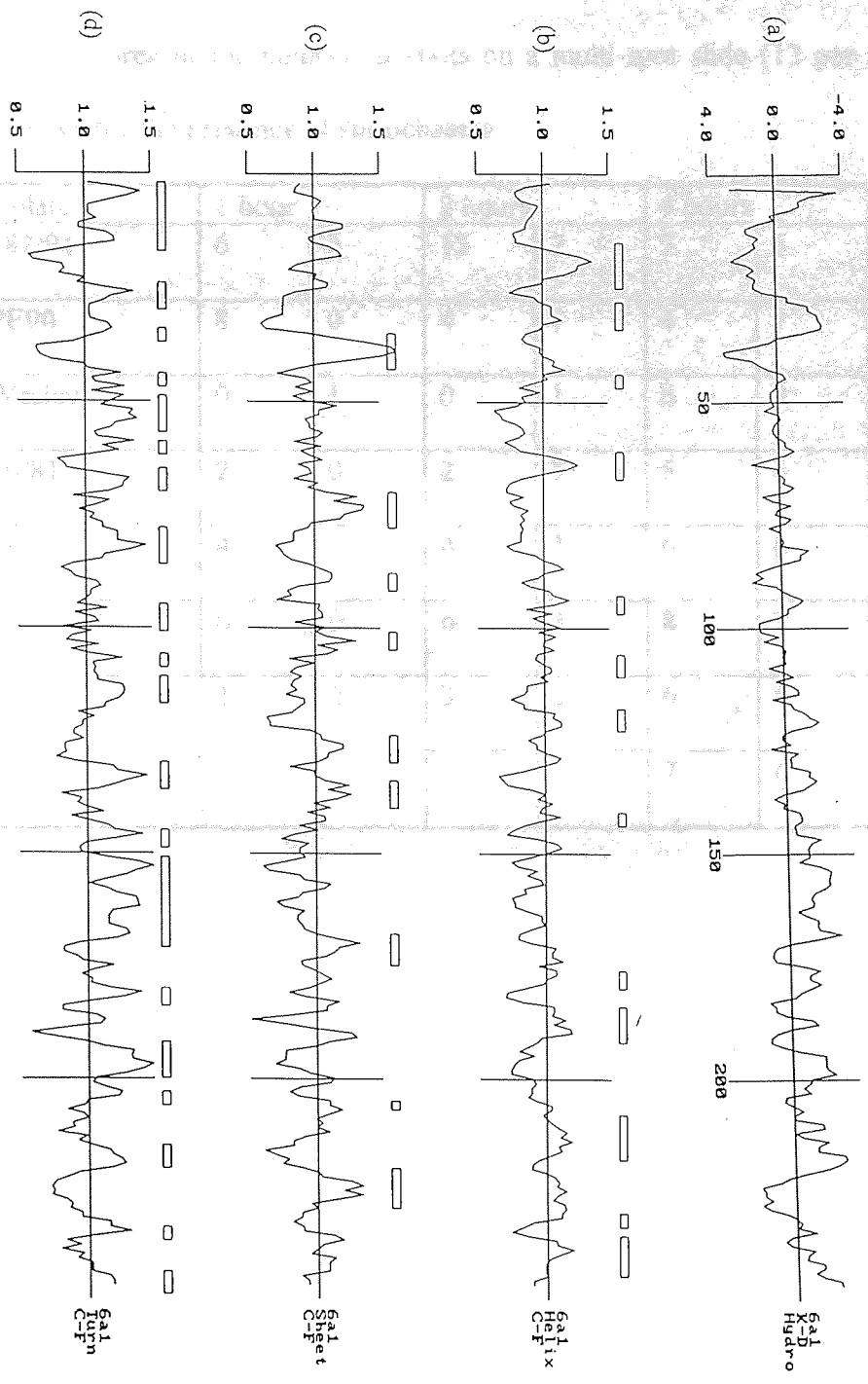
Appendix 11: Kyte-Doolittle hydropathy plot (a) and Chou-Fasman plots (b-d) of α-helix (b), β-pleated sheet (c) and turns (d) (which are represented by open boxes) of sequence 3a2.

Appendix 12



Appendix 12: Kyte-Doolittle hydropathy plot (a) and Chou-Fasman plots (b-d) of α-helix (b), β-pleated sheet (c) and turns (d) (which are represented by open boxes) of sequence 3a3.

Appendix 13



Appendix 13: Kyte-Doolittle hydropathy plot (a) and Chou-Fasman plots (b-d) of α-helix (b), β-pleated sheet (c) and turns (d) (which are represented by open boxes) of sequence 6a1.

Appendix 14

Table showing the penetration of Caco-2 cells by intestinal spirochaetes after 1hour, 2 hours, 4 hours and 6 hours. Each experiment was conducted in duplicate and the figures shown represent the number of spots on a multi-spot slide (12 per slide) which were positive for the presence of spirochaetes.

Isolate	1 hour		2 hours		4 hours		6 hours	
382/91	6	0	12	7	7	6	11	12
PE90	8	0	4	1	4	1	11	8
Wesley	0	1	0	1	6	9	0	7
80/81	2	0	2	3	5	6	5	12
28/94	4	2	4	3	6	6	7	6
P43/6/78^{T}	6	0	6	3	8	3	11	5
60	1	2	3	2	6	6	8	9
RA87	0	0	0	2	7	6	12	6