CLONING, SEQUENCING AND PHYSICOCHEMICAL CHARACTERIZATION OF A HEMAGGLUTININ FROM *Escherichia coli* 09:H10:K99

By

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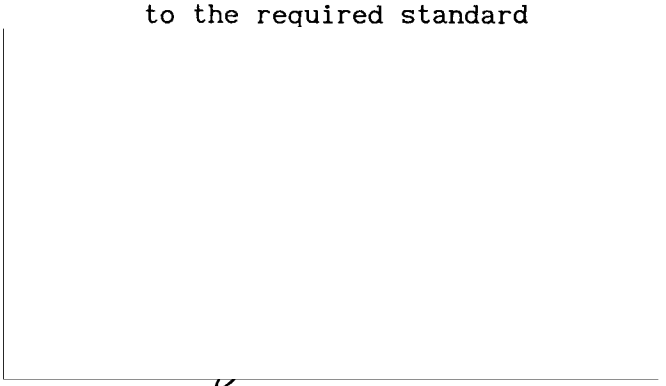

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Abstract

A mannose-resistant hemagglutinating (erythrocyte aggregating) protein was cloned from *Escherichia coli* 09:H10:K99. This hemagglutinin was determined to be different from the two mannose-resistant hemagglutinins that this strain was known to possess, F41 and K99, and was named Heat Resistant Agglutinin 1 (HRA1) on the basis of one of its physical properties. The HRA1 gene present on the recombinant plasmid pETE1 was localized and identified unambiguously by subcloning. The nucleotide sequence of the gene was determined and found to consist of a 792 base pair open reading frame coding for a protein of 29 kDa. This protein has a predicted prokaryotic N-terminal secretory signal sequence. The protein sequence derived from the genetic information showed no significant similarity to database protein sequences. Physical and chemical attempts to isolate HRA1 from the cell membrane met with limited success. N-terminal sequence analysis of a pronounced 25 kDa band present on polyacrylamide gels of crude membrane preparations of bacteria harbouring pETE1 correlated with the predicted N-terminal amino acid sequence of HRA1 after cleavage of the signal peptide. Four other open reading frames were found on the cloned DNA fragment. Three of the deduced proteins from these regions contained predicted signal sequences and membrane associated areas and one showed 50 % identity with *E.coli* lipoprotein, suggesting that a region of DNA associated with outer membrane structure and function was cloned.

Partition in various aqueous two-phase polymer systems showed that expression of HRA1 caused pronounced cell-surface changes in host bacteria. Viscometric analysis showed that the agglutination event

mediated by HRA1 was less pronounced than that of F41. This was likely due to the fact that F41 is known to be an exposed high molecular weight multivalent adhesin, whereas evidence suggests that HRA1 is a monovalent molecule that is closely associated with the bacterial membrane.

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1. Introduction

1.1. General Background

Bacterial infections are ubiquitous in humans and other species and have been the focus of intense scientific scrutiny for many years. At present, although much has been learned about bacterial physiology, pathogenic bacteria still present an enormous medical and veterinary problem. For example, it has been estimated that one third of the deaths of children under 5 in developing countries are due to enterotoxigenic bacterial infection (1).

An important initial step in bacterial pathogenesis is the adherence of the bacterial cell to the host tissue. This adherence involves recognition of specific receptor molecules on the host cell surface by specialized bacterial membrane structures called adhesins. A large number of these bacterial systems have been described and some of their receptors elucidated. There follows an overview of the bacteria cell surface environment with special reference to adhesive agents present on the surface of the Gram-negative bacterium, *Escherichia coli*.

1.2. The Gram-Negative Bacterial Membrane

Bacterial membranes are very complex systems. The important features of a Gram-negative (e.g. *E.coli*) membrane are shown in Figure 1. There are two lipid/protein membrane bilayers, an inner

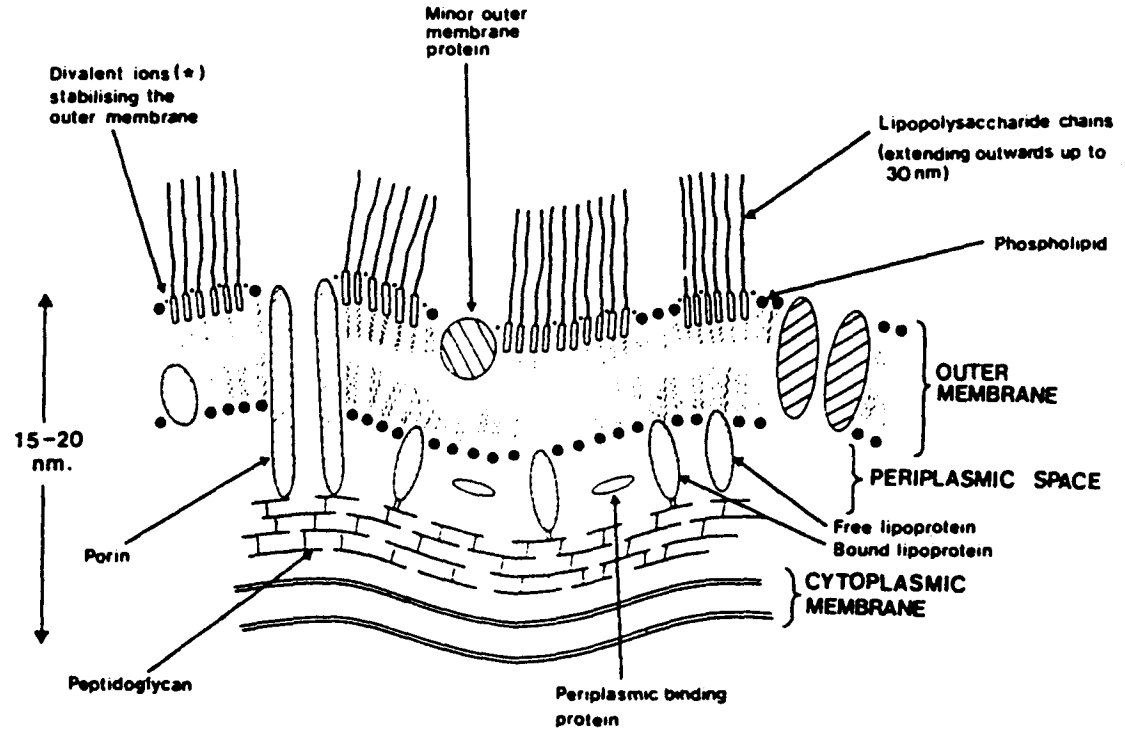


Figure 1. The envelope of gram-negative bacteria.

(from reference 68)

(cytoplasmic) membrane containing the enzymes of the electron transport chain and various transport proteins, and an outer membrane containing integral porins and other proteins with lipopolysaccharide (LPS) chains anchored on the external side extending outwards. It is the LPS that gives rise to the O serotype designation (serotyping is a method of identification and classification of bacteria based upon the antigenic identity of the outer membrane). The periplasmic space between the membranes contains peptidoglycan, lipoprotein and various periplasmic proteins.

Almost all periplasmic and outer membrane proteins are synthesized in the cytosol with a secretory signal peptide sequence of 20-30 amino acids at the N-terminus. These signal peptides have very little amino acid sequence identity but always consist of a positively charged N-terminal followed by a long sequence of hydrophobic residues before a short processing site usually containing alanine residues (68)(Fig. 2). This signal sequence is required for passage of the protein through the inner membrane and is cleaved at the outer face of the membrane by the inner-membrane associated enzyme signal peptidase.

The outer membrane also anchors flagella, which are locomotory organelles consisting of a basal body extending through the membrane connected to a hook region which in turn anchors the filament. The filament contains 95 % of the mass of the flagellum and consists of a polymer of a single protein subunit, flagellin (60 kDa in *E.coli*) that is some 20 nm in diameter and 10-20 μm long. The flagellum comprises the H antigen in serological analysis.

The K serological determinants are generally polysaccharide moieties, sometimes forming a glycocalyx (capsule) around the bacteria. Another class of surface antigens expressed by some *E.coli* strains are

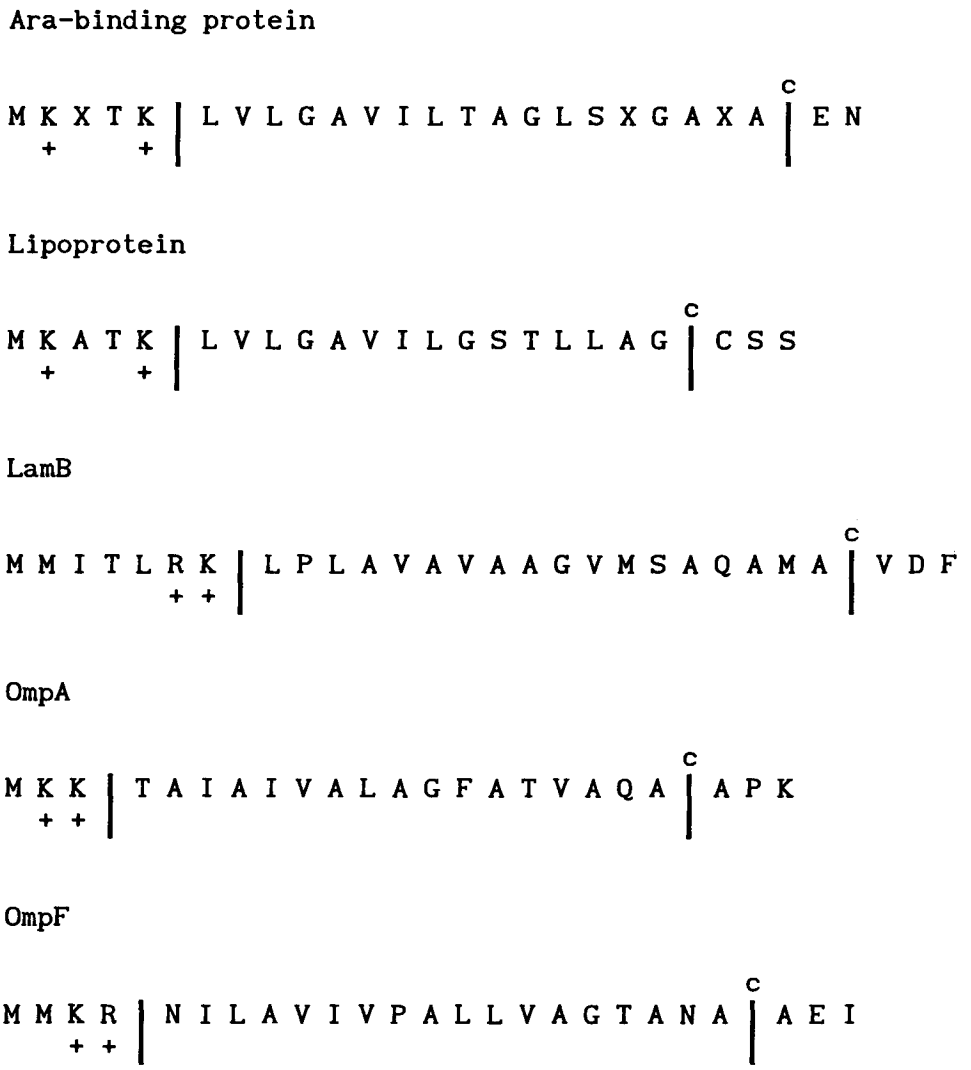


Figure 2. *E.coli* protein secretory signal sequences (adapted from 68)

Amino acids are shown in single letter code (see Appendix); X denotes undetermined residues. All sequences contain a positively charged N-terminal region followed by a long region of hydrophobic residues. The signal peptidase cleavage point is designated "c".

known as curli (82). Curli are coiled wiry fibres with a diameter of about 2 nm, made of a 17 kDa structural subunit, curlin. The curlin subunit is very unusual as it appears to have no signal peptide; its N-terminal region is highly homologous to a subunit protein of thin, aggregative fimbriae in a *Salmonella enteriditis* strain (83). Curli mediate binding to fibronectin, and it is suggested that they play a role in colonization.

Fimbriae, often known as pili or adhesins, constitute another class of bacterial surface structure. Many variations in these organelles have been described. Generally they consist of smaller filaments than the flagellae, made up of repeating protein subunits; these structures mediate adhesion to host cells and other surfaces. Non-fimbrial surface adhesins have however also been described. Often, a related side-effect of these adhesins is agglutination of different species' erythrocytes (hemagglutination) due to the presence of appropriate receptor molecules on the red cell surface (12). In fact, this effect was first noticed by Guyot in 1908 (86), long before mechanisms of bacterial pathogenesis were elucidated. Hemagglutination by pathogenic bacteria has simplified their study and has led to adhesin molecules often being described as hemagglutinins.

1.3. Bacterial Adhesins

Virulence factors in many Gram-negative bacteria have been described. Those found in *E.coli* will be reviewed in Section 1.4. *Pseudomonas Aeroginosa*, an opportunistic agent of local infections and a major cause of lung infection in cystic fibrosis patients, produces pili that have been shown to bind to specific receptors on host epithelial

cells (2). These pili consist of a single protein subunit of 15 kDa called pilin, which is chromosomally encoded. The pilin gene has been cloned and sequenced (3) and is translated as prepilin, with a leader peptide that is removed enzymatically leaving an N-terminal phenylalanine which is methylated. A whole class of N-methyl-phenylalanine pili has been described (5). In the related bacteria, *Pseudomonas cepacia*, it has been shown that the respiratory mucin-binding adhesin is a 22 kDa protein associated with surface pili that is dissimilar to the epithelial-binding pilin molecule (4).

Helicobacter pylori, a Gram-negative human gastric pathogen, produces an adhesin with a subunit of 19,600 Da that appears to be afimbrial, mediates agglutination of human and rabbit erythrocytes and shows a limited sequence homology with an adhesin from *Vibrio cholerae* (6). Type 1 mannose sensitive adhesins (i.e. adhesion inhibited by mannose in solution) have been studied in *Salmonella enterica* (7) and *Shigella flexneri* (8) and *Moraxella lacunta*, a causative agent of conjunctivitis, is known to express pili involved in pathogenesis (9). Other Gram-negative geni where fimbrial systems have been explored include *Neisseria, Klebsiella, Serratia* and *Proteus* (10). Clearly, adhesive surface structures are a near-universal feature of the Gram-negative bacteria.

Few Gram-positive strains possessing adhesive structures have been described. One genus, that of the *Corynebacterium*, show production of bundles of fimbriae that have been correlated with increased virulence (11). These fimbriae show very limited mannose-resistant hemagglutination against trypsinized sheep erythrocytes (13).

1.4. *Escherichia coli* Adhesins

Escherichia coli, a Gram-negative bacterium, is the most common organism found in the large bowel of humans and many other species. It generally exists in a symbiotic relationship with the host, although it is also an opportunistic pathogen commonly associated with gastrointestinal and urinary tract infections (14).

Escherichia coli produces some fourteen or more families of adhesin distinguishable by morphological, serological, chemical and receptor binding characteristics (15). These can be grouped into two classes - mannose-sensitive (type 1) and mannose-resistant adhesins. After attachment of bacteria to the host cell, disease can be caused in several ways: invasion of the host cells by the bacteria, production of toxins or other unelucidated pathways. In *E.coli* infections of the gut, this leads to a classification of bacteria as enteroinvasive, enterotoxigenic (ETEC) or enteropathogenic (EPEC).

1.4.1. Type 1 (Mannose-sensitive) Adhesins

1.4.1.1. Biochemistry

The majority of *Escherichia coli* stains express type 1 fimbriae and these structures have been extensively studied. Morphologically, type 1 pili average 7 nm in width and 2 μm in length (17) The assembly of the pili has been studied by immunoelectron microscopy to determine that subunits are added to the base of the growing pilus (18) . Duguid *et al* (12) determined that the fimbriae mediate strong mannose-sensitive

agglutination of erythrocytes from most species, including pig, dog, horse, guinea-pig and rabbit. Human erythrocytes are agglutinated moderately, sheep weakly, and only ox red blood cells show no agglutination. Brinton *et al* (16) purified type 1 pili and found that as well as the major 17 kDa structural pilin monomer there are three minor proteins present as integral components with one of these proteins (28 kDa) being the actual mannose-binding adhesin. Recent research has shown cryptic mannose binding sites present at intervals along the pilus that are inactive under normal conditions but that are exposed by freeze-thaw cycles (19). Immunological variation of type 1 adhesins has been described by Klemm *et al* (33). They found three variants with very similar N-terminal amino acid sequences which they named 1A, 1B and 1C. The expression of type 1 fimbriae is known to be phase-variable (expression controlled at the genetic level), with cells being either piliated or non-piliated. Isolates appear to phase-vary randomly in liquid culture but many are non-piliated when grown on plates (20). Variation with temperature is also known, with 37 °C favoured over 30 °C for fimbriae production (22).

1.4.1.2. Genetics

The operon (that is, the region of DNA) responsible for the correct expression of type 1 fimbriae was mapped on the bacterial chromosome by Schmidt *et al* (26) and cloned by Orndorff and Falkow (21). They showed that the operon has eight open reading frames (sequences of uninterrupted DNA prefaced by a start codon and terminated with a stop codon, usually encoding a single protein) determining the production of proteins involved in the biosynthesis and expression of pili (Fig. 3).

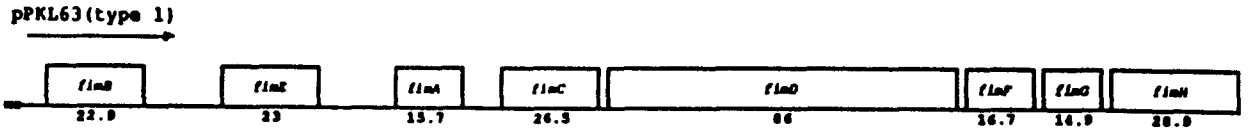


Figure 3. The type 1 fimbriae operon (from 101).

Numbers indicate size of protein products (x 1000 Da). Arrow indicates direction of transcription/translation.

The protein product of *fimA* (italics indicate genes, standard case with capitalized initial letter denotes protein) was found to be the structural pilus subunit and has since been determined to be under thermal expression control (22). The products of *fimB* and *fimE* are involved in regulation of expression; *fimE* has been shown to stimulate the *fim* invertible control element from on to off when bacteria are grown on solid media (23). The proteins FimC and FimD are involved in location of the fimbria, with FimC acting as a periplasmic chaperone (a protein that aids the folding and location of other proteins) molecule (24) and FimD being anchored in the outer membrane (28). The tip adhesin protein was determined to be the product of the *fimH* gene (25). The genes *fimF* and *fimG* appear to regulate the length of the pilus (29).

1.4.1.3. Receptors

The mannose inhibition of hemagglutination that type 1 adhesins exhibit shows that the fimbriae probably recognize mannose-like sugar residues on erythrocytes and other cells. Nakazawa and coworkers (30) have studied attachment of *E.coli* expressing the type 1 adhesin to the small intestine of piglets, Durno *et al* (31) have shown that type 1 fimbriae mediate attachment of bacteria to α-mannosyl residues on surface glycoproteins of rabbit epithelial cells and Dal Nogare has found that type 1 pili bind to a mannose-containing glycoprotein on tracheal epithelial cells (32).

1.4.2. Mannose-resistant Fimbrial Adhesins

Many mannose-resistant (MR) fimbrial agglutinins have been described. Generally, the occurrence of MR adhesins of certain types is correlated with somatic O antigens, the lipopolysaccharide complexes of the bacterial outer membrane. Production of most MR agglutinins is temperature sensitive. Synthesis is usually repressed when grown at 18 °C and heating bacteria to 65 °C most often inactivates the adhesin (60). Important MR systems are reviewed below.

1.4.2.1. Colonization Factors

Colonization Factor Antigen I (CFAI) was found on an *E.coli* strain causing diarrhea in humans (34). Related, but immunologically distinct, mannose-resistant hemagglutinins CFAII, CS (coli surface)I, CSII and CSIII were later described (35), and CSIV and CSV have recently been

identified (36,37). The CFAII and CSIII adhesins are afimbrial (see section 1.4.3.). McConnell *et al* (39) have shown that polyclonal rabbit antisera prepared to these antigens showed significant cross-reactivity between types.

Colonization factor antigen 1 has been isolated and purified to ascertain a subunit molecular weight of 15 kDa (38). The organization of the operon was found to be somewhat different to other *E.coli* adhesin operons, with two coding regions separated by 40 kbp (kilo base pairs); the first region contains the structural determinants and the second region the regulator (40). The first part of the CFAI operon has been cloned and the four genes it contains sequenced (40).

The receptors for these adhesins are largely undetermined, although Evans *et al* (44) have shown that the erythrocyte receptor for CFAII is more common among black donors than non-black donors.

1.4.2.2. S Fimbriae

The S fimbria, a subset of a larger heterologous group of oligosaccharide-binding (X) adhesins, are primary determinants in *E.coli* mediated meningitis in newborn infants (41). The pili consist of a fimbrial subunit protein polymer and a tip adhesin, the genes of which have been cloned and sequenced (42). Other involved genes have also been identified (42). The adhesin mediates MR hemagglutination of human erythrocytes and the O-linked sialyloligosaccharide on glycophorin A, a major erythrocyte membrane glycoprotein, has been identified as the receptor on the red blood cell (43). The binding behavior of S-fimbria to cultured human umbilical vein endothelial cells has been studied (44). Interestingly, adhesion of S-fimbriated *E.coli* to

buccal epithelial cells is inhibited by human milk fat globule membrane components, leading to the suggestion that these components inhibit bacterial growth in the entire intestine, providing protection from infection (45).

1.4.2.3. P Fimbriae

Fimbriae mediating adhesion to the uroepithelium in bacterial infection of the urinary tract are known as P or pap (pili associated with pylonethritis) fimbriae. The genetic structure of this system is very well known, as are its receptors: digalactoside-containing glycolipids found on the surface of erythrocytes and uroepithelial cells. The chromosomal operon mediating expression of these fimbriae was cloned by Hull *et al* (46) and is shown in Figure 4.

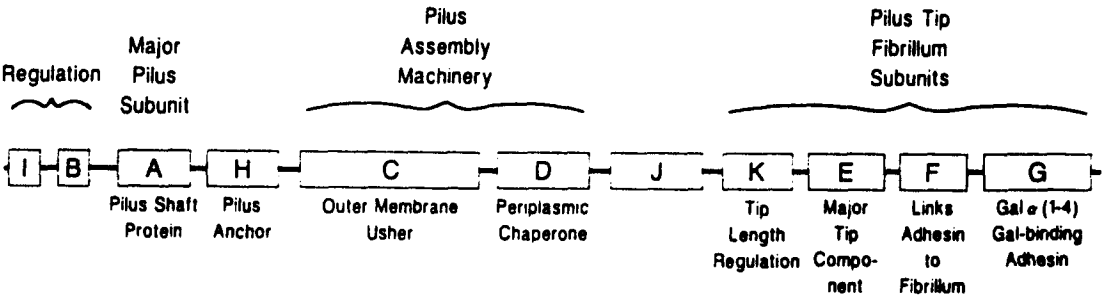


Figure 4. The *pap* operon (from 49).

The organization of this operon is very similar to that of type 1 fimbriae. The product of *papA* is a 16,500 Da protein that forms the pilus subunit; about 1,000 of these subunits are arranged helically to form hair-like appendages on the outside of the cell (47). It has been shown that mutants lacking PapA do not produce fimbriae, but do still bind the receptor (53). The digalactoside-binding adhesin is PapG (48), which is located on the tip of the fimbriae. The tip itself is mainly composed of repeating PapE protein subunits, with small amounts of PapF and K also present (49). The PapF protein is required for the correct presentation of the adhesin at the distal end of the tip structure; PapK joins the tip to the major part of the fimbria and regulates its length (55). All of these proteins are translated with secretory signal peptides. Figure 5 is a diagram detailing the biosynthesis of the pap fimbriae.

The molecular structures of some of these proteins are known in some detail. The three-dimensional structure of the periplasmic chaperone PapD has been solved to a resolution of 2.5 Å and a PapA binding area similar to that of an immunoglobulin has been proposed (50). The 88 kDa protein PapC has been given the name of "molecular usher", since it regulates an ordered assembly of the correct components on the outer membrane. This protein seems to be conserved in pilus-producing bacteria and interacts with the chaperone-subunit complexes on the periplasmic side of the outer membrane (51). Normark *et al* have studied the structure of PapG, the digalactoside-binding protein and have found that it consists of two domains, one for receptor binding (the amino-terminal end) and one that is involved in incorporation of the protein into the pilus (52). The amino-acid

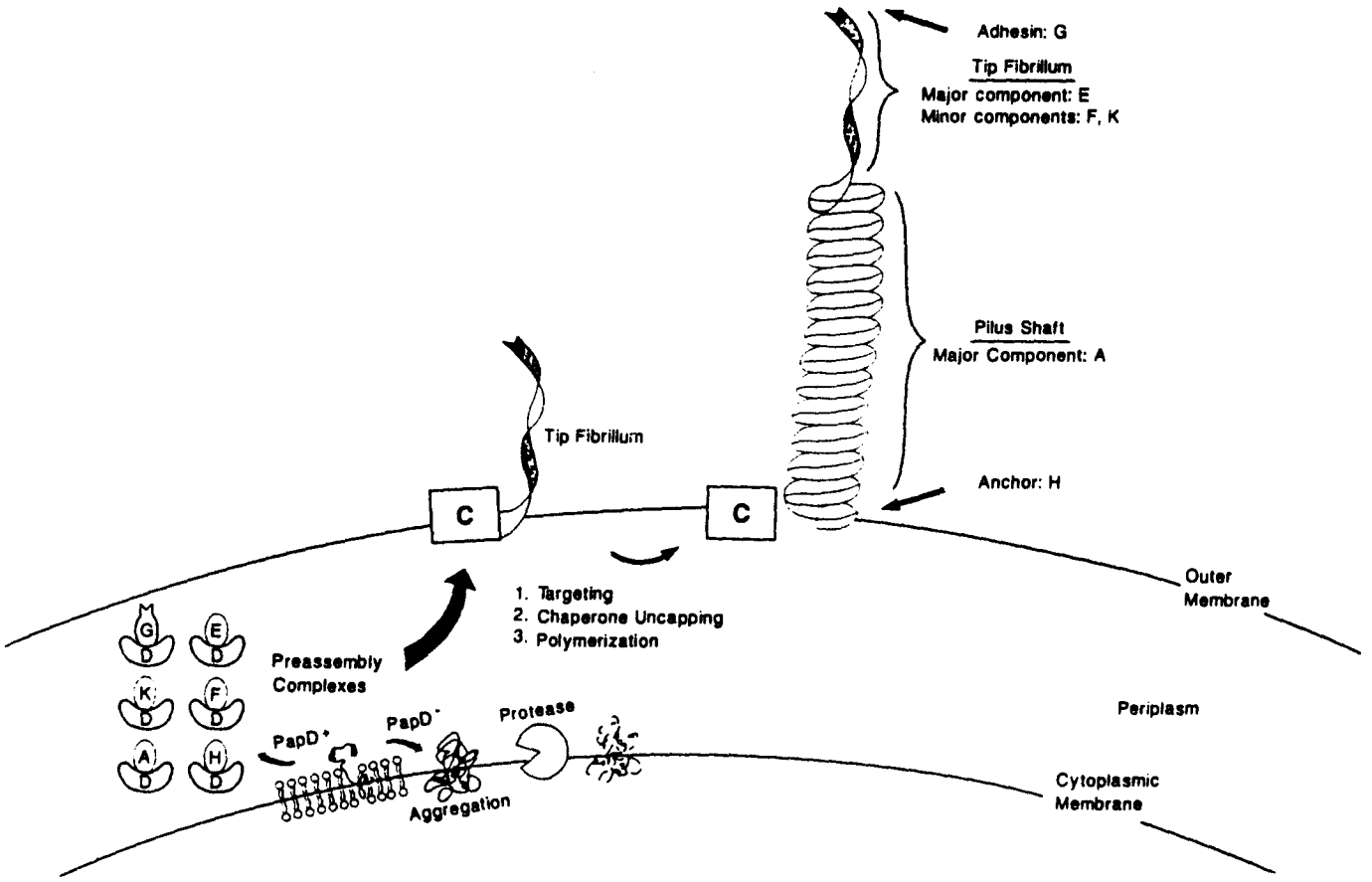


Figure 5. Putative assembly mechanism of pap pili (from 49).

primary structure has been deduced from the nucleotide sequence and shows some homology with the CFAI protein group (54).

The receptor for the pap pili is the α-D-galactopyranosyl-(1-4) -β-D-galactopyranose moiety present on the glycolipids on the surface of cells lining the urinary tract (56). The production of pap pili is phase variable; there is evidence to suggest that, as well as the pap operon regulatory proteins, DNA methylation upstream of the regulatory region of the operon occurs, controlled by the mbf (methylation blocking factor) locus (57) and involving Lrp (leucine-responsive regulatory protein) (58).

1.4.2.4. The K88 Antigen

The K88 adhesive antigen was discovered by Orskov *et al* (59) who also distinguished two variants K88ab and K88ac. The K designation was assigned although it was found that the antigen was in fact a protein rather than a polysaccharide (88). A third variant K88ad has since been described (61). The major fimbrial subunit has a molecular weight of between 23.5 kDa and 27.5 kDa depending on the variant. It has a 21 amino acid N-terminal signal sequence and contains no cysteine residues, which implies hydrophobic or electrostatic interactions hold the subunits together in the pili (60). The plasmid-encoded genetic determinant of these fimbriae was cloned (62) and is found to be similar to other fimbriae determinants, with components for structure, adhesion, construction and expression grouped in the operon. However, despite this similarity it appears that, in contrast to other systems, the major component of the fimbriae also carries the adhesive property (63). Jacobs *et al* modified the K88 fibrillar subunit by site-directed

mutagenesis and found that the replacement of phenylalanine 150 with serine resulted in production of fimbriae that were essentially indistinguishable from wild-type, but which had lost the adhesive capacity of the pili (64). They ascertained that the region containing this phenylalanine residue shared some sequence identity with a conserved region in the gonococcal pilin and in the B-fragment of diphtheria toxin, supporting a common receptor binding function. The receptor for K88 is unknown, though there is some evidence indicating galactosyl residues are important (60).

1.4.2.5. The K99 Antigen

The K99 fimbriae have a helical structure with a diameter of 4.8 nm, made of structural protein subunits of 18,500 Da (65). Production of fimbriae is dependent upon utilizing minimal growth media The operon codes for seven proteins involved in biosynthesis and deletion mutants constructed by de Graaf and coworkers (65) indicated that all polypeptides were required for MR agglutination of horse erythrocytes. The adhesive function was determined to reside on the structural subunit (105) and was found to be specific for carbohydrates on erythrocytes (66), with sialic acid residues (sialic acid is, strictly, N-acetyl neuraminic acid (Fig. 6) although the term "sialic acids" generally comprises all N- and O-acyl derivatives of neuraminic acid isolated from natural materials) being implicated. The equine erythrocyte receptor was determined by Smit *et al* (72) to be the glycolipid Neu5Gc-α(2,3)-Gal-β(1,4)-Glc-β(1,1) -ceramide (where Neu = neuraminic acid, Gal = Galactose and Glc = glucose). This specificity was confirmed by Lindahl and Carlstedt (67) by studying binding of purified K99

fimbriae to sections of pig small intestine. The fimbriae were found to bind to high molecular weight mucin glycopeptides and the binding was abolished when these glycopeptides were enzymatically desialated. The N-terminal amino acid sequence of the structural subunit shows some identity with the K88 fimbrial protein and limited identity with the F41 adhesin described below (69) (Figure 7).

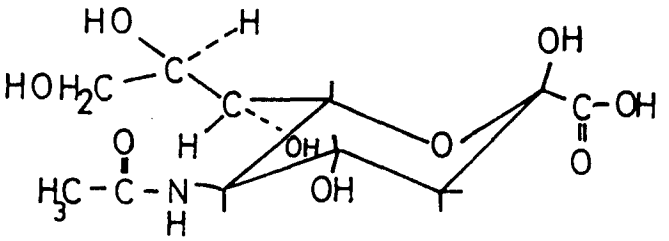


Figure 6. Sialic acid (N-Acetyl-β-D-neuraminic acid)(from 102)

1.4.2.6. The F41 adhesin

This MR adhesin was discovered on a K99 negative mutant of the K99 reference strain B41M (serotype O101:K(A):NM) (69). As with K99, the production of this antigen was found to be dependent on the composition of the growth media with biosynthesis being repressed by rich media and optimum production occurring when a minimal salt medium was used. The agglutinin molecule was very easy to detach from the cell by simply vortexing a bacterial suspension for five minutes; denaturing polyacrylamide gel electrophoresis showed that the fimbriae were

composed of subunits of molecular weight 29,500 Da. The production of this antigen appeared to be restricted to the serotypes 09 and 0101. The amino acid composition was determined, as was the N-terminal amino acid sequence, which was compared to those of other adhesins (Fig. 7). Limited sequence identity was found with K99, but not with K88, CFAI or type 1.

The F41 chromosomal genetic determinant was cloned from a different porcine enterotoxigenic *E.coli* strain (VAC1676, 0101:K30:F41:H-) by Moseley *et al* (70). Their results showed that there was significant homology between the genes encoding F41 and those encoding K88, as DNA probes specific for the K88 operon hybridized to the cloned F41 determinant. In later work, Moseley and Anderson described the genetic organization of the operon, detailing several proteins that together mediated full expression of the F41 fimbriae. The accessory proteins showed high sequence identity with the equivalent proteins in the K88 operon but there was little similarity in the fimbrial subunits themselves apart from the signal peptide sequence (71). Moseley and Anderson conjecture that the homology between adhesin synthesis and assembly systems indicates the ability of the bacterium to rapidly evolve new fimbria differing in antigenic structure. The gene coding for the fimbrial subunit was sequenced and its deduced amino acid structure was found to differ significantly from the amino acid composition determined by analysis of the purified F41 structural unit from *E.coli* B41M reported by de Graaf and Roorda (69), although the N-terminal sequences are almost identical.

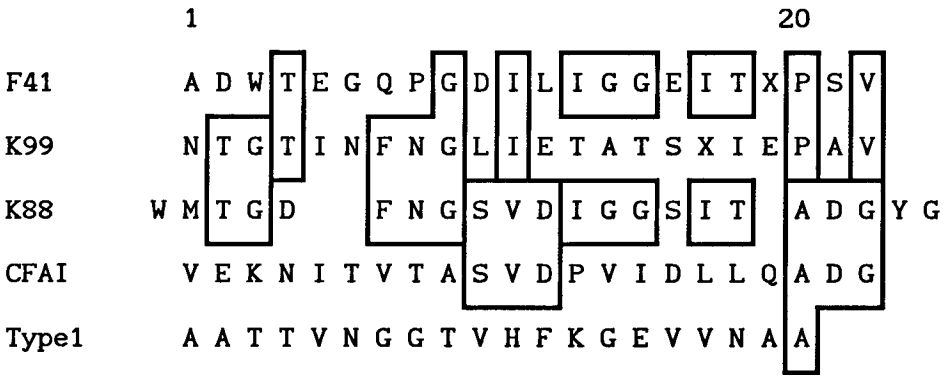


Figure 7. Comparison of adhesin N-terminal sequences (adapted from 69). Regions of identity are boxed. For clarity, not all identity relationships are shown.

The receptor for the F41 adhesin on human erythrocytes is glycophorin A (73). The agglutinating activity of the purified adhesin is greatest when the erythrocytes used are of blood group MM and least when the blood group is NN (76). The M/N antigen lies in the first and fifth amino acid residues of the N-terminal region of glycophorin A as shown in Figure 8 (74). Glycophorin MM has an N-terminal serine and a glycine at position five; glycophorin NN has leucine and glutamic acid in these respective locations. Neuraminidase treatment of the erythrocytes (which removes the terminal sialic acid residue) also decreases agglutination showing that both the amino acid sequence and the carbohydrate at the N-terminus are important in the recognition of the receptor by the adhesin.

A thermodynamic study of the binding of radiolabelled purified F41 adhesin to radiolabelled glycophorin A^{MM} utilizing an aqueous two-phase polymer system has indicated a stoichiometry of 1 adhesin monomer to 1 glycophorin molecule. This indicates that the F41 system does not require a specialized tip adhesin, but that binding sites are an integral part of the structural subunit (76). This suggestion was recently strengthened by epitope mapping of the adhesin with monoclonal antibodies, where 23 antibodies recognizing five highly conserved epitopes were produced (77). The antibody binding was studied by immunoelectron microscopy and showed that the epitopes were equally distributed along the fimbrial structure. The binding of F41 to glycoproteins in the bovine and porcine colostrum was studied by Mats Lindahl (75). He found that the adhesin was carbohydrate specific, binding to periodate-sensitive oligosaccharides present on the glycoproteins.

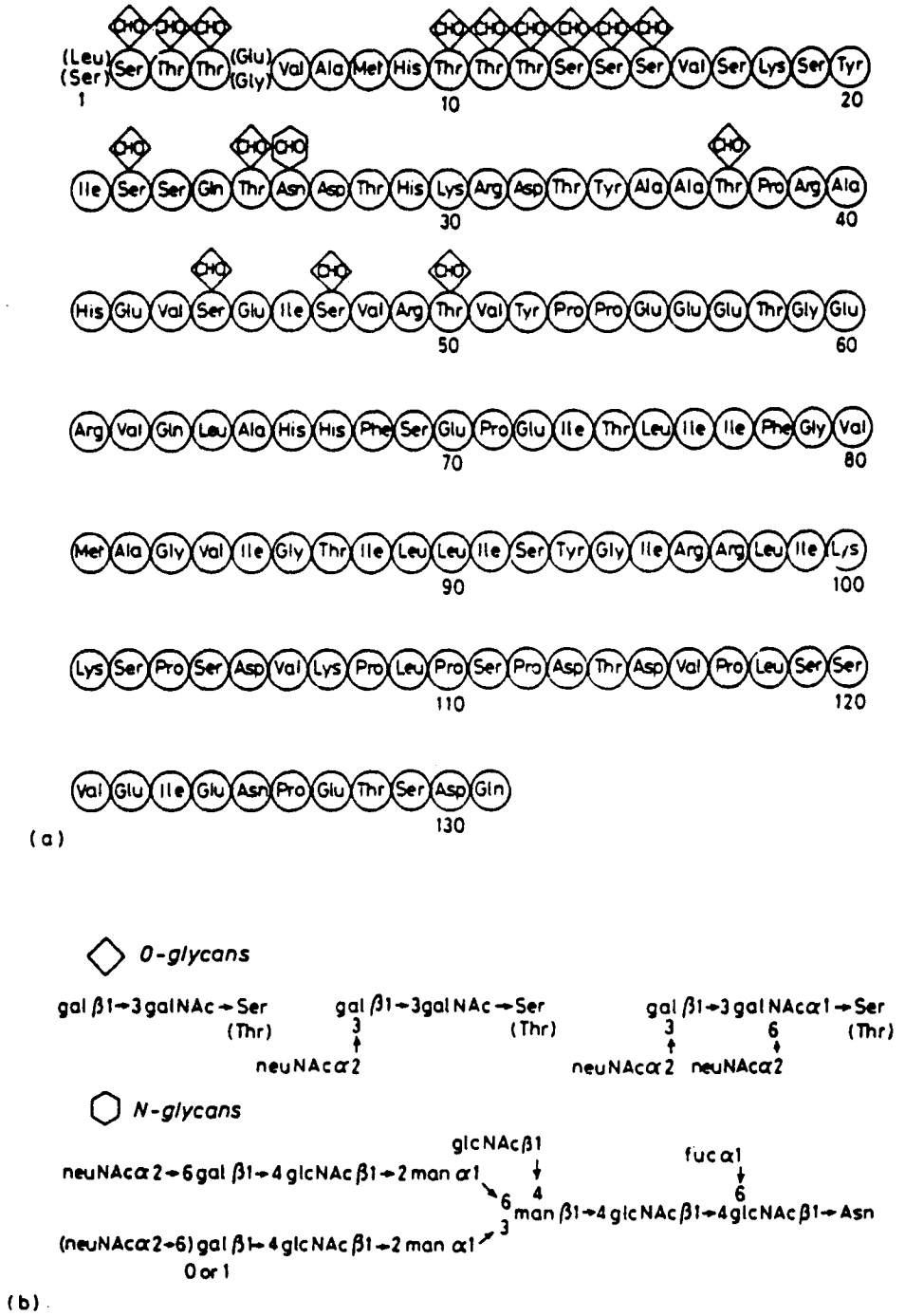


Figure 8. Glycophorin A (from 103)

(a). Primary structure showing glycosylated amino acid residues

(b). Generalized structures of the carbohydrate units

1.4.2.7. Other *E.coli* Fimbrial Adhesins

Other fimbrial adhesins include CS31A, present on *E.coli* isolates from diarrheic or septicemic calves. The CS31A determinant is encoded on a 105 MDa plasmid. The fimbriae produced are thin (2 nm) and consist of a structural subunit of 29,500 Da (78). Amino acid sequencing of the N-terminus of this protein showed that it shares a high degree of sequence identity with K88 (Fig. 9) however relocation of the plasmid into a non-hemagglutinating strain does not confer hemagglutination even though CS31A fimbriae are produced.

CS31A	G	T	T	G	D	F	N	G	S	F	D	M	N	G	T	I	T	A	D	A	T	K		
K88	W	M	T	G	D	F	N	G	S	V	D	I	G	G	S	I	T	A	D	G	Y	G		

Figure 9. N-terminal homology of CS31A and K88
(Adapted from 78)

The CS31A determinant was cloned from genomic DNA using F41 determinant DNA probes (79), indicating that the entire determinant was related to that of F41. The clone harbouring the recombinant CS31A antigen was able to adhere to cultured epithelial cells.

The 987P fimbriae are composed of subunits of 20,000 Da and are morphologically identical to type 1 pili, although they show a MR hemagglutination pattern (60). The plasmid encoded 987P operon has been cloned and shown to contain open reading frames for eight proteins (80).

Analysis of these genes has shown that, although all gene products are involved in pili biosynthesis, the lack of expression of a single protein never resulted in the separation of fimbriation from adhesion, strongly suggesting that the fimbrial subunit is also the receptor binding adhesin (81).

The bundle-forming pilus present on some enteropathogenic *E.coli* strains is so-called because filaments of the repeating 19.5 K subunit aggregate into rope-like bundles that bind together individual bacterial cells (85). This effect occurs when the bacteria are grown on blood agar. The structural gene was cloned (167) and it was found that when this gene was used as a DNA hybridization probe, all enteropathogenic *E.coli* strains showed homologous regions, as did most of the *Salmonella* strains tested. The authors suggest that this pilus is involved in initial colonization early in the infective process.

1.4.3. Afimbrial Adhesins of *Escherichia coli*

Much less is known about these structures, several of which have been identified in the last ten years. Afimbrial adhesins were first described by Duguid *et al* in 1979 (84). In a survey of 387 strains of *E.coli*, they found that approximately ten percent of strains that cause hemagglutination do not appear to produce fimbriae when viewed under the electron microscope. These bacteria are thought to express non-fimbrial (or afimbrial) adhesins. Since then several afimbrial systems have been investigated, some of which are detailed below.

1.4.3.1. The Z Antigens.

Three uropathogenic MR-hemagglutinating *Escherichia coli* strains were studied by Orskov *et al* (87). They found that the strains possessed a common surface antigen (Z1) when grown at 37 °C, but not at 18 °C. One of the strains produced an additional antigen Z2. Unlike other MR strains, heating to 100 °C did not destroy the agglutination. A mutant that produced much less of the Z1 antigen was non-adherent, so the Z1, and possibly the Z2 moieties were held responsible for the hemagglutination. The Z1 antigen was identified as a 14,400 Da. protein. Immunoelectron microscopy of the embedded, sectioned bacteria showed that the protein formed a capsule around the bacterium. In electron microscopy studies after staining the bacteria with uranyl acetate, this capsule appeared as a mesh of fine filaments. The authors tied their results in with results of a group who had isolated a nonfimbrial MR hemagglutinin from two enteropathogenic *E.coli* strains, of monomer molecular weight of about 14,500 Da (89). This group had noticed a capsule on the cells of both strains despite the fact that no polysaccharide K antigens were included in the serotype designations; it was proposed that this may indicate a similar adhesive protein capsule.

1.4.3.2. The NFA Group of Afimbrial Adhesins

Non-fimbrial adhesin (NFA)-1 and NFA-2 were first reported by Goldhar and coworkers (92) as consisting of soluble proteins of molecular weights 21,000 and 19,000 Da respectively. Both mediate a mannose resistant hemagglutination of human erythrocytes and were shown

to bind to cultured human kidney cells. Biosynthesis of both is halted when the bacteria are grown at 18 °C. Adhesive bacteria of the *E.coli* strain expressing NFA-1 showed an extracellular capsule-like layer, whereas non-adhesive cells did not. The hemagglutinating strain expressing NFA-2 did not have this layer. The proteins tended to form aggregates of high molecular weight, and Goldhar *et al.* state that the proteins recognize some glycoprotein on the erythrocyte. The adhesins NFA-3 and NFA-4 have been described (95,96). The NFA-3 protein has a molecular weight of 17,500 Da and is specific for glycophorin^{NN}. Non fimbrial adhesin 4 is 28,000 Da and binds glycophorin^{MM}, but shows no sensitivity to the presence of sialic acid residues.

1.4.3.3. AIDA-I (Adhesin Involved in Diffuse Adherence I)

This adhesin was cloned from a large plasmid present in an enteropathogenic *E.coli* strain (90). A 100 kDa protein was identified as the product of the smallest subclone that still mediated adherence to HeLa cells. This molecular weight is similar to that of another afimbrial adhesin, EAF (enteropathogenic adherence factor), also coded on a large plasmid (91). These adherence factors do not show hemagglutination and seem to belong to a separate family of adhesins being discovered on enteropathogenic *E.coli* strains.

1.4.3.4. AFA-1 (Afimbrial Adhesin-1)

The adhesin AFA-1 exists on the bacterial surface and as an aggregate in culture supernatants. It has a monomer molecular weight of 16,000 Da and has been found to agglutinate only human and gorilla

erythrocytes. Glycophorin A is not bound by AFA-1 (93). The operon encoding AFA-1 biosynthesis was cloned from chromosomal DNA (94) and found to contain genes coding for five polypeptides. The nucleotide sequence of the structural subunit gene, *afaE*, was determined and was found to have no similarity to known fimbrial or afimbrial adhesins. Nowicki *et al* (97) have studied the receptor binding specificities of AFA-1 and the related afimbrial adhesins AFA-3, the Dr hemagglutinin and F1845. They have identified the common erythrocyte receptor as the Dr antigen, a cell membrane protein called DAF (decay accelerating factor). The protein takes part in the regulation of the complement cascade. The researchers, using enzyme treated erythrocytes and monoclonal antibodies, found that different regions of the protein were recognized by the different adhesins, showing that the agglutinins were distinct, but related.

1.5. Viscometric Studies on Bacterial Adhesins

Although bacterial adhesins are the subject of much research, little attention has been paid to the effect of the *in vivo* physiological environment in which these adhesins act. Bacteria are able to colonize mucosal surfaces despite fluid and mechanical shear caused by blood, mucus, urine and gastrointestinal content flow. The viscometer, a device that measures the viscosity of a liquid under shear, provides a convenient *in vitro* method for studying bacteria-erythrocyte interactions under these conditions (98).

1.5.1. Viscometric Analysis of Erythrocyte Suspensions

Einstein showed that the viscosity of a very dilute suspension of non-interacting particles under shear was a linear function of the volume fraction occupied by these particles (149). This equation only holds true for suspensions where the volume fraction of the particles is less than 2 %. For higher particle concentrations, the relationship is based upon higher order terms in the volume fraction (150), and theory exists to model the viscosity of non-interacting suspensions with particle volume fractions of the order of 10 % (151). In the viscometric experiments described from the literature below, and for those in this work, the viscosity of a concentrated (≅50 %) erythrocyte suspension is measured. These suspensions behave as a complex non-Newtonian fluid, with the cells being non-spherical, interacting electrostatically and deforming under shear. There is as yet no theory that allows modeling of such a complex system. It is known experimentally, however, that all other factors being equal, red cell aggregation can greatly enhance the viscosity of the erythrocyte suspension (152).

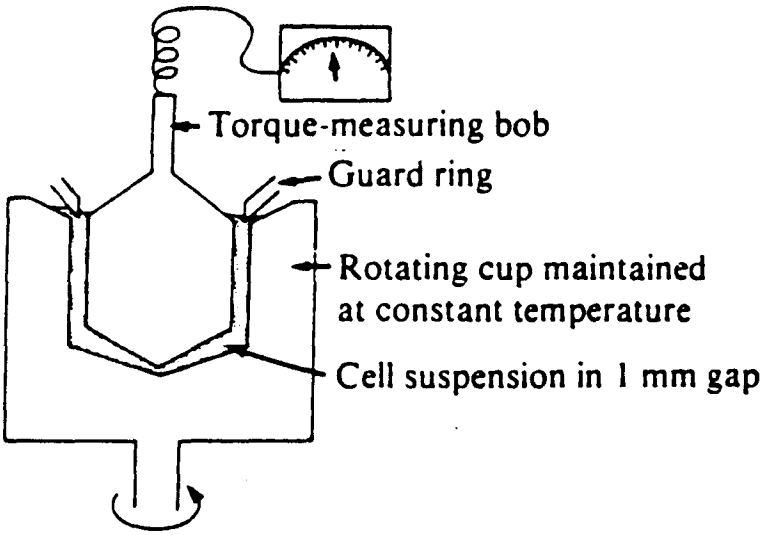
The viscometer consists of a rotating cup and a central bob (Fig. 10). There is a small gap between the cup and bob into which the solution under test is placed. The cup is then rotated at a constant angular velocity, producing a roughly constant shear rate (velocity gradient) throughout the sample, assuming Newtonian flow. The central bob is held still by the application of an electrical torque that is proportional to the shear stress exerted upon it by the liquid, and hence proportional to the viscosity of the liquid (viscosity = shear stress/shear rate). A signal proportional to the shear stress is

provided continuously by the instrument. Normally a liquid will show a viscosity that will not vary over time. Samples of material that aggregates will show an increase in apparent viscosity relative to the non-aggregated condition. If the sample is a cell suspension aggregated by multivalent ligands, the viscosity may increase as shear continues. The hemagglutination event that occurs between erythrocytes and bacteria is an example of this. The apparent viscosity of the bacteria-erythrocyte mixture at constant shear rate is increased over that of a non-aggregating suspension because bonds between the cells are being formed and energy is required to break them if the suspension is to flow in the velocity gradient between the cup and the bob. This energy dissipation is the source of the extra shear stress and therefore the increase in viscosity.

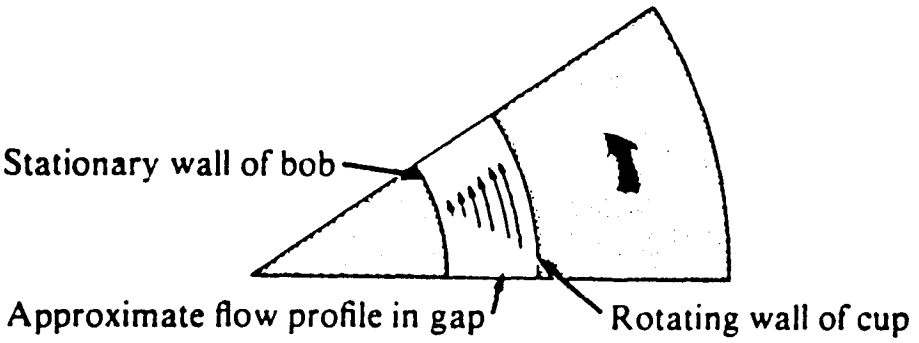
The technique of viscometry is useful for the study of bacteria-erythrocyte interactions because a quantitative index of the degree of agglutination (and hence the strength of the agglutination event) can be expressed by the value R, which is the ratio of the shear stress in the presence of aggregation to that in its absence.

1.5.2. Viscometric Analysis of *Aeromonas salmonicida* 438.

The hemagglutination event mediated by *Aeromonas salmonicida* 438 was studied by measuring the viscosity of a mixture of erythrocytes and bacteria under shear, and it was found that adhesion was enhanced by shear forces, as indicated by a viscosity increase (99). The adhesion reaction was found to consist of two phases, a fast (10 s) initial viscosity rise followed by a slower increase which reached a plateau at approximately 30 min. The slower, later phase of the reaction was



Cross-section of concentric cylinder viscometer



Transverse section showing flow profile

Figure 10. Diagrammatic view of a couette type viscometer

determined to be induced by the shear, as bacteria-erythrocyte mixtures that were preincubated without shear for certain lengths of time always showed lower viscosity when shear was applied than mixtures that had been subjected to shear for the entire time.

1.5.3. Viscometric Analysis of *E.coli* Adhesins

When erythrocytes were sheared with purified F41 adhesin the same effect was noticed as for the *Aeromonas salmonicida* bacteria (Figure 11). A model for this shear enhancement involving a shear induced rearrangement of the membrane proteins involved was put forward (100). This model states that after initial contact and adhesion takes place, there is a shear induced rearrangement of the receptor proteins in the fluid membrane increasing the probability of more adhesive interactions occurring. As these interactions build, the adhesion becomes progressively stronger.

Viscometric analysis was also used to determine the M/N blood group specificity of the F41 adhesin (76). Although the agglutination difference between MM and NN erythrocytes was unnoticed in a microtitre assay at room temperature, at 37 ^{o}C in the viscometer the F41 mediated agglutination of NN erythrocytes was noticeably less than that of the cells carrying the MM antigen (Figure 12).

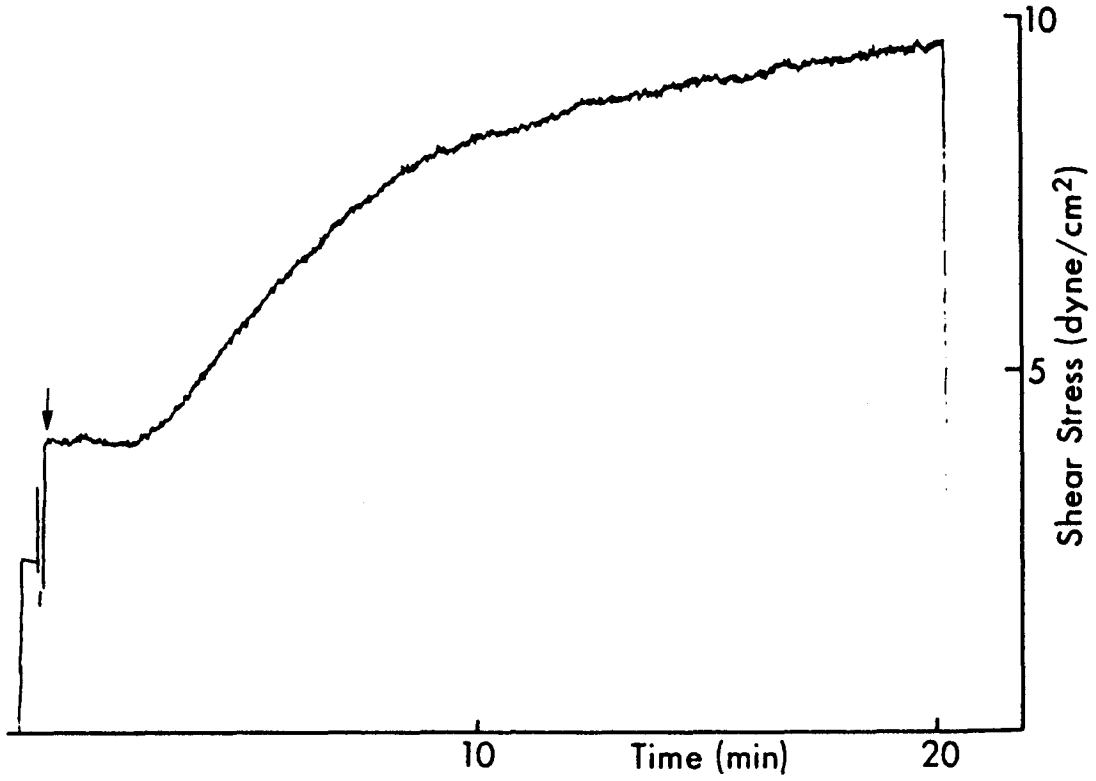


Figure 11. Adhesion event between *E.coli* F41 adhesin and erythrocytes as determined by viscometry (from 100). Adhesin added to erythrocyte suspension at the arrow.

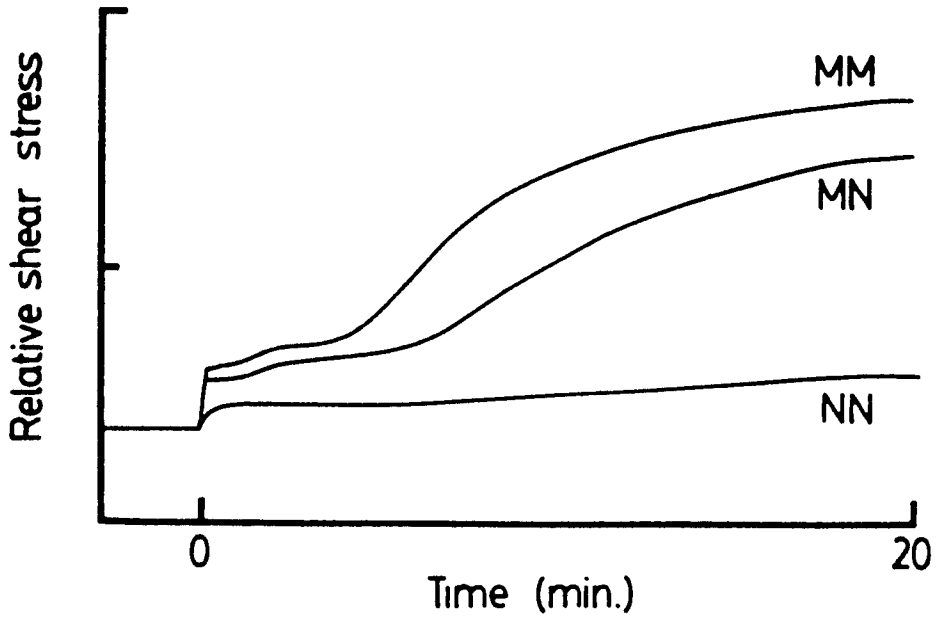


Figure 12. Differential agglutination of MM and NN erythrocytes by F41 adhesin as determined by viscometric assay (from 76). (shear rate = 49 s^{-1})

1.6. Partition of Bacterial Cells in Aqueous Two-phase Systems

1.6.1. Principles of Aqueous Two-phase Polymer Partition

Aqueous solutions of polymers are often incompatible above certain concentrations such that if two are mixed they will rapidly separate into two phases, each of which is enriched in one of the polymers, with the denser solution becoming the lower layer. An example of this phenomenon is the phase system composed of dextran (Dx, Fig. 13) and poly(ethylene glycol) (PEG, Fig. 14), which consists of a dextran-rich lower phase, and a PEG-rich upper. Another system used in this thesis is dextran\Ficoll (Ficoll is a synthetic branched polymer made by the copolymerization of sucrose and epichlorohydrin, (104)). A general phase diagram for the PEG/Dx system is shown in Appendix 2. These phase systems can be made up in buffers to suit a wide range of physiologically sensitive applications. When cells such as erythrocytes are added to these systems, the cells partition unequally among the upper and lower phases and the interface, which acts as a third compartment (107). Some ions, such as phosphate, show preferential partition in one phase over the other, leading to an electrostatic potential difference between phases. Such systems are termed charge-sensitive and can be useful for separations based on the electrostatic properties of cell surfaces (108).

The partition of cells in phase systems can be influenced by a wide range of properties of the cell surfaces. In charge-sensitive systems, the charge on the cells is an important factor in their partition (108), and other determinants such as cell surface-polymer

interactions (including polymer adsorption to cells) also appear to be significant (108). From the hundreds of papers that have been published on cell partition it is clear that the cell distribution in a given system is very sensitive to both the properties of the system and of the cell surface. There is as yet no theory that allows the cell surface structure to be predicted from the partition behavior, however (109).

1.6.2. Bacterial Partition

The first partitioning studies with bacteria were performed by Albertsson (who also developed the partitioning technique). He used the multiple partition method of countercurrent distribution in PEG\dextran systems to separate what was thought to be a single strain of *E.coli* into two populations based upon differences in their surface characteristics (110). In *E.coli*, the O and K antigenic determinants have been investigated as sources of partition differences. The capsular polysaccharide K antigens on the surface of Gram-negative bacteria contain a large amount of acidic carbohydrate material and effectively render the bacteria negatively charged, which to a great extent dictates partition in charge-sensitive systems. In these systems the bacteria tend to show affinity for the phase which is relatively positive in potential (the PEG-rich phase in dextran/PEG charge-sensitive systems). Lipopolysaccharide (LPS) structure varies considerably between strains of bacteria and has been shown to influence partition in non charge-sensitive systems. In one set of experiments, isolated LPS from two different *Salmonella typhimurium* strains was partitioned in a dextran\PEG system. The LPS partitioned very differently, with one type strongly favouring the PEG rich upper phase

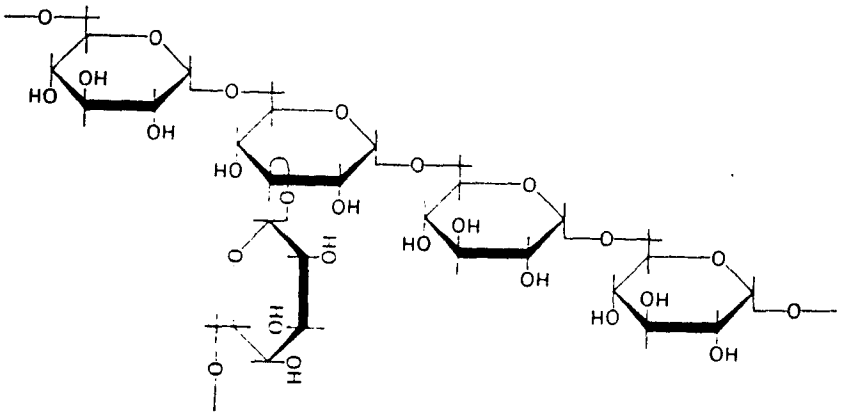


Figure 13. Dextran

$$H-[-O-CH_2-CH_2-]_n-OH$$

Figure 14 Poly(Ethylene Glycol)

and the other strongly favouring the dextran rich lower phase. This difference was also seen when the whole bacteria were partitioned, indicating that the LPS was the major determinant of the partition difference (111)

The role of fimbriae in determining partition has also been studied. Using a PEG\dextran system with differing amounts of the hydrophobic PEG-palmitate derivative in the upper phase, it was found that the partition of piliated strains of *Neisseria gonorrhoeae* was less affected by the PEG-palmitate (and hence said to be less hydrophobic) than non-piliated strains (112). Kihlstrom and Magnussen (113) found that hemagglutinating properties (probably mediated by fimbriae) of *Yersiniae* species correlated with increased tendency to hydrophobic interaction. As mentioned earlier, the valency of the F41 adhesin was determined using a PEG/dextran phase system to generate an equilibrium binding isotherm based on the differing partitions of the F41 adhesin and its receptor, glycophorin.

2. Cloning of Hemagglutinin Gene

2.1. Background and Aims

The *Escherichia coli* strain 09:H10:K99 is known to express at least two mannose-resistant agglutinins, K99 and F41 (114). As described earlier, the K99 antigen is an 18,500 Da protein that is plasmid encoded and agglutinates horse erythrocytes, but not human (65), whereas F41 has a molecular weight of 29,500 Da, is chromosomally encoded and agglutinates human erythrocytes as well as several other species (69).

The original aim of this study was to clone and investigate the F41 adhesin, which has since been cloned by Moseley (70). To this end the cloning procedure was designed to take advantage of the fact that only the F41 adhesin agglutinates human red blood cells. A simple microtitre assay was used to screen quickly insert-containing colonies. This initial assay was then backed up with colony blots using antibodies raised against the purified F41 and K99 adhesins and by an oligonucleotide probe designed from the N-terminal amino acid sequence of the F41 protein. On the basis of the results of these assays, a clone was chosen for further study which showed the interesting property of exhibiting agglutinating activity with absence of both the K99 or F41 antigens, and that did not hybridize to the oligonucleotide probe.

2.2. Materials and Methods

2.2.1. Bacterial Strains and Growth Conditions

E.coli 09:H10:K99 ($K99^+$,$F41^+$) was originally isolated by R.E. Isaacson (115) and was kindly supplied by T. Wadström. Hereafter, this strain is referred to as " F41 ". *E.coli* JM101 (116) was used as the host strain for cloning and maintenance of recombinant plasmids. Both strains were grown in Luria-Bertani (LB) broth (117), which was supplemented with Difco agar (1.5 %) if solid media was required. Cells were grown aerobically at 37 °C, and the media was supplemented with ampicillin (100 μg/ml) for maintenance of plasmid-containing clones. Tetracycline (15 μg/ml) was added for the original screening of the library for clones that contained a foreign insert in the pBR322 plasmid.

2.2.2. Enzymes and Reagents

All restriction enzymes were purchased from either Boehringer Mannheim, Laval, Quebec, New England Biolabs, Beverly, MA, USA, or Bethesda Research Laboratories (BRL), Gaithersburg, MD, USA. Modifying enzymes came from the same sources. Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridium bromide) was obtained from Sigma, St. Louis, MO, USA. For probe labelling, γ-^{32}P-ATP was purchased from Amersham, Arlington Heights, IL, USA. X-Phosphate (5-bromo-4-chloro-3-indolyl-phosphate) and nitroblue tetrazolium, the colour development reagents used in alkaline phosphatase conjugated

antibody assays, were purchased from Bio-Rad (Richmond, CA, USA). All other chemicals used were of analytical grade and obtained from chemical suppliers.

2.2.3. Buffers

Restriction enzyme buffers used were those supplied by the manufacturers with the enzymes. Buffers for DNA suspension, ligations, and modifying reactions were as described by Maniatis *et al* (117). For gel electrophoresis of DNA, tris-acetate-EDTA (TAE, 0.04 M tris-acetate, 0.002 M EDTA, pH 8.0) was used (117). Phosphate-buffered saline (PBS, 130 mM NaCl, 16.7 mM Na_2HPO_4 and 3.0 mM NaH_2PO_4, pH 7.4) was used to wash and suspend erythrocytes in the hemagglutination assay, and for washing filters in the antibody assay. Where desired this was supplemented with 10 mM mannose.

2.2.4. Plasmid

The plasmid pBR322 (118) was used for the cloning procedure. This plasmid utilizes the ColE1 origin of replication and contains genes that code for proteins that make the host cell resistant to ampicillin and tetracycline.

2.2.5. Antibodies

The monoclonal antibodies against the F41 adhesin were made by Nancy Hamilton in the Brooks laboratory and the K99 antisera was kindly donated by Dr. Steve Acres from the Veterinary Infectious Diseases

Organization (VIDO), Saskatoon, SK.

2.2.6. Gel Electrophoresis of DNA

Agarose gel electrophoresis for visualization and preparation of restriction digested DNA was performed in submerged horizontal gel systems (117). Gel composition was routinely 0.8 % agarose / 0.5 μg/ml ethidium bromide in TAE buffer. Ethidium bromide complexes with DNA by intercalating between stacked base-pairs and allows visualization of the DNA as it fluoresces orange under ultraviolet illumination when bound. The applied voltage and electrophoresis time varied with experiment. The gels were photographed with a Polaroid 57 4x5 Land Film camera with orange filter and were illuminated for photographs with a short-wavelength UV transilluminator.

2.2.7. Gel Electrophoresis of Proteins

Protein samples were fractionated on denaturing polyacrylamide gels by a modification of the procedure of Davis (119) and Ornstein (120). The gels were cast and run on the Bio-Rad Mini Protean II slab gel system. Acrylamide:bis solution (30:08) was made up as follows: Acrylamide (60 g) and N,N'-Methylene-bis-acrylamide (1.6 g) were dissolved in distilled water to a final volume of 200 ml. The solution was filtered through Whatman #1 filter paper and stored at 4 °C in the dark. For the resolving gel, the acrylamide:bis stock solution (12.5 ml) was mixed with 1.875 M tris-base pH 8.8 (6 ml), 0.2M EDTA (300 μl), water (10.9 ml) and SDS (300 μl) and degassed under vacuum. To this solution was added 300 μl of freshly-made ammonium persulphate

($(NH_4)_2S_2O_8$, 10 %) and 15 µl TEMED, and the solution was carefully pipetted between the glass plates of the casting apparatus (1 mm spacers). Water was layered onto the solution surface and the polymerization was complete in 30 min. The water was removed, the gel top rinsed, a sample comb inserted and a stacking gel composed of acrylamide:bis (2.5 ml), tris.HCl pH 6.8 (1.88 ml), 0.2 M EDTA (150 µl), 10 % SDS (150 µl), 10.3 ml H_2O, 10 % ammonium persulphate (150 µl) and 7.5 µl TEMED was pipetted on top of the resolving gel. The quantities of solutions described were sufficient to pour two gels each. When polymerization was complete (30 min), the comb was removed and the gel loaded into the running chamber. The chamber was filled with running buffer, a 4:1 dilution of the stock solution, 14.4 g/l glycine and 1 g/l SDS in 0.05 M tris-base.

Protein samples were prepared for electrophoresis by mixing with an equal volume of sample buffer (4.0 ml water, 1.0 ml 0.5 M tris-HCl, pH 6.8, 0.8 ml glycerol, 1.6 ml 10 % SDS, 0.4 ml 2-mercaptoethanol and 0.2 ml 0.05 % (w/v) bromphenol blue.) and boiling for 5 min. The bromphenol blue was omitted when preparing samples to be sent for N-terminal sequencing. Samples (5-20 µl) were applied to the gel and a current of 150 mA was applied until the tracking dye reached the bottom of the gel. Gels were then removed from the apparatus and were either stained or western blotted as described below. Staining the gels was performed for two hours or overnight with Coomassie brilliant blue R-250 (2 g/l) in 35 % EtOH, 10 % acetic acid. The gels were destained with two changes of 35 % EtOH, 10 % acetic acid over several hours. The gels were generally then allowed to destain further overnight in water before being photographed with a Polaroid 57 4x5 Land Film camera and dried for three hours on a Bio-Rad 543 gel drier.

2.2.8. Western Blotting

Protein samples size-fractionated on SDS-PAGE gels were blotted onto either nitrocellulose (NC, Bio-Rad) or PVDF (polyvinylidene fluoride, 148) (Immobilon^{TM}, Millipore) using a Bio-Rad western blotting apparatus, by an adaption of the method of Towbin *et al* (121). For nitrocellulose blotting, the transfer buffer used was tris-base (3.025 g/l, pH 8.3) and glycine (14.41 g/l) in 20 % methanol. For Immobilon blotting the buffer used was CAPS (2.213 g/l 3'-[cyclohexylamino]-1-propanesulphonic acid in 10 % methanol). The membrane was wetted (NC in blotting buffer, PVDF in MeOH) and sandwiched against the gel in the blotting clamp, using filter paper pads to keep the two in tight contact. The apparatus was set up so that the membrane was nearest to the positive electrode, and the gel was electroblotted for two hours at 1 A, or overnight at 0.5 A. Usually, prestained protein standards were run on the SDS-PAGE gel so that the success of the blotting could be ascertained. After blotting, the protein samples were probed with antibodies or utilized in other manners described later.

2.2.9. DNA Extractions

For total cellular DNA, *E.coli* 09:H10:K99 cells were grown overnight in 500 ml LB, harvested by centrifugation at 4 °C, washed in 20 mM tris pH 8 / 100 mM NaCl, and resuspended in 40 ml 20 mM tris / 100 mM NaCl / 5 mM EDTA. Lysozyme was added to 2 mg/ml, and the cells were incubated for 30 min at 37 °C with gentle mixing. Sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5 % and the cells

were incubated for 30 min at 65 °C, after which time 10 mg Proteinase K (BRL) was added. The lysed cells were incubated at 30 °C for 3 h and were extracted three times with an equal volume of phenol (saturated with TNE buffer - 10 mM tris.Cl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0). To remove RNA, 10 mg/ml RNase was added to a final concentration of 50 μg/ml, incubated for 30 min at room temperature, then extracted twice with phenol:chloroform (1:1). The purified DNA was then precipitated from the aqueous phase with 95 % ethanol (2 volumes), washed with 70 % ethanol by resuspension followed by centrifugation, air-dried and resuspended in sterile distilled H_2O. The concentration and purity of the DNA was determined by measuring the absorbance of a dilute solution at 260 and 280 nm. The 260 nm reading gives the concentration of DNA (1 a.u. ≅ 50 μg ml^{-1}), and the ratio A_{260}/A_{280} gives a measure of its purity. Pure preparations of DNA have an A_{260}/A_{280} ratio of 2.0, contamination with protein or phenol lowers this ratio (117).

Large scale plasmid preparations were made by ethidium bromide-CsCl density gradient ultracentrifugation. Overnight cultures (1000 ml) were harvested by centrifugation at 6000 g for 15 min and resuspended in 20 ml GET (50 mM glucose, 25 mM tris.Cl pH 8.0 and 10 mM EDTA). The cells were lysed gently with NaOH (0.2 N) and SDS (1 %) to allow the cells to release plasmid DNA yet retain most high molecular weight chromosomal DNA associated with the cell debris. Potassium acetate (7.5 ml, solution is made up with potassium acetate and acetic acid so that the final concentration of potassium is 3 M, acetate is 5 M.) was added to precipitate the cell debris, which was removed by centrifugation.

The covalently closed circular plasmid DNA was isolated from the cleared lysate supernatant by centrifugation to equilibrium in a CsCl density gradient (2 g/ml) containing ethidium bromide (25 μg/ml). The ethidium bromide serves two purposes - it allows visualization of the DNA bands in the gradient and it separates the covalently closed plasmid DNA from the remaining chromosomal DNA and linearized plasmid DNA bands. It does the latter by decreasing the density of the DNA as it intercalates. Intercalation of ethidium bromide forces a partial unwinding of the DNA helix and since closed circular plasmid DNA has more topological constraints than open-ended DNA it binds less and retains a higher density. After 40 h at 42,000 rpm in a Beckman ultracentrifuge (Titanium 70.1 rotor), two ethidium bromide stained bands were seen, the lower corresponding to the covalently closed plasmid DNA. The plasmid band was extracted by puncture of the polyallomer centrifuge tube with a 25 gauge needle and syringe and purified by precipitation with two volumes 95 % ethanol, centrifugation and washing of the pellet in 70 % ethanol. The DNA was redissolved in tris.Cl pH 8.0-EDTA (TE) buffer and its concentration and purity assessed by reading the absorbance of a dilute sample at 260 and 280 nm.

Plasmid minipreps were performed by gentle alkaline lysis of the cells as above, followed by ammonium acetate protein precipitation, phenol / chloroform extraction and ethanol precipitation of the DNA (117). Precipitated DNA was dissolved in TE containing RNase (50μg/ml) and purity and concentration were estimated by agarose gel electrophoresis.

2.2.10. Construction of *E.coli* 09:H10:K99 Chromosomal DNA Library

An *E.coli* 09:H10:K99 genomic DNA library was constructed using a fragmentation cloning procedure (Figure 15), where random chromosomal DNA fragments are packaged into plasmid vectors and introduced into host bacteria. The bacteria are then grown on plates so that each clone containing a plasmid incorporating foreign DNA gives rise to a single colony, which can then be assayed for production of the desired gene product (117). Genomic DNA from the above strain was partially digested with the restriction enzyme *Sau*3AI to create a spectrum of DNA size fragments. These fragments were then ligated into the complementary *Bam*HI site of pBR322 with T4 DNA ligase (2 Weiss units) at 16 °C overnight. The plasmid pBR322 codes for proteins that confer resistance to the antibiotics ampicillin and tetracycline. *Escherichia coli* JM101 cells were made competent (ready to take up foreign DNA) by $CaCl_2$ treatment (135). The cells (5 ml) were grown overnight, then 500 µl of the culture was diluted into 25 ml LB and incubated at 37 °C for 2-3 h until the optical density at 650 nm of the bacterial suspension was between 0.2 and 0.4 (exponential phase). The cells were centrifuged at 6,000 rpm in a Sorvall centrifuge at 4 °C for fifteen minutes. The bacteria were resuspended in 10mM sterile NaCl (10 ml) to wash and centrifuged to pellet. The cells were then suspended in 12 ml 30 mM sterile $CaCl_2$ and incubated on ice for 20 min, centrifuged and resuspended in 1.5 ml 30 mM $CaCl_2$. Ligation mix (12 µl) was added to 200 µl of the competent cells and the mix was incubated on ice for 30 min. The cells were then subjected to heat-shock at 42 °C and incubated in LB-0.2 % glucose at 37 °C for 1.5 h before plating on LB ampicillin

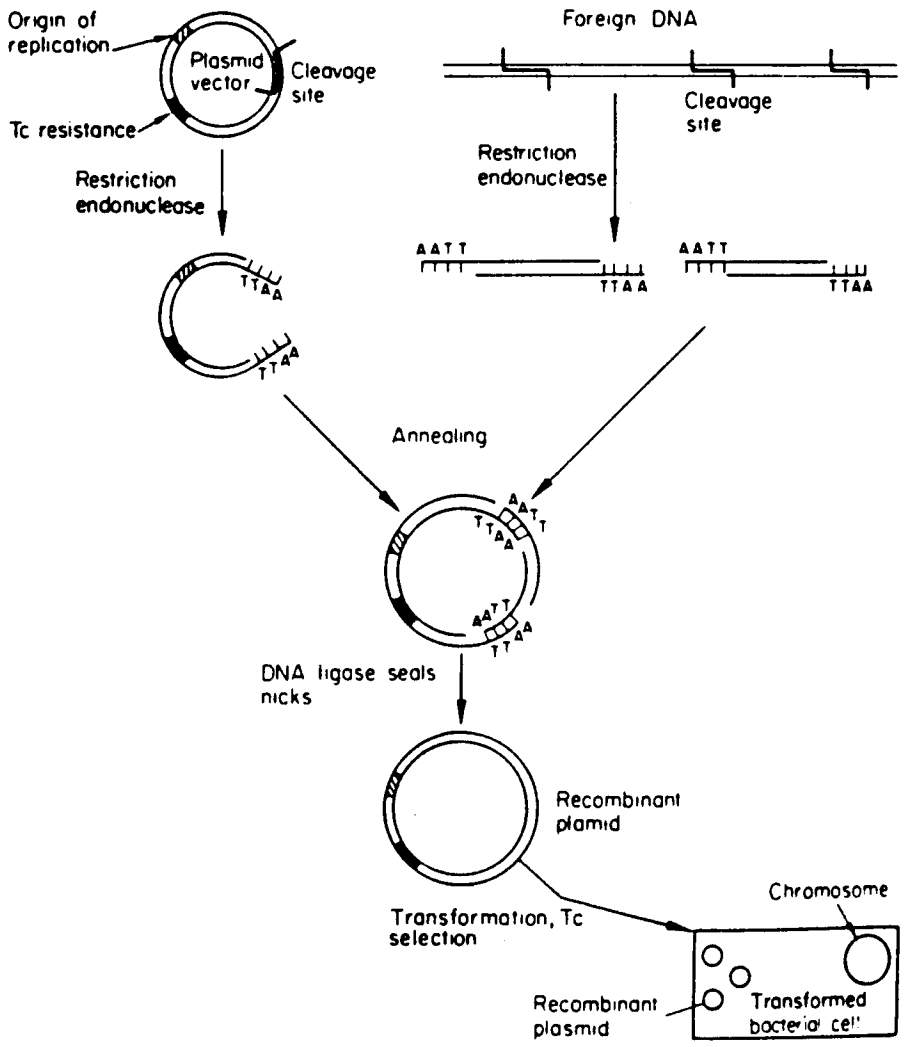


Figure 15. Diagrammatic representation of random fragment cloning procedure (from 122)

plates and overnight growth at 37 °C.

Plasmid-containing colonies were selected for foreign inserts by transferring colonies with a sterile toothpick to a grid position on both an LB ampicillin and an LB tetracycline plate, since cloning into the *Bam*HI site of pBR322 generally inactivates the plasmid's tetracycline resistance gene. After overnight growth, those colonies that grew on ampicillin but not tetracycline were selected for further screening.

2.2.11. Library Screening

2.2.11.1. Hemagglutination Assay

For the initial clone screening, bacterial colonies were scraped from a plate with a sterile toothpick and mixed in a round-bottomed microtitre well with 50 µl of a 1 % v/v fresh human erythrocyte suspension (3x washed in PBS,). The plates then stood for 2 h at 23 °C before being read. Unagglutinated erythrocytes formed a tight button in the bottom of the well, whereas agglutinated cells were spread over the whole well surface by adhesive interactions with neighbouring cells. Clones of interest were then titred against the erythrocytes by adding doubling dilutions of the bacteria in PBS (initial concentration 3.9×10^9 cells/ml) to 50 µl of red cells in PBS-mannose in microtitre plates until no agglutination was seen.

2.2.11.2. Oligonucleotide Probe

A 20-nucleotide oligonucleotide pool was designed to a seven amino acid region of the published N-terminal sequence of the F41 protein (76). This entailed working backwards from the amino acid sequence to the sequence of three nucleotide codons that determine each amino acid. Since most amino acids are coded by more than one codon, points where there is more than one base possibility will occur. At these points, proportions of each possible base are synthesized into the nucleotide chain, and hence a pool of oligonucleotides is built up. The seven amino acid region with the fewest possible codons was chosen as the template for the probe. The nucleotide probe sequence is shown in section 2.3.2.1. The oligonucleotide pool was synthesized by the UBC Nucleotide Synthesis Facility on an Applied Biosystems DNA synthesizer, and purified by chromatography using a C_{18}SEP-PAK^{TM} (Millipore).

The oligonucleotide probe was checked for specificity by hybridization to F41 chromosomal DNA that had been partially digested with *SalI*, electrophoresed on a 0.7 % agarose gel and blotted to nitrocellulose by the method of Southern (123). The hybridization was carried out in an analogous manner to the colony hybridization described below.

The insert-containing clones were grown on plates in grids of 100, colonies were transferred to nitrocellulose filters and alkaline lysed with 0.5 M NaOH, 1.5 M NaCl. The nitrocellulose filters were then neutralized in 1.5 M NaCl, 0.5 M tris.Cl pH 8.0 and the DNA permanently immobilized on the filter by baking at 80 ^{o}C for 2h (117). An 80 pmol sample of the probe was labelled with ^{32}P using γ-^{32}P-ATP and T4

polynucleotide kinase, and hybridized to the filters as previously described (117). The filters were soaked in 6x SSC (20x SSC = 175.3 g/l NaCl, 88.2 g/l sodium citrate, pH 7.0) for 5 min then incubated for 2 h in prewashing solution (50 mM tris.Cl pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1 % SDS). Sites on the nitrocellulose that may bind DNA non-specifically were then blocked by 5 h incubation in prehybridization solution (5x Denhardts solution (50x = 5 g ficoll, 5 g polyvinylpyrrolidone, 5 g BSA in 500 ml H_2O), 5x SSPE (174 g/l NaCl, 27.6 g/l $NaH_2PO_4.H_2O$, 7.4 g/l EDTA, pH 7.4), 0.1 % SDS and 100 µg/ml denatured salmon sperm DNA). The DNA probe was denatured (100 °C, 5 min), was added to the filters and incubated overnight. The probe binds to any regions of the immobilized bacterial DNA to which it is complementary. Filters were then washed with high stringency in 1 and 2x SSC, dried and autoradiographed overnight. Any colonies that showed a dark spot on the x-ray film were chosen from replica plates for further study.

2.2.11.3. Antibody Colony Blots

This assay was adapted from Helfman and Hughes (124). A general diagram for enzyme-conjugated antibody assays is shown in Fig. 16. Briefly, bacterial colonies grown in grids on plates were lysed with chloroform onto a nitrocellulose filter and the DNA enzymatically degraded with DNase 1. The filters were then blocked with bovine serum albumin (BSA) to prevent non-specific antibody adsorbtion, washed in PBS and introduced to the antiserum (1/1000 dilution) in PBS-1% BSA for 3 h at room temperature. The antibodies in the serum bind very specifically to any proteins present against which they were raised. The filters

were washed 3x in PBS-0.5% tween 20 (poly(oxyethylene)$_{20}$ -sorbitan-monolaurate, a detergent) and transferred to a Petrie dish containing either alkaline phosphatase-conjugated goat anti-rabbit IgG (for polyclonal primary antibodies), or alkaline phosphatase-conjugated goat anti-mouse IgG (for monoclonal primary antibodies), for 3 h at room temperature. These secondary antibodies are specific for regions of the primary antibody, and bind to them. The washes were repeated, then the filters were incubated in 0.1 M tris.Cl (pH 9.6) to which was added X-phosphate (50 μg/ml) and nitroblue tetrazolium (10 μg/ml), pseudosubstrates of alkaline phosphatase that are digested to yield an intense purple colour. After developing for 2-20 min, the filters were washed once in PBS and air-dried.

2.2.11.4. Immunoblotting of SDS-PAGE Samples

Protein samples were electrophoresed as in Section 2.2.6. and transferred to nitrocellulose as in Section 2.2.7. The proteins on the nitrocellulose sheet were then incubated with antibody and developed in the same manner as described in Section 2.2.11.3. above.

2.2.11.5. Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay or ELISA is very similar in principle to the above assay, with the exception of the detection enzyme and the fact that the bacteria are bound intact to flat-bottomed wells in a 96 well plastic microtitre plate. This means that only proteins expressed on the surface of the bacteria will react with the primary antibody. Low-binding micro-ELISA plates (Falcon/Becton Dickinson,

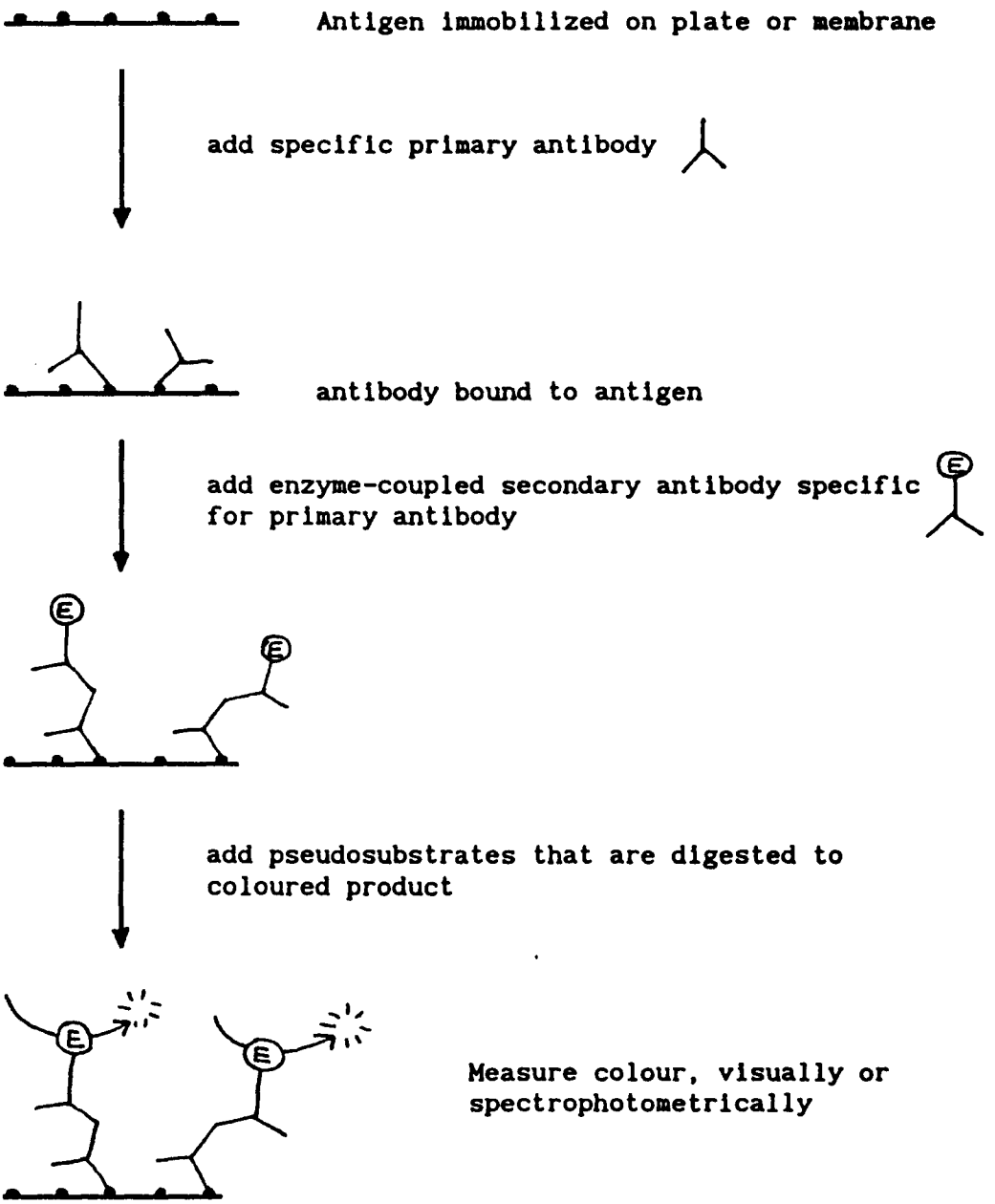


Figure 16 Diagrammatic representation of a generalized enzyme-linked immunoassay

Lincoln Park, NJ, USA) were coated overnight at 4 °C with whole bacteria (100 µl of 3.9×10^9 cells/ml in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6, per well). The plates were washed 3x in PBS-tween and blocked with 3 % BSA in PBS at 23 °C for 30 min. Diluted antibody (usually 1/2000 dilution, 100 µl per well) in PBS-tween was added. After incubation for 1 h at 37 °c, the plates were washed and blocked with 0.5 % BSA in PBS-tween for 30 min at 37 °C. The plates were again washed and 100 µl of horseradish peroxidase-conjugated second antibody was added at 1/2000 dilution to each well. The plates were incubated for 2 h at 37 °C, washed and 200 µl freshly made tetramethylbenzidine solution (Sigma), (0.04 % in 25 mM citric acid, 51 mM Na_2HPO_4, pH 5.0) was added per well. The reaction was stopped after 2-10 min by addition of 50 µl 4 M H_2SO_4, and the plates were read at 450 nm on an SLT EAR 400 AT ELISA plate reader.

2.3. Results

2.3.1. Random Fragment Cloning

From the ampicillin-resistant colonies that resulted from the transformation of *E.coli* JM101 with the ligation products of the cloning experiment, 5000 were plated onto grid positions on LB amp and LB tet plates. Of these clones, approximately 1500 did not grow on tetracycline, indicating a foreign DNA insert. The insert-containing clones were screened for hemagglutination. Two were found that showed partial agglutination and one that showed strong agglutinating activity (clone 8-82) was chosen for further study. The plasmid that the strongly agglutinating clone harboured was named pETE1 and serial dilution hemagglutination experiments showed that this bacterium was capable of full agglutination of 1 % Hct human erythrocytes in the presence of mannose, at concentrations of 6.0 $x10^7$ cells/ml. This figure is equivalent to that for F41 grown on minca plates, and indicates agglutination at erythrocyte:bacteria ratios of about 2:1. A photograph of the microtitre serial dilution assay is shown in Figure 17. The plasmid pETE1 conferred the hemagglutination phenotype onto *E.coli* JM101, B23 and C600 when purified and retransformed into these strains.

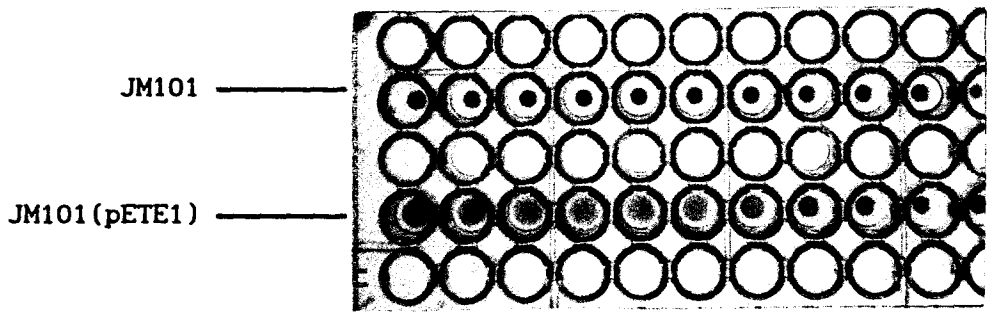


Figure 17. Hemagglutination microtitre assay

Assay was carried out as described in section 2.2.8.1. with initial bacteria concentrations of 3.9 x10^9 cells/ml. Well 6 from the left was judged to be the last well where full agglutination occurred.

2.3.2. Subsequent Library Screening

2.3.2.1. Oligonucleotide Probe Hybridization

The oligonucleotide probe pool synthesized is shown in Figure 18. The last nucleotide of the final codon was omitted as it was an ambiguity point which the nucleotide synthesizer could not synthesize at the 3' end. The probe was designed to hybridize to the non-coding strand of the F41 gene.

H_2N - Ala - Asp - Trp - Thr - Glu - Gly - Gln -

```
5' - G C A G A T T G G A C T G A A G G T C A  -3'
       C     C          C     G     C
       T                A           A
       G                G           G
```

Figure 18. N terminal amino acid sequence of F41 and oligonucleotide probe pool designed to it.

The ^{32}P-labelled probe was first hybridized to a Southern blot of F41 genomic DNA, partially digested with *SalI*, to check its specificity. The results (Fig. 19) showed that the probe did recognize F41 DNA and not JM101 DNA, although hybridization to the bacteriophage λ DNA molecular weight standards was seen. The library was then screened and two colonies showed dark spots on X-ray film (Fig. 20), the colonies 28-61 and 12-07. Colony 8-82 (JM101(pETE1)) did not hybridize to the probe.

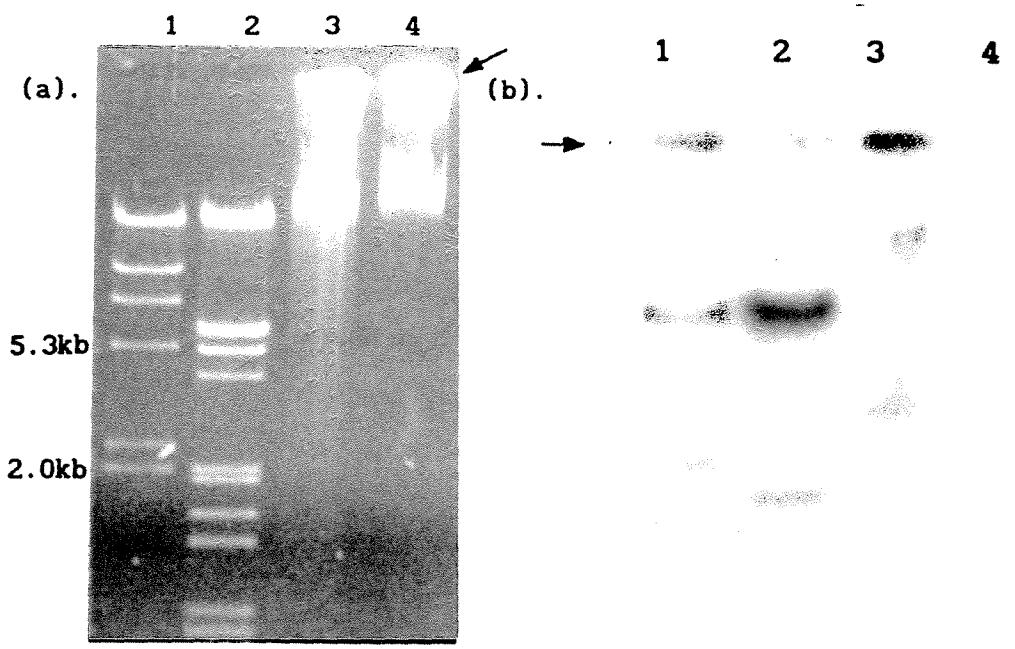


Figure 19. Southern blot of probe hybridized to partially digested F41 chromosomal DNA.

(a). Original 0.7 % agarose gel (ethidium bromide stained)

(b). Autoradiograph of membrane-blotted DNA from (a) after hybridization with probe as described in section 2.2.8.2.

Reproductions are not the same size. Gel wells are arrowed.

lane 1 : λ-*Hind*III molecular weight standards,

lane 2 : λ-*Hind*III/*Eco*RI molecular weight standards,

lane 3 : F41 total DNA *Sal*I digest

lane 4 : JM101 total DNA *Sal*I digest.

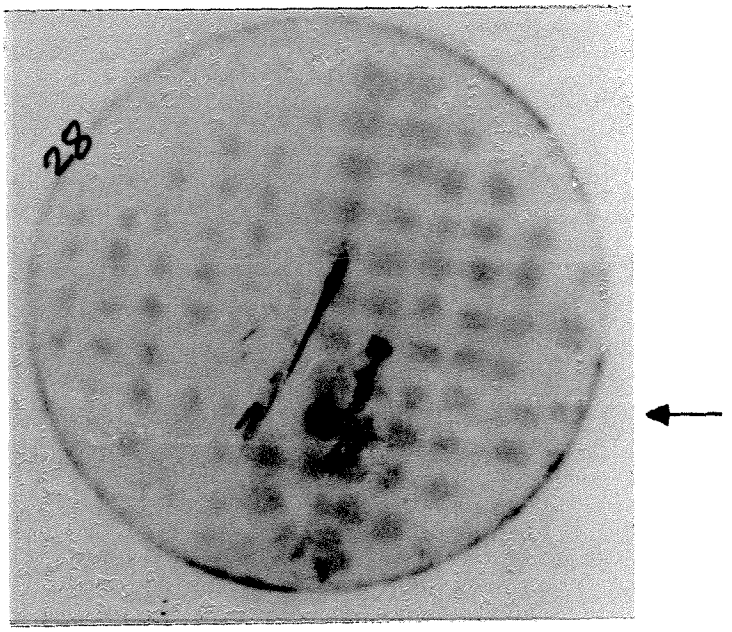


Figure 20. Autoradiogram of blotted library colonies after hybridization with oligonucleotide probe.

Experiment carried out as described in Section 2.2.10.2. Arrow points to positive colony 12-07.

2.3.2.2. Antibody Colony Blots

The library colonies lysed on nitrocellulose filters were incubated with anti-F41 mouse monoclonal antibodies. After incubation with the alkaline phosphatase-conjugated secondary antibody and development, one colony, 3-49, showed faint purple colour (Fig. 21). This weak colour was seen when the experiment was repeated. The colonies that were positive in the oligonucleotide probe assay were negative in this assay, as was JM101(pETE1).

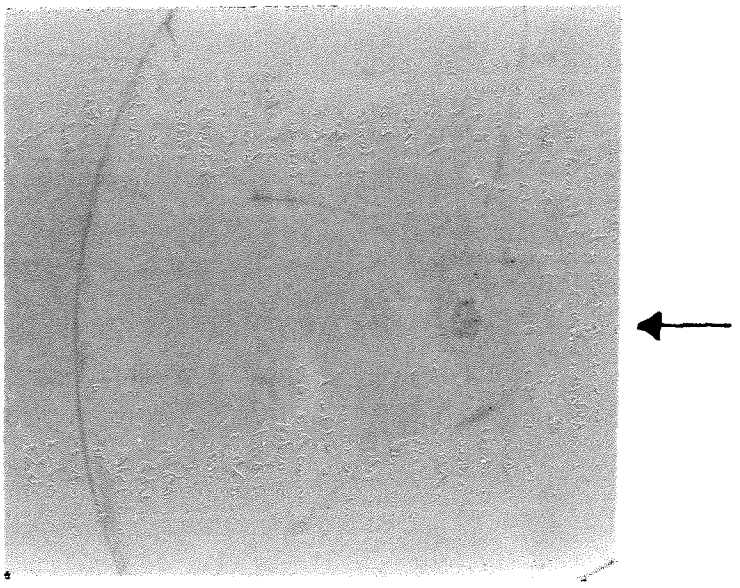


Figure 21. Photograph of antibody colony blot

Experiment was performed as in Section 2.2.10.3. This filter shows a weak positive reaction from colony 3-49 (arrowed).

2.3.2.3. Western Blotting

When electrophoresed and blotted against α-F41 monoclonal antibodies as described earlier, no library clones showed any positive bands. Blotting against α-K99 antibody produced several non-specific bands common to both JM101 and JM101(pETE1). Purified F41 adhesin showed a minor 18 kDa band against α-K99 antibody This data is shown in Figure 22.

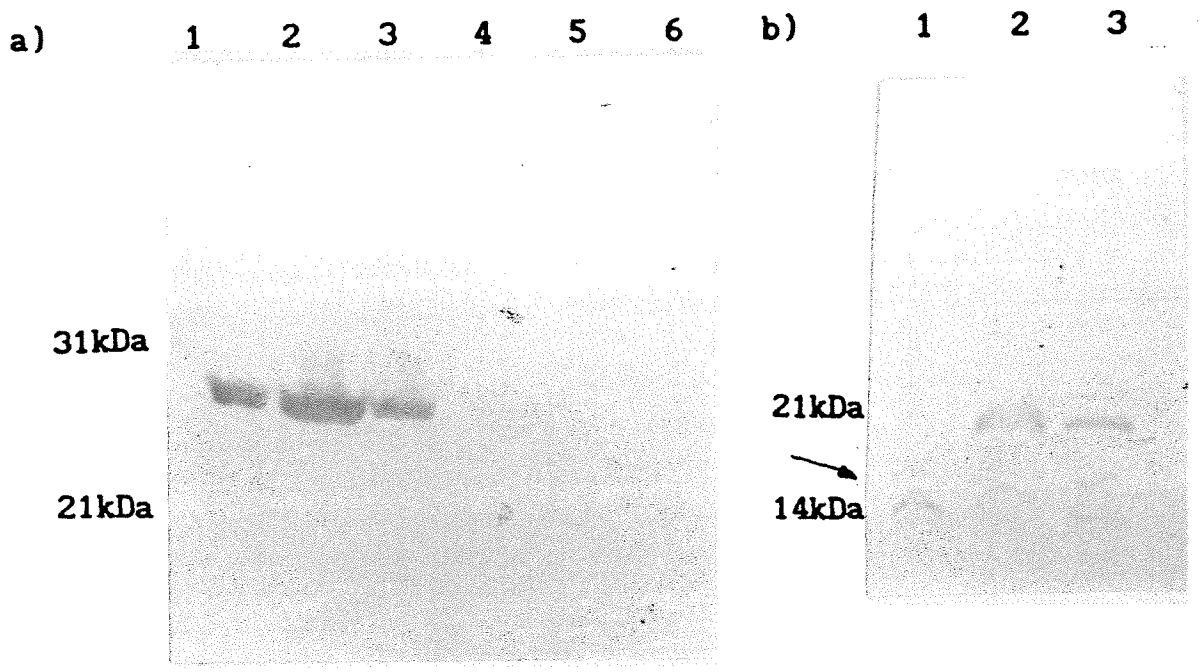


Figure 22. Western blotting of bacteria against a) anti F41 and b) anti K99 antibodies.

Experiment performed as described in Section 2.2.10.4

a) 1 = purified F41 adhesin
   2 = F41 bacteria (minca grown)
   3 = F41 bacteria (LB grown)
   4 = JM101
   5 = JM101(pETE1)
   6 = clone 3-49

b) 1 = F41 purified adhesin
   2 = JM101
   3 = JM101(pETE1)

Anti-K99 band in F41 preperation is arrowed.

2.3.2.4. Enzyme-linked Immunosorbent Assay

Test ELISAs were carried out using dilutions of F41 bacteria in either carbonate-bicarbonate or glyoxal buffer as positive controls and JM101 as a negative control on both high binding and low binding plates. The results are shown in Fig 23. As the assay appeared to be satisfactory for identifying bacteria expressing the F41 antigen, library clones were tested at 1:500 dilution. No clones were found that showed a significant level of accessible F41 antigen expression. The clones found to be positive in the assays described above were negative in this assay including clone 3-49 and JM101(pETE1).

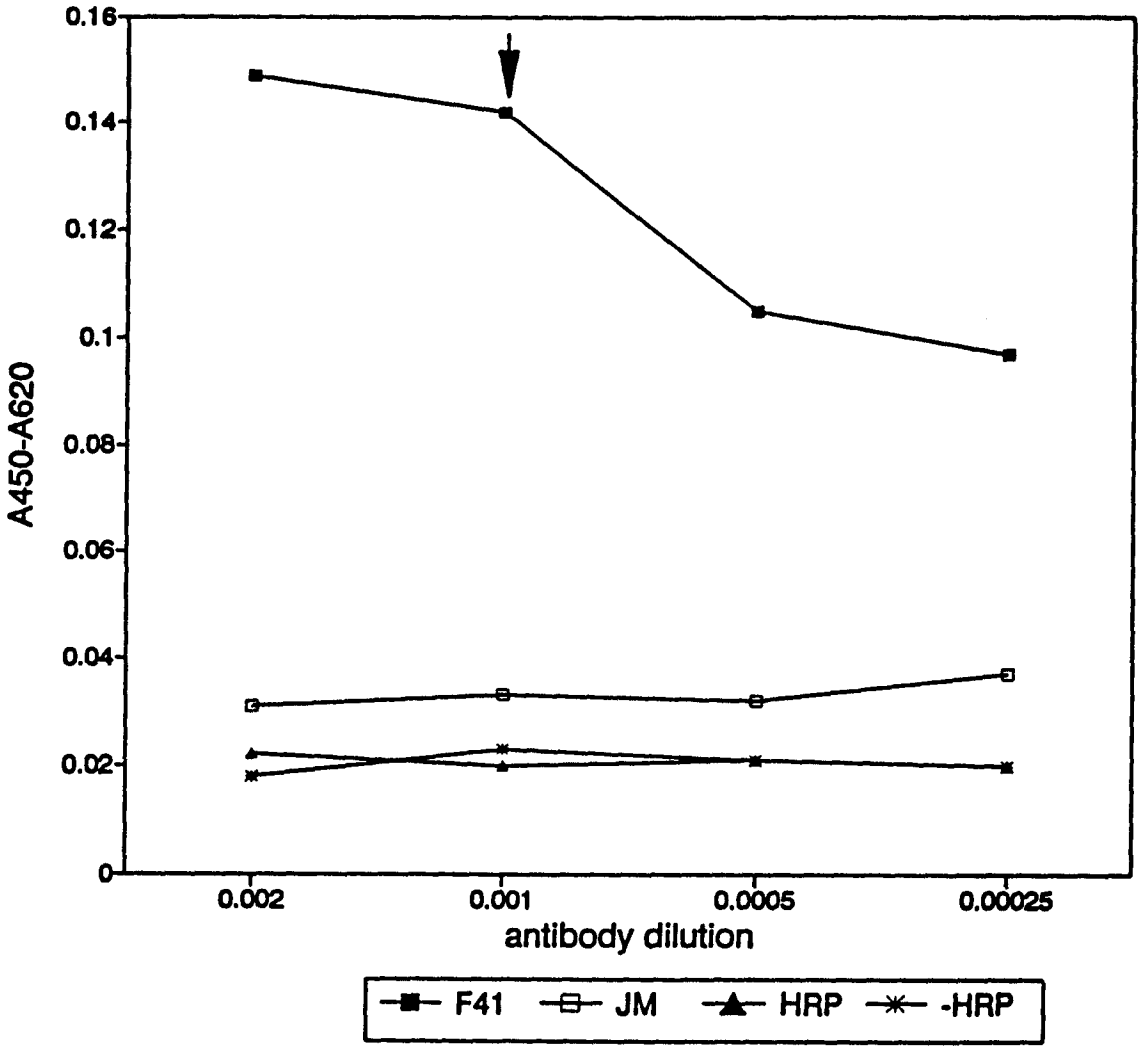


Figure 23. Graph of absorbance against antibody dilution for whole-cell ELISA experiment as described in Section 2.2.10.5.

Concentration of antibody used for subsequent screening is arrowed. No test clone showed an absorbance significantly greater than that of JM101.

F41 = *E.coli* 09:H10:K99, JM = *E.coli* JM101

HRP = horseradish peroxidase alone blank, -HRP = blank without HRP but with bacteria

2.4. Discussion

The cloning experiments were not successful in the generation of a recombinant plasmid that coded for an intact, functional F41 hemagglutinin protein that was expressed on the cell surface. The clones that hybridized with the oligonucleotide probe probably did contain some portion of the genetic determinant but not the full sequence of information necessary for construction and surface expression of the protein. Hybridization seen between the probe and the bacteriophage λ DNA molecular weight standards is unusual given the statistical specificity of a 20-base DNA sequence. Ambiguity points in the probe, where more than one base could complete the correct codon, raise the probability of non-specific hybridizations. This effect was not seen in the library screening as JM101 chromosomal DNA showed no sequence homology with the radioactive oligonucleotide.

The clone 3-49, which showed a repeatable weak positive signal in colony blots, was negative in the ELISA assay possibly indicating that the F41 antigen was being expressed inside the cell but not exported to the surface and assembled. This conjecture cannot however explain the Western blot results where whole cell lysates of 3-49 electrophoresed, blotted onto nitrocellulose and incubated with α-F41 antibodies showed no antibody-recognized band. The denaturing conditions used in the colony blots (chloroform) were different from those used for the electrophoresis (SDS, β-mercaptoethanol, heat) and could possibly have created a cross-reacting false α-F41 epitope on a recombinant protein present in a library colony.

Since these experiments were performed, Moseley (70) has cloned the F41 determinant and has shown that a number of genes are necessary for the full expression of the adhesin, as is the case with many other adhesin systems. It may be that ligation of a simple partial digest of F41 DNA into pBR322 generates a large proportion of recombinant plasmids containing small inserts that do not contain the necessary genetic information for correct expression. An approach based upon selection of F41 chromosomal DNA fragments of greater than 6 kb for ligation into a vector would seem to have a greater chance of success.

The experiments did however allow discovery of a mannose-resistant hemagglutinin that was different from either the F41 antigen or the K99 antigen that the parent strain is known to express. This hemagglutinin was capable of agglutinating human erythrocytes in a microtitre assay to the same extent as the original F41 bacteria. Since the *E.coli* strain used for this work, O9:H10:K99, is different from the F41 strains used by most other researchers (*E.coli* B41 (O101:K99) (60,69) and *E.coli* VAC1676 (O101:K30:F41:H^-)(70), it was postulated that the agglutinin coded by pETE1 was an as yet uncharacterized surface protein on this strain and as such was worthy of further investigation.

3. Subcloning and Sequencing of pETE1

3.1. Background

Once the recombinant plasmid pETE1 had been identified as the source of a gene coding for a protein that caused mannose-resistant agglutination of human erythrocytes, the process of restriction mapping the plasmid was undertaken. This led to the construction of a number of subclones based on the pTZ19R vector, some that retained the hemagglutination determinant and some that lost the genotype. The nucleotide sequences of some of the pTZ-based recombinant subclones were then determined by the dideoxy-chain termination method of Sanger *et al* (125). Single-stranded DNA templates were generated by utilizing the M13 origin of replication present in pTZ to grow the plasmid as bacteriophage. The sequence was then determined by utilizing a series of custom oligonucleotide primers to "walk along" the DNA. Sequencing results (processed using PCGene) were used to generate more subclones with specific properties.

3.2. Materials and Methods

3.2.1. Bacterial Strains, Growth conditions and Plasmids

The bacterial host for plasmids was JM101, grown as described in Section 2.2.1. Plasmid pTZ19R (117) was used for subcloning and to generate single-stranded sequencing templates. This plasmid is also known as a phagemid, a name that reflects its ability to propagate as a double-stranded plasmid or a single-stranded bacteriophage.

For preparation of single-stranded DNA for sequencing from pTZ plasmids, cells were grown in TPY broth (16 g tryptone, 16 g yeast extract, 5 g NaCl and 2.5g K_2HPO_4 in 1 l distilled water, autoclaved 15 min) in the presence of kanamycin (70 μg / ml).

When pUC-based plasmids were used (pTZ), isopropyl β-D-thiogalactoside (IPTG, Sigma) (5 mM) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma) (20 μg/ml) were added to LB agar plates for blue/white screening of transformants. The IPTG induces the *lacZ* gene present on pUC based plasmids, which codes for the enzyme β-galactosidase. The X-gal is a pseudosubstrate for the enzyme that produces a blue coloured product which stains the bacterial colony. When the *lacZ* gene is disrupted by the cloning of foreign DNA into pUC's multiple cloning site, β-galactosidase is not produced and the colony remains off-white.

3.2.2. Electroporation

As an alternative to $CaCl_2$-transformation of bacteria, plasmids were often introduced to host cells by electroporation. Electroporation is a technique that transiently permeabilizes cell surfaces with an electric pulse permitting the highly efficient uptake of DNA (153). Electrocompetent cells (made by P. Miller using the method of Dower *et al* (154)) were electroporated typically with 1 μl of ligation mix and pulsed at 25 kV, 0.3 s in a Bio-Rad Gene Pulser electroporation apparatus. The cells were rescued by incubation in LB broth (1 ml) for 1 hour at 37 °C. The cells (2-200 μl) were then plated on LB amp and grown overnight.

3.2.3. Sequencing Polyacrylamide Gel Electrophoresis

Radiolabelled products of DNA sequencing reactions were fractionated according to size on urea-polyacrylamide gels. The gels were formulated by adding 7.5 ml acrylamide-bisacrylamide (40 % : 0.8 % (Bio-Rad)) to 42.5 ml TBE, giving a final acrylamide concentration of 6 %. Urea (25 g) was dissolved in the solution by heating to 55 °C, and the solution was degassed under vacuum. Polymerization was initiated by addition of 25 µl TEMED (N,N,N',N', tetraethylmethylene diamine, Bio-Rad) and 300 µl ammonium persulphate (100 mg/ml), the acrylamide solution was poured between glass gel plates (40.0 cm x 85 cm, with a gap (gel thickness) of 4 mm) and left to polymerize fully. The gels were run vertically in a Bethesda Research Laboratories SI model electrophoresis system for up to 8.5 hours at 1500 V. The running buffer used was TBE.

3.2.4. Restriction Mapping

Restriction analysis of plasmids of interest was undertaken for size estimation and for subcloning. This involved digests of the plasmid DNA with a range of restriction enzymes that cut infrequently (i.e. those with a six-base recognition sequence), followed by agarose gel electrophoresis to count the bands produced and estimate their molecular weights. These primary digests (after phenol:chloroform extraction and resuspension in the correct buffer if necessary) were then digested further with a range of restriction enzymes and the cleavage pattern and molecular weights of products noted. In this way

cleavage sites were arranged in an internally consistent manner in unambiguous locations. The restriction enzymes used are shown in Table 1.

Table 1. Restriction enzymes utilized during restriction mapping and subcloning.

Enzyme	5'- site -3'	Cuts in pBR322	Cuts in pETE1
*Bam*HI	G|GATCC	1	0
*Eco*RI	G|AATTC	1	2
*Hin*dIII	A|AGCTT	1	1
*Pst*I	CTGCA|G	1	3
*Apa*LI	G|TGCAC	3	4
*Apa*I	GGGCC|C	0	0
*Kpn*I	GGTAC|C	0	0
*Nco*I	C|CATGG	0	2
*Stu*I	AGG|CTT	0	0
*Xho*I	C|TCGAG	0	0
*Acc*I	GT|MNAC	2	3
*Pvu*I	CGAT|CG	1	2
*Xma*I	C|CCGGG	0	2
*Sma*I	CCC|GGG	0	2
*Xba*I	T|CTAGA	0	0
*Sal*I	G|TCGAC	1	2
*Hinc*II	GTPy|PuAC	2	4
*Sph*I	GCATG|C	1	2

M = A or C; N = T or G Py = T or C; Pu = A or G

3.2.5. Subcloning

Subclones of the plasmid pETE1 were generated using the enzyme *Hinc*II, which recognizes the hexanucleotide $^{5'}$G T Py Pu A C$^{3'}$ (Py = Pyrimidine, either Thymine (T) or Cytosine (C) and Pu = Purine, either Adenine (A) or Guanine (G)). This enzyme cuts after the third nucleotide and creates blunt ends. In pBR322 the enzyme makes two cuts, whereas in pETE1 it makes four, meaning there are two *Hinc*II sites in

the foreign DNA region. Plasmid DNA (approx 2.3 μg) was digested with *Hinc*II (8 U) in a volume of 40 μl for three hours at 37 °C. Stop dye (10 μl) was added and the DNA was fractionated by electrophoresis on a 0.8 % agarose gel. Of the four bands that are obtained by electrophoresis of *Hinc*II-digested pETE1, one (3.0 kb) is entirely pBR322 DNA, two (2.3 kb and 1.3 kb) contain both vector and foreign DNA, and one (1.0 kb) is entirely foreign DNA.

The three insert DNA-containing bands were excised from the gel with a scalpel and purified using the Geneclean kit (Fisher). This protocol involves melting the gel in 750 μl 6 M NaI and addition of 5 μl Glassmilk™, a suspension of silica matrix in water. In high salt concentrations, DNA adsorbs to the glass microspheres that compose the matrix. The glassmilk was then pelleted by centrifugation for 30 s at high speed in a benchtop microfuge. The pellet was washed 3x by resuspension in 750 μl NEW wash (NaCl/Ethanol/Water) followed by centrifugation at high speed for 5 s. After the final wash, the pellet was resuspended in 10 μl sterile distilled H_2O, heated to 65 °C for two minutes to elute the DNA from the silica, then centrifuged for 30 s. The purified DNA was then transferred in the supernatant to a clean eppendorf tube and checked for purity and yield by agarose gel electrophoresis.

The vector chosen for religation and recircularization of the fragments was pTZ19R, a pUC-based plasmid with ampicillin resistance and the *lacZ* gene. The vector also has a single *Hinc*II site in the multiple cloning site. Cesium-pure pTZ19R (5 μg) was digested with 1 U *Hinc*II overnight at 37 °C in a volume of 20 μl. The overnight digest was diluted to 200 μl with TE and extracted once with phenol:chloroform then once with chloroform, precipitated with ethanol, and redissolved in

100 μl TE. The concentration was estimated by electrophoresis and ligations were set up with each of the Genecleaned pETE1 fragments as follows. In an eppendorf tube were mixed 10μl pTZ18R-*Hinc*II, 8 μl pETE1 fragment, 6 μl 5x ligation buffer, 1 μl 10 mM ATP and 20 Weiss units T4 DNA ligase (high conc. 15 U/μl), with sterile distilled H_2O to 30 μl. The ligations were incubated overnight at room temperature then transformed into $CaCl_2$-competent JM101 (see detailed description Section 2.2.10.). Transformation mix (50 μl) was plated onto LB-amp-IPTG-X-Gal plates (4 for each fragment ligation) and incubated at 37 °C overnight. White colonies were selected and stocks were made by as in Section 2.2.1. Agarose gel electrophoresis of *Hinc*II-digested subclones confirmed the ligations were successful.

Hemagglutination studies on the subclones (see 2.3.1.) led to the decision to generate another subclone containing the 1.3 kb and 1.0 kb *Hinc*II fragments together in pTZ18R. To do this, pETE1 DNA (2.3 μg) was digested with *Hinc*II for one hour in a total volume of 40 μl. Aliquots (5 μl) were taken at 0, 30 s, 1 min, 2 min, 5 min, 10 min, 30 min and 1 h, added to stop dye (4 μl) and run on an agarose gel. A digest time (5 min) was chosen such that the plasmid DNA was partially digested and the transient band running at about 2.3 kb (just below the 2.3 kb band that is present in a complete digest) was at a maximum. A digest was then performed under exactly the same conditions for 5 min and the entire digest was subjected to agarose gel electrophoresis. The 2.3 kb band was excised, Genecleaned and ligated into the *Hinc*II site of pTZ18R, and the plasmid was then transformed into competent JM101. Clones that gave rise to off-white colonies on LB-amp-IPTG-X-Gal plates were cultured. Restriction digests with *Hinc*II of isolated plasmids from these clones followed by electrophoresis showed that some bacteria

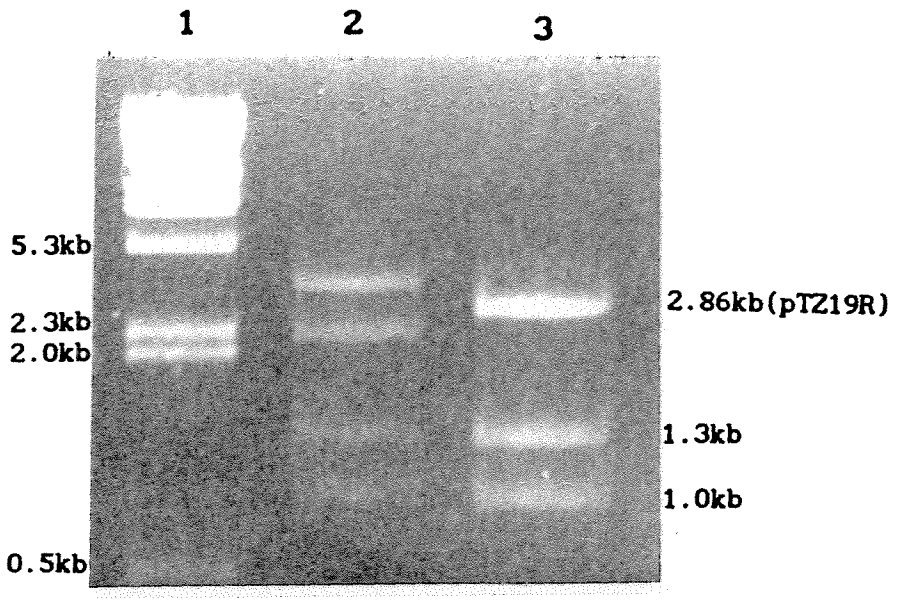


Figure 24. Construction of pPL7.

Lane 1 : high Mwt standards, lane 2 : *Hin*cII digest of pETE1

Lane 3 : *Hin*cII digest of pPL7

harboured a recombinant plasmid that released a 2.3 kb fragment, and one clone harboured the desired product, a plasmid that released both the 1.3 kb and 1.0 kb regions. The plasmid size was confirmed to be approximately 6.2 kb (2.86 (pTZ) + 2.3 + 1.0) by digestion with *Sal*I, which cuts the plasmid once only. This plasmid was named pPL7 (Figure 26).

Once nucleotide sequencing had determined important open reading frame areas in the subclones' DNA, further subclones were produced in similar manner to investigate these regions. The subclones and their properties are shown in Figure 29, Section 3.3.2.

3.2.6. Nucleotide Sequencing

3.2.6.1. Strategy

It was decided to sequence the F41 DNA stretches in the subclones pPL2, pPL3 and pPL4 as these plasmids are based on pTZ19R, which contains the intergenic (origin) region of bacteriophage M13 (126). This means that when the bacteria are grown in the presence of kanamycin with the helper phage M13KO7, virions containing single-stranded phagemid DNA are produced. Bacteriophage M13K07 is a derivative of M13 with a mutated gene II, a plasmid origin of replication (p15A) and a kanamycin resistance gene. When M13K07 infects bacteria that harbour pTZ plasmids, the incoming single-stranded DNA is converted into double stranded by bacterial enzymes. This form then uses its plasmid origin of replication to replicate without the need for viral gene products. However, M13K07 does code for all the proteins necessary for propagation as a virus, and as these gene products build up, they begin to generate

progeny single stranded DNA. The mutated gene II product of M13K07 interacts less efficiently with its own origin of replication than it does with the origin present on the pTZ plasmids so there is preferential production of single-stranded phagemid DNA, which can be purified for use as a sequencing template.

The dideoxy chain-termination method (125) was used for sequencing. This method entails firstly producing the DNA to be sequenced as a single strand. A single stranded oligonucleotide primer (usually 18-21 bases) complementary to a known region of the template is then annealed to the DNA strand. The Klenow fragment of the enzyme DNA polymerase (contains the full polymerase activity but lacks the 5'-3' exonuclease activity of the intact enzyme (117) then synthesizes a complementary strand to the template in a 5' to 3' direction, starting at the 3' end of the primer. The enzyme needs the four deoxy bases as substrates, and one of them (usually dATP) is radioactively labelled so that the synthesized strand can be visualized by autoradiography. The enzyme can also use dideoxy bases as substrates, but when they are incorporated into the growing chain they halt synthesis, as they have no free 3' hydroxyl group. The polymerization is performed in four tubes, each tube having a low concentration of one of the four dideoxy nucleotides included. As the reaction continues, each tube develops a population of radioactive DNA strands of varying lengths,having a common 5' end and varying 3' ends dependent upon incorporation of the dideoxy analogue of a specific base. These DNA populations are denatured and electrophoresed side by side, with visualization of the bands by autoradiography. The sequence is then read directly from the bottom of the gel (shortest fragments) upwards.

Reverse primer, a primer that is complementary to a sequence in

pTZ19R just to the 5' side of the *Hinc*II site, was used to sequence around 300 - 350 bp into the fragments. After that the DNA was sequentially sequenced by synthesizing custom oligonucleotides complimentary to the sequenced DNA about 50 nucleotides from the end of readable sequence from each primer. In this way the recombinant DNA fragments were "walked along" until vector DNA or information from a primer synthesized in the opposite direction was encountered.

3.2.6.2. Growth and purification of single-stranded DNA sequencing templates

Escherichia coli JM101 strains harbouring the pTZ-based sequencing phagemids were inoculated into 2 ml TPY medium containing ampicillin and the helper phage M13K07 ($1x10^9$ pfu (plaque-forming units)) and incubated at 37 °C for one hour. Kanamycin (70 μg/ml) was added and incubation continued overnight. Cells (1.5 ml) were centrifuged for 5 min in a microfuge and the supernatant (which contained the virions) was transferred to a clean tube. Poly(ethylene glycol) (PEG) 6000 (200 μl of 25 % in 3.75 M ammonium acetate) was added to the supernatant, which was vortexed and stored on ice for 10 min. Centrifugation at 17,000 rpm for 10 min, 4 °C and removal of all liquid left a tight pellet of bacteriophage, which was resuspended in TE (20 μl). To this was added 200 μl of 4M $NaClO_4$, which allows the DNA to bind to a glass fibre filter in the next step. Whatman GF/C filters were cut with a number 3 cork borer to make 7 mm diameter discs. These discs were placed into wells in an ELISA microtitre plate which had each had a small hole bored in the bottom by a hot 21 gauge needle. The microtitre plate was attached to a base that was connected to a vacuum pump, samples were

loaded into the wells and the vacuum was slowly applied. The vacuum drew the liquid through the filter paper, leaving the DNA bound. The DNA was washed 4x by passing 200 µl of 70 % ethanol through the filters and gentle suction was continued until the filters were dry. The filters were placed into capless 0.5 ml eppendorf tubes with needle punctures in the bottom then each 0.5 ml tube was placed inside a capless 1.7 ml eppendorf. Sterile distilled water (20µl) was carefully added to the filter and left at room temperature for 5 min, then both tubes were centrifuged for 30 s and the eluted single-stranded DNA was recovered from the large tube. The quality and quantity of the DNA was checked by agarose gel electrophoresis.

3.2.6.3. Sequencing Reactions

For the annealing of the primer to the template, 5 µl of the single-stranded DNA prepared by the glass fibre filter method was incubated with 1 pmol of primer (concentration adjusted so that 1 pmol = 1 µl) at 68 °C for 5 min in annealing buffer (56 mM tris.Cl, 20 mM $MgCl_2$ and 70 mM NaCl, pH 7.5, final volume = 10 µl). The initial reverse primer was purchased from Boeringer Mannheim, subsequent primers were custom synthesized on an Applied Biosystems DNA synthesizer, and purified by chromatography on a C_{18} Sep-Pak™. The annealing mixture was allowed to cool slowly to room temperature then 1 µl of 0.1M dithiothreitol (DTT) was added, along with 2µl of nucleotide labelling mix (3.0 µM deaza-GTP, 1.5 µM dCTP and 1.5 µM dTTP). The radiolabel, ^{35}S-α-thio-dATP (0.5 µl, 10 µCi / µl, Amersham, Arlington Heights, IL) and finally 3 U of T7 DNA polymerase (Sequenase version 1.0 (United States Biochemical, Cleveland, Ohio), diluted to 1.5 U / µl) were added

and the reaction allowed to continue for five minutes at room temperature. After this time 3.5 μl of the reaction mix was added to each of the four termination mixes. The termination mixes were made up as 8 μM concentrations of dideoxy nucleotides in 20 mM NaCl and 2.5 μl of each mix was pipetted into an individual well of a Nunc microtitre plate (i.e. one well for ddATP, one for ddCTP etc.). The termination reaction continued for five minutes until stopped by addition of 4 μl stop buffer (20 mM EDTA (pH 7.5), 0.05 % (w/v) xylene cyanol FF, 0.05 % (w/v) bromphenol blue in 95 % deionized formamide) to each well. The two dyes in this mix allow tracking of the electrophoresis. The final mixes were heated at 95 °C for two minutes and 2 μl of each sample was loaded onto a denaturing urea (50 % w/v) polyacrylamide (6 %) gel in adjacent wells in the order A,C,G,T. The electrophoresis took place at 1500 V for up to 8.5 h.

Usually, to gain the most information from one primer, three sets of samples were loaded and run for 3, 5, and 8.5 h. The reactions that were run for the shortest time (i.e. those giving information on sequence closest to the primer) were often produced using a 10x dilution of the labelling mix to retard the T7 polymerase activity and produce smaller labelled DNA fragments. The incubation times for both labelling and termination reactions were reduced to 3 min in these cases also.

After electrophoresis, the gel was backed with Whatman filter paper and dried for two hours under vacuum in a Bio-Rad gel drier. The dry gel was then exposed to X-ray film (Amersham) overnight and developed in a Kodak automatic developer. The autoradiograph was read manually on a light box.

3.2.6.4. Sequence Data Handling

Sequence data from autoradiographs was entered into the program PC Gene™ version 4.0 (Intelligenetics) for open reading frame determinations, nucleotide to protein translations, consensus sequences, prokaryotic protein secretory signal sequences and secondary structure prediction. Searches for homology with known sequences were made using the non-redundant SwissProt + PIR + SPUpdate + GenPept + GPUpdate database and the non-redundant GenBank + EMBL + EMBLupdate + GBupdate database by sending sequences by electronic mail to the BLAST server at the National Institute of Health, Bethesda, Maryland, USA.

3.3. Results

3.3.1. Restriction map of pETE1

From agarose gel electrophoresis, the plasmid pETE1 was found to be approximately 8.0 kb in size, indicating an F41 DNA insert of around 3700 bases. The restriction map generated by single and double enzyme digests is shown in Figure 25.

3.3.2. Subcloning and the localization of the agglutinin gene

The subclones generated are shown in Table 2 and figure 26.

Table 2. Constructed pETE1 subclones

Name	Description	HA
pPL2(A)	pETE1 1.3kb *Hinc*II fragment in pTZ19R vector	-
pPL2(B)	as above, but opposite orientation	-
pPL3(A)	pETE1 1.0kb *Hinc*II fragment in pTZ19R vector	-
pPL3(B)	as above, but opposite orientation	-
pPL4(A)	pETE1 2.3kb *Hinc*II fragment in pTZ19R vector	-
pPL4(B)	as above, but opposite orientation	-
pPL7	pETE1 1.3 + 1.0 *Hinc*II Fragments in pTZ19R	+++
pPL7N	pPL7 with a 395bp *Nco*I deletion in ORF1 (see Section 3.3.2) (construction Figure 32)	-
pBA	A control plasmid for partitioning studies. pBR with a 17bp *Ban*II disabling deletion in the *tet* gene	-

HA = hemagglutination activity in microtitre assay

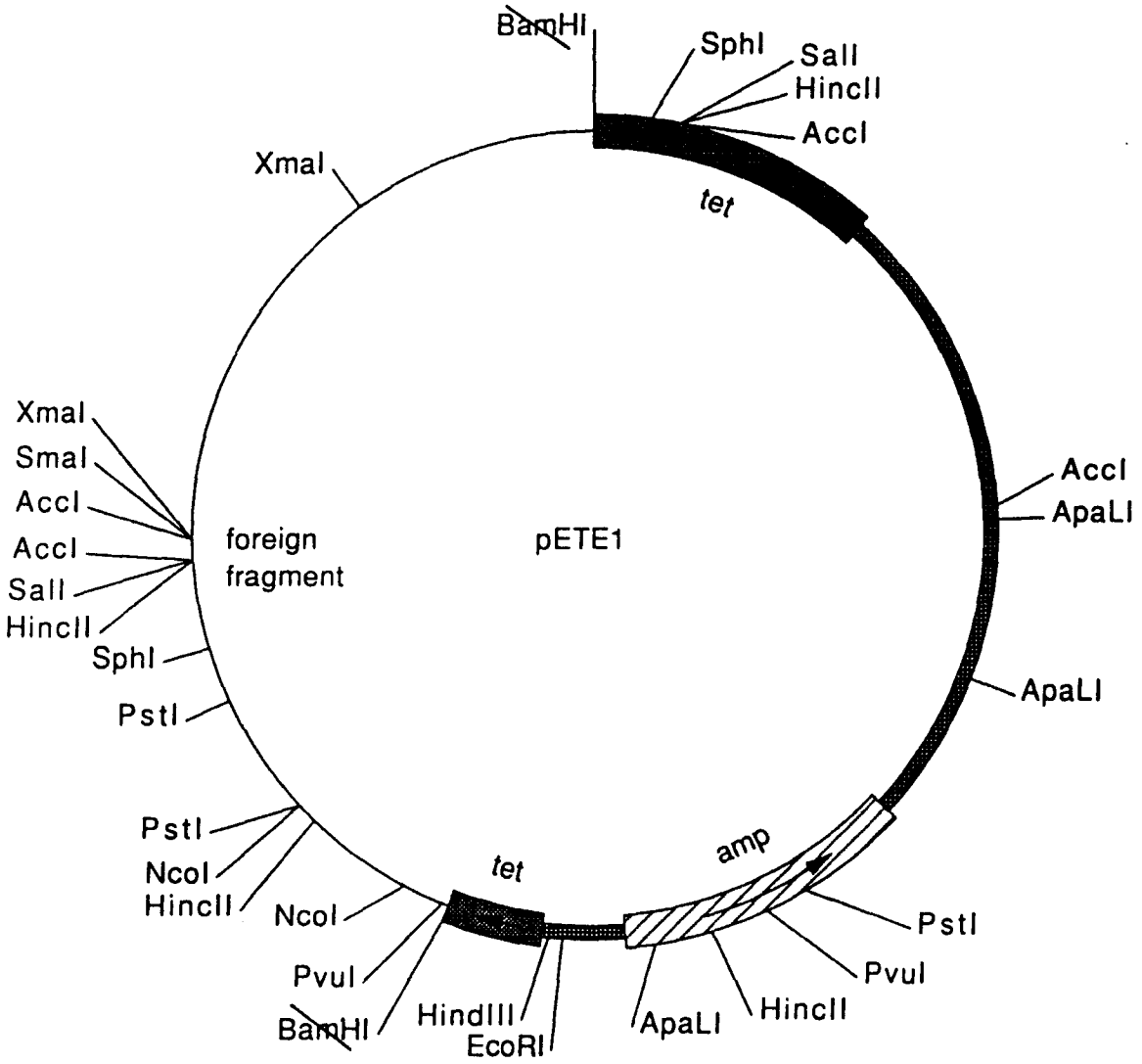


Figure 25. Restriction map of pETE1

F41 DNA insert is shown as thin line. ~~*Bam*~~HI = *Bam*HI sites that were not regenerated upon incorporation of foreign fragment.

The first subclones were made from *Hinc*II fragments of 2.3, 1.3 and 1.0 kb ligated into the *Hinc*II site of pTZ19R. The subclones were tested for hemagglutination with negative results, therefore a subclone containing both the 1.3 and 1.0 fragments was made (Figure 24, Section 3.2.5.). This clone, named pPL7, showed a mannose-resistant agglutination of human erythrocytes in a microtitre assay to a level equivalent to the bacteria containing the pETE1 plasmid. It was decided to sequence the foreign DNA present in pPL2 and pPL3. Location of an incomplete open reading frame in pPL7, interrupted by the 1.0\2.3 fragment *Hinc*II junction, led to the sequencing of the pPL4 plasmid also.

3.3.3. Identification of Open Reading Frames

Open reading frames were discovered in several locations on the plasmids (Fig. 26). The interrupted tetracycline resistance gene from pBR322 runs into the insert DNA for 47 bases until a stop codon is reached. After just one base there is an initiation codon (ATG) followed by a 792 base-pair open reading frame that would code for a protein of 29,024 Da. The initial region of DNA sequence is shown in Figure 27. This putative protein was named plprot1. The open reading frame spans the *Hinc*II site between the 1.3 and 1.0 fragments. The start codon does not appear to have a consensus promoter/initiation site or ribosome binding site (RBS) (131). This suggests that an ATG codon later in the reading frame could be the true starting codon. There are two such codons close downstream, the first of which has a putative ribosome binding site (GGAG). The nucleotide sequence and its translation are shown in Figure 28, amino acid composition shown in Table 3.

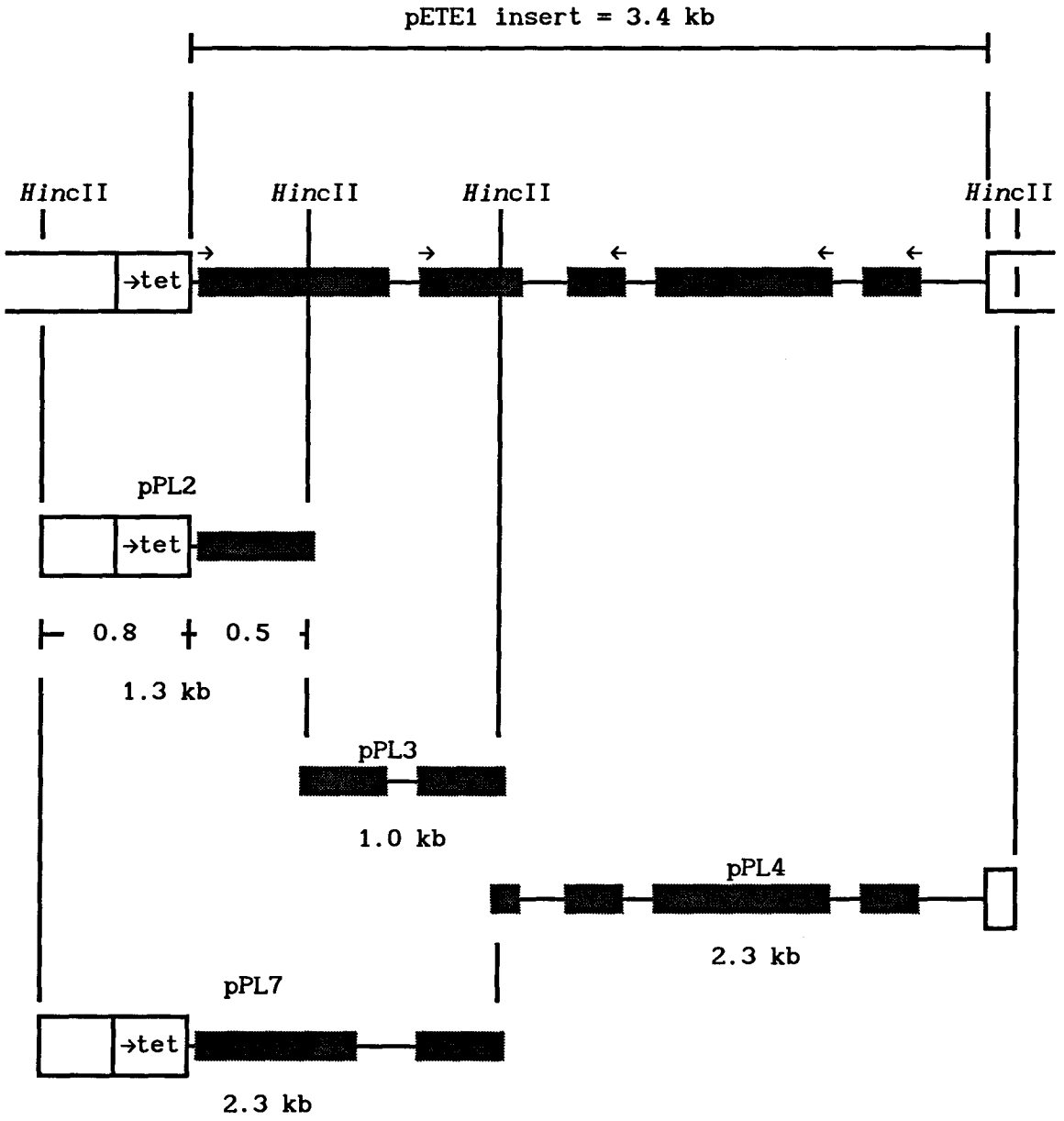


Figure 26. Subclones of pETE1 showing open reading frame areas

*Hinc*II fragments were ligated into *Hinc*II site of pTZ19R

■ = open reading frame

□ = pBR322 DNA; tet = tetracycline resistance gene (truncated)

← ,→ = direction of putative transcription/translation

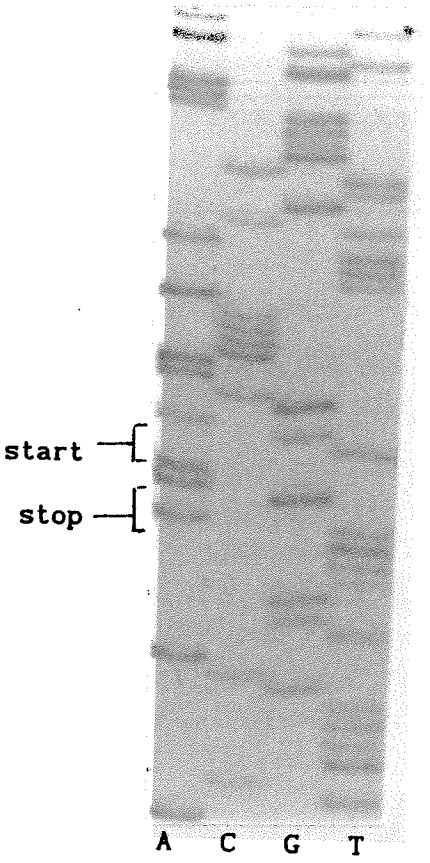


Figure 27. DNA sequence in the region of the plprot1 putative start codon

```
from bottom - ATCTTTTGCATGGTTTTAGAATGAGCAACCCCATTTATCGTTCGGGG.....
                S  F  A  Y  F  *   M  S  N  P  I  Y  R  S  G....
                                   plprot1→
```

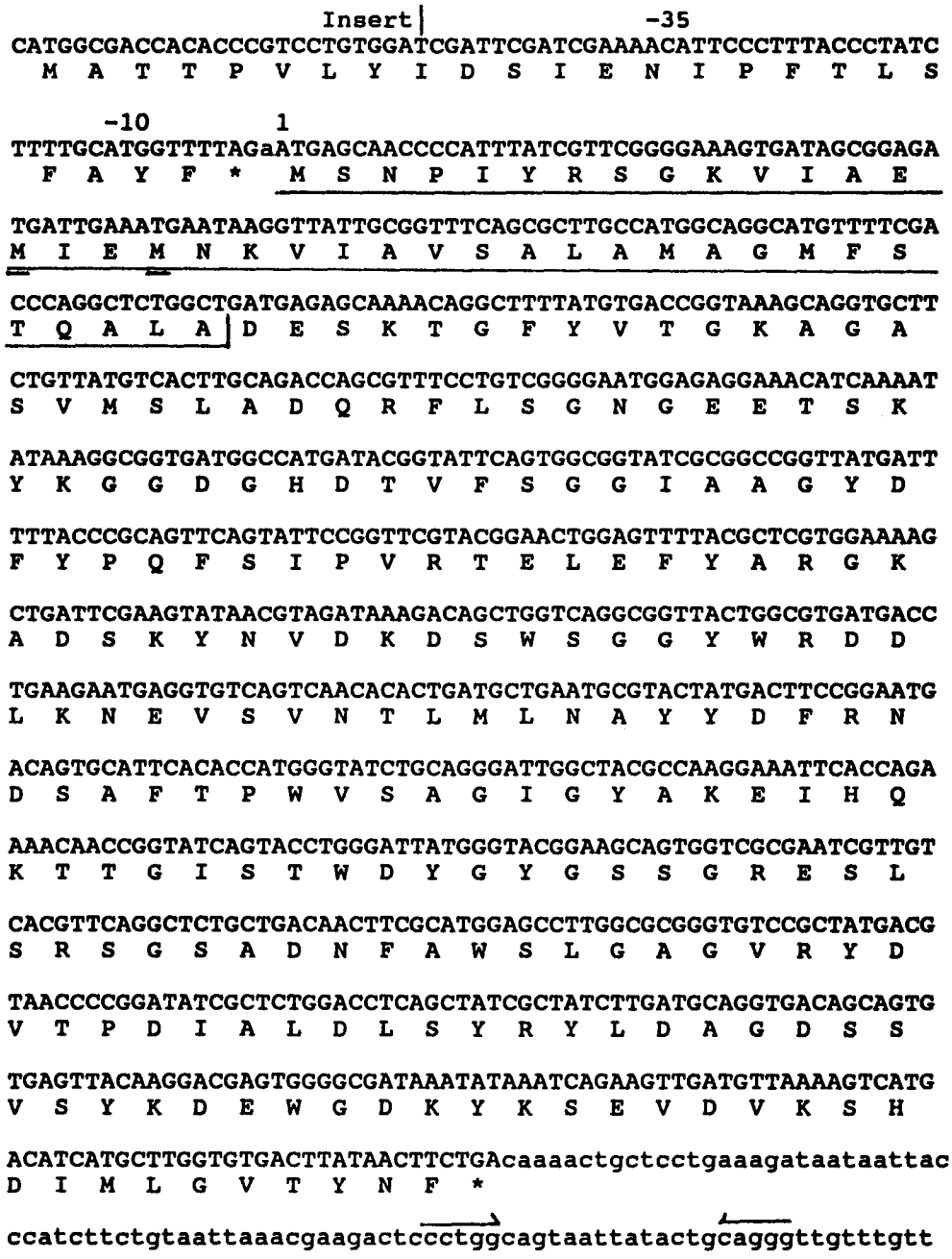


Figure 28. Nucleotide sequence and deduced amino acid structure of plprot1.

Predicted secretory signal sequence is underlined. Arrows show possible inverted repeats; * = termination codon; a = single nucleotide between stop codon of possible tet-fusion protein and start codon of plprot1 ORF Other potential plprot1 start codons are double underlined.

Table 3. Predicted amino acid composition of plprot1

Residue	No. of aa's	%age
Ala	23	8.7
Arg	10	3.7
Asn	10	3.7
Asp	23	8.7
Cys	0	0
Gln	4	1.5
Glu	12	4.5
Gly	28	10.6
His	3	1.1
Ile	11	4.1
Leu	14	5.3
Lys	16	6.0
Met	8	3.0
Phe	11	4.1
Pro	5	1.8
Ser	31	11.7
Thr	13	4.9
Trp	6	2.2
Tyr	19	7.1
Val	17	6.4

(%age = number of residues/total residues in protein)

The predicted protein contains three putative secretory signal sequences as predicted by the method of Von Heijne (127). The potential signal sequences are given a score based on a survey of known signal peptides. The highest-scoring plprot1 predicted signal sequence is shown below.

```
6       10                  20                  30              39|40
|       |                   |                   |               |
y r s g k v i a e m i e m n k v i a v s a l a m a g m f s t q a l a|D E S
  +     +                   +                                     |
```

Figure 29. plprot1 predicted secretory signal sequence

Amino acids shown in single letter code (see abbreviations). Vertical line indicates predicted cleavage point, "+" denotes positively charged residues.

The lower scoring predictions also identified this region but predicted cleavage between residues 30-31 and 37-38. This signal sequence is fairly long, but there are other potential start codons (coding for methionine residues) present in the signal region, one of which appears to have a consensus ribosome binding site (Met at position 15). This codon could be the true initiation codon of plprot1. This would not affect the size or amino acid composition of the mature protein. The mature plprot1 protein would contain 225 amino acids and have a molecular weight of 24,896 Da. All of the predicted signal sequences conform to the -3,-1 rule which states that the amino acid residues in the -3 and -1 positions relative to the cleavage point should be small and uncharged.

The predicted protein plprot1 was scanned for potential membrane associated regions by the methods of Klein *et al* (128) and Eisenberg *et al* (155). No section of this protein had a sufficiently large concentration of hydrophobic residues to be positively identified as a putative membrane spanning sequence. This point is discussed further in Chapter 6. An analysis of the possible secondary structure by the method of Garnier (129) predicted a predominantly extended form with appreciable helical content, whereas a prediction according to the method of Gascuel and Golmard (130) indicated a predominantly coiled form. (Table 4 and Figure 30).

Table 4. Comparison of plprot1 secondary structure predictions

conformation	Garnier	Gascuel and Golmard
Helical	31.4 % (83 aa)	36.7 % (97 aa)
Extended	45.8 % (121 aa)	14.3 % (38 aa)
Turn	11.7 % (31 aa)	
Coil	10.9 % (29 aa)	48.8 % (129 aa)

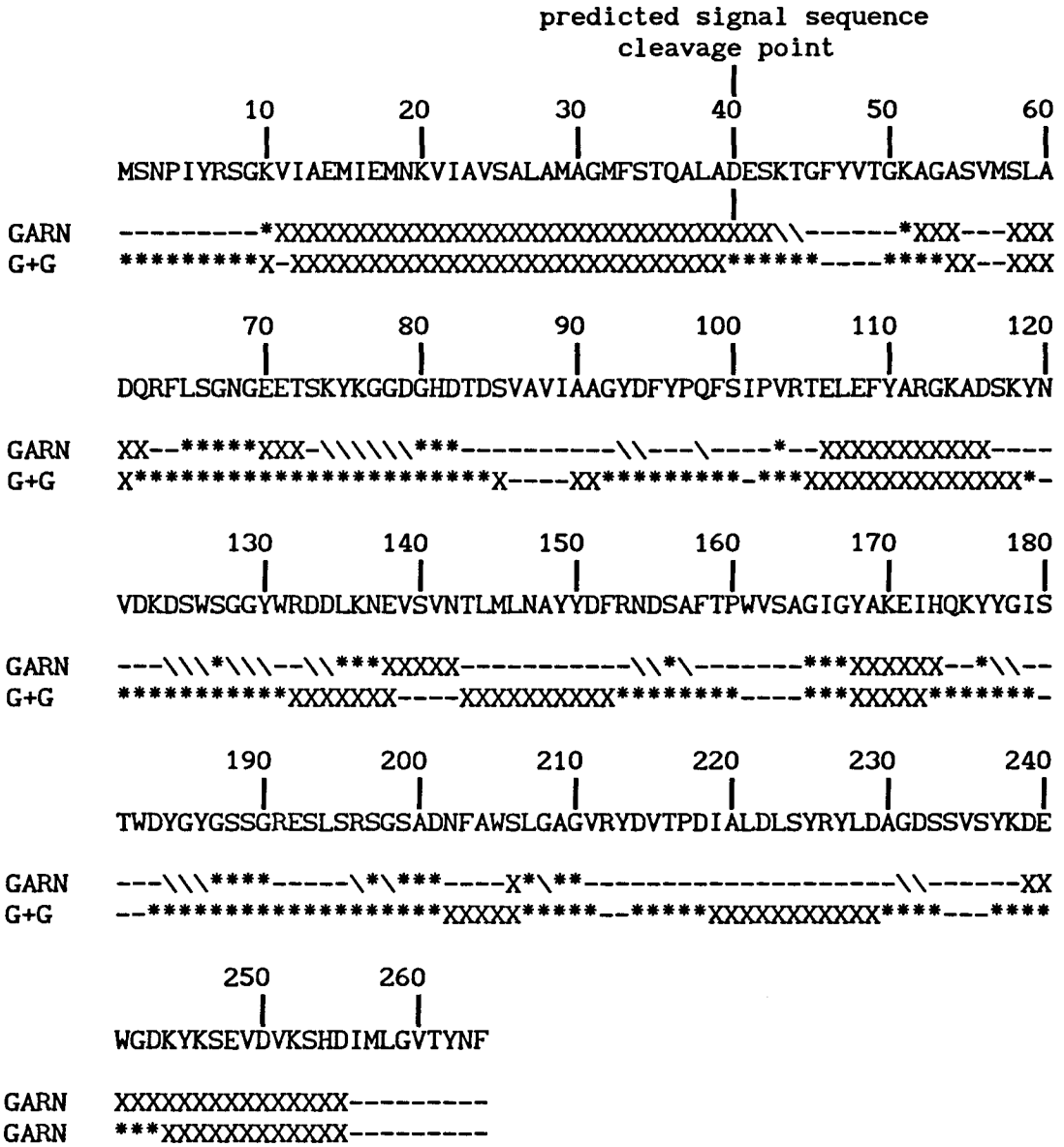


Figure 30. Semi-graphical display of secondary structure prediction for plprot1 by the methods of Garnier and Gascuel and Golmard

GARN = Garnier prediction; G+G = Gascuel and Golmard prediction

X = Helical Conformation; - = Extended Conformation; * = Coil Conformation: \ = Turn Conformation

The second open reading frame begins 169 bp later, is 522 bp and codes for a putative protein of 18,394 Da (named plprot2). This open reading frame has consensus transcription/translation initiation and termination sites and spans the *Hin*cII site between the 1.0 and 2.3 fragments. The nucleotide sequence and translation of this open reading frame are shown in Figure 31.

Hemagglutination observations for the plasmid pPL7 can be rationalized with the preceding information as follows. Bacteria harbouring the plasmid pPL2, containing just the 1.3 kb *Hin*cII fragment from pETE1, do not agglutinate red cells. Included on the 1.3 fragment is the *tet* promoter from pBR322, which promotes transcription/ translation of a truncated tetracycline resistance protein (156). This truncated protein was therefore not responsible for the agglutination activity present in pPL7, even though the protein signal sequence is intact so presumably the truncated form is being exported through the bacterial inner membrane but perhaps is not inserted into the outer membrane. The gene coding for plprot2 is not complete in pPL2. In pPL7, the full plprot1 open reading frame is present, but only part of the gene encoding plprot2. The pPL7 plasmid carries the hemagglutinin determinant, therefore the likelihood was that the hemagglutinin was the protein product of the complete open reading frame (ORF), plprot1. It was also possible however that the truncated product of the incomplete ORF could be mediating the adhesion.

To resolve this uncertainty, the plasmid pPL7N was constructed (see Figure 32). This plasmid has a deletion in the plprot1 open reading frame and cells containing this plasmid are completely inactive in a microtitre hemagglutination assay, even with very high initial

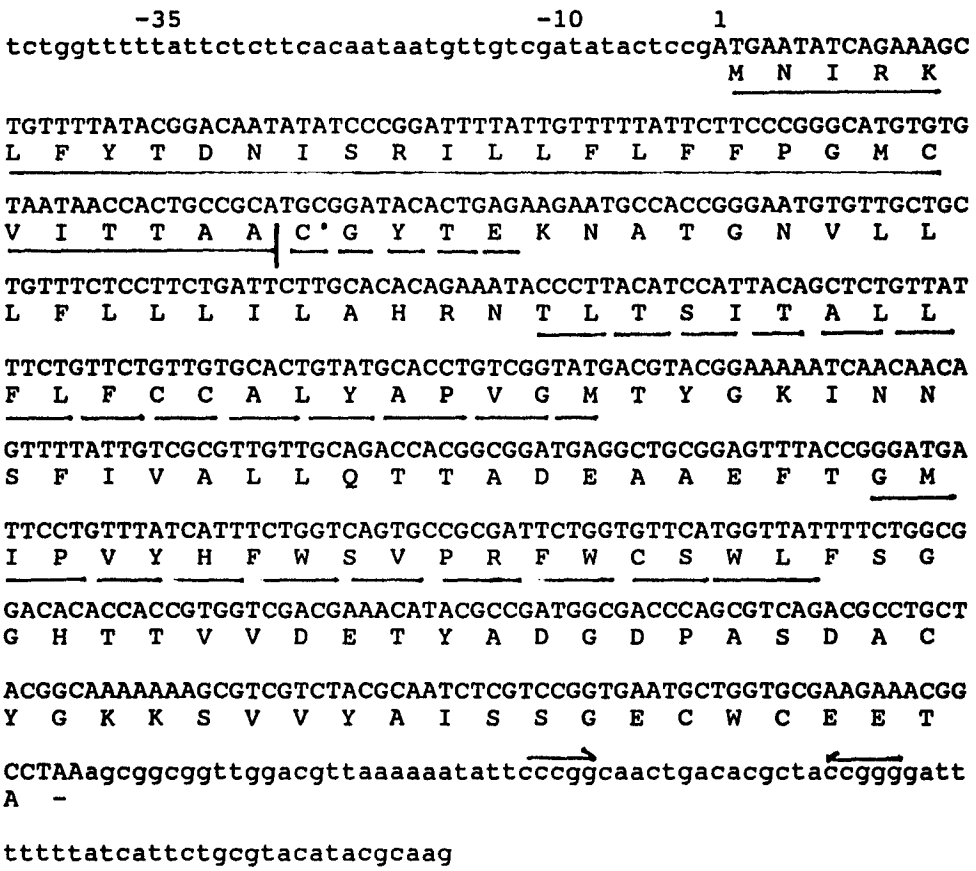


Figure 31. Nucleotide sequence and deduced amino acid structure of plprot2

Predicted signal sequence : solid underlining

Predicted membrane spanning segments : dashed underlining

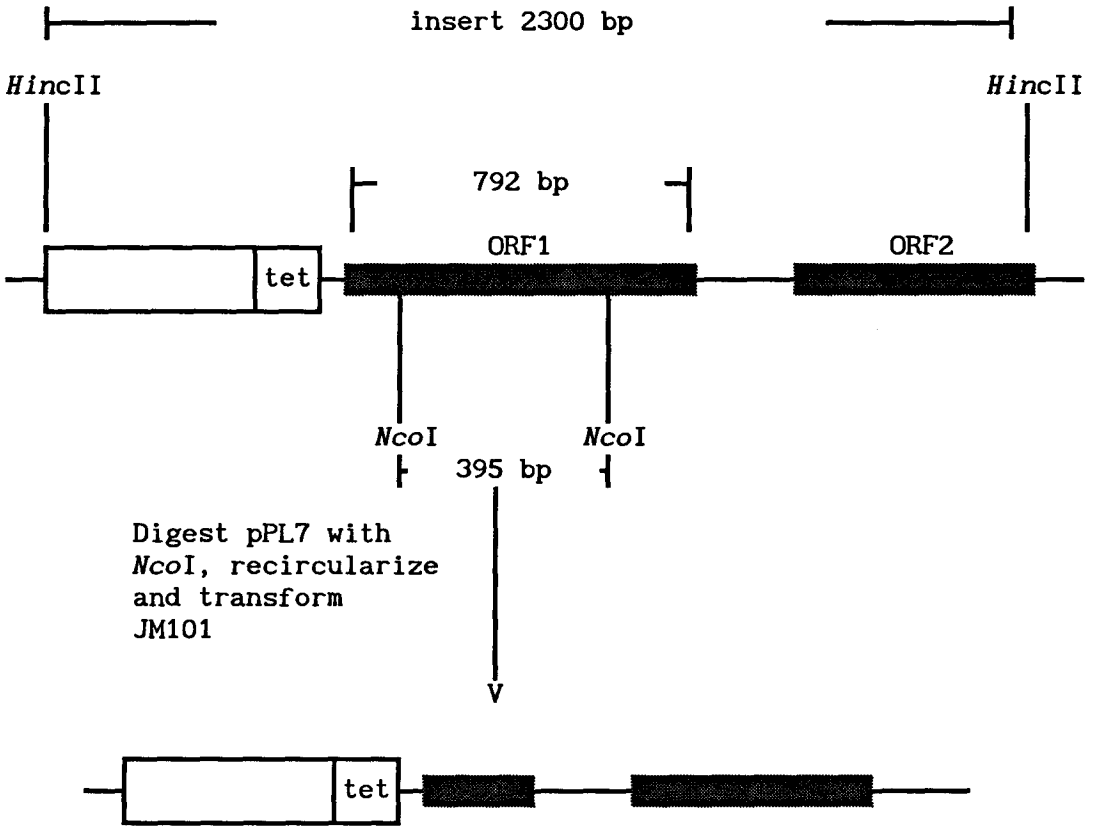


Figure 32. Construction of plasmid pPL7N

= pBR322 DNA; tet = truncated tetracycline resistance gene

= Open Reading Frames (not to scale)

pPL7 = pETE1 *Hinc*II fragment (from partial digest) inserted into pTZ19R *Hinc*II site.

JM101(pPL7) shows strong mannose-resistant hemagglutination

JM101(pPL7N) shows no hemagglutinating activity

concentrations of bacteria. This data localizes the agglutinin to the plprot1 open reading frame.

The putative plprot2 protein was predicted to be an integral membrane protein with a prokaryotic secretory signal sequence, which when cleaved would leave a mature protein of 135 amino acids, 14,802 Da. Interestingly, plprot2 was also predicted to have a membrane lipoprotein lipid attachment site, a site which generally occurs just after the signal sequence. The enzyme signal peptidase II recognizes a conserved sequence and cuts in front of a cysteine residue to which a glyceride-fatty acid lipid is attached (132). This consensus sequence appears in plprot2 as shown below.

```
-Ile-Thr-Thr-Ala-Ala-CYS-Gly-Tyr-Thr-Glu-Lys-
 32                  37                  42
```

The sequencing of the 2.3 kb *Hinc*II fragment revealed three open reading frames in the opposite direction (Fig. 26) that code for proteins of 24,623 Da (plprot3), 9,555 Da (plprot5) and 7,801 Da (plprot4). The nucleotide sequences and deduced amino acid sequences for these putative proteins are shown in Figures 33,34 and 35. The plprot3 and plprot5 proteins each contain predicted signal peptides which, when cleaved, would create mature proteins of 22,681 Da and 8,079 Da respectively. Although these proteins were not responsible for any of the agglutination effect (because pPL4, which contains only this fragment, shows no agglutinating activity), they were scanned for homology with other proteins in the genebank.

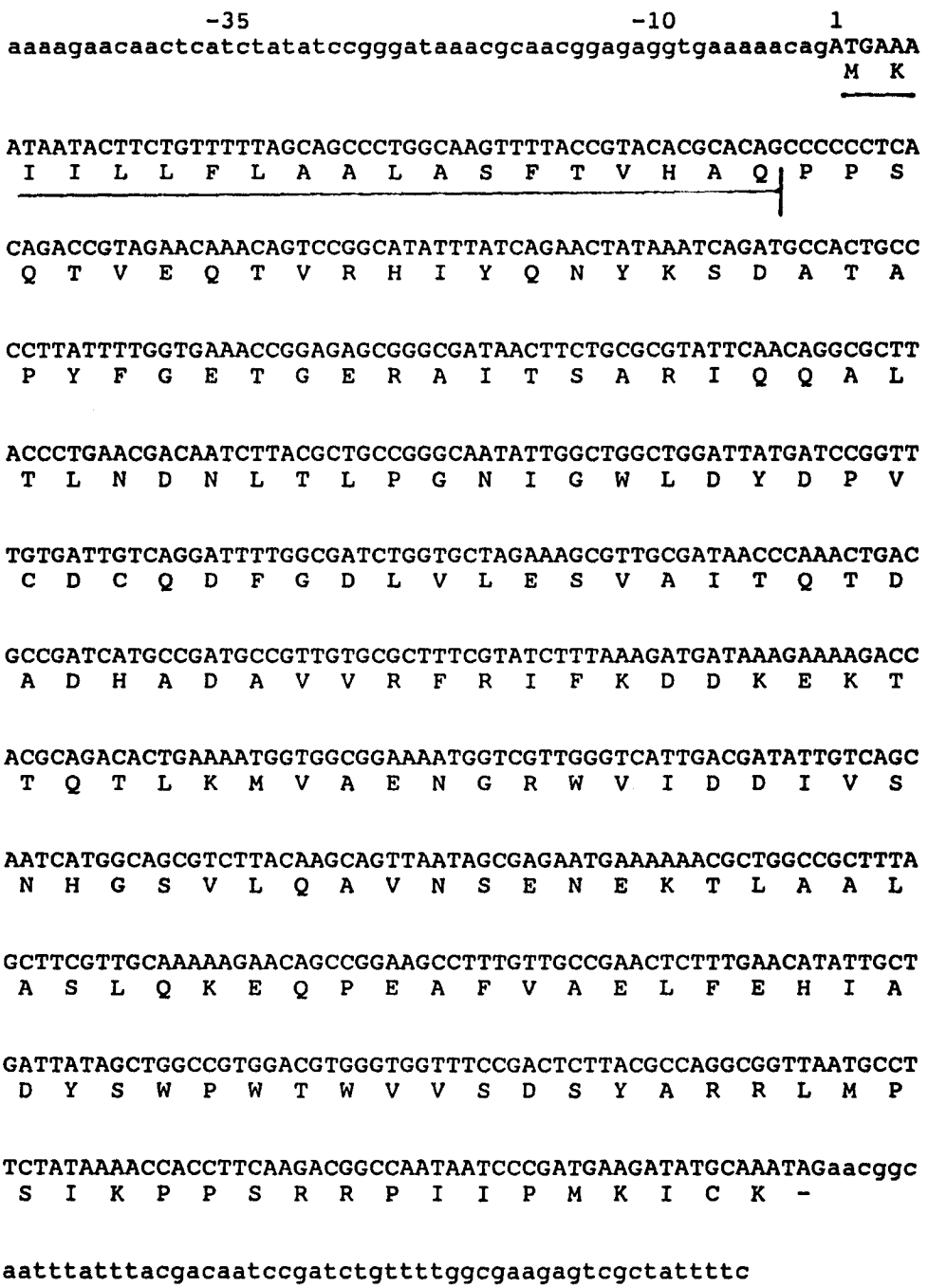


Figure 33. Nucleotide sequence and deduced amino acid structure of plprot3

Putative signal sequence is underlined.

```
              -35                          -10          1
ggcctggtggtgatcccgaaatcggtcacaccttcacgtattgccgaaactttgATGTCT
                                                       M  S

GGGATTTCCGTCTCGACAAAGACGAACTCGGCGAAATTGCAAAACTCGATCAGGGCAAGC
 G  I  S  V  S  T  K  T  N  S  A  K  L  Q  N  S  I  R  A  S

GTCTCGGTCCCGATCCTGACCAGTTCGGCGGCTAACATGCAAATTCTCCCGGTGGCGGTA
 V  S  V  P  I  L  T  S  S  A  A  N  M  Q  I  L  P  V  A  V

ATGTTCCGCTACCGGACTTTTCAGAAATCATTTATTCCCCTCGCGTCCCGCCCGTTGTTA
 M  F  R  Y  R  T  F  Q  K  S  F  I  P  L  A  S  R  P  L  L

CTCTTCCTTGTTCAGGAATGCCAAATATAAggacatcatcatgcagagccggaagctctt
 L  F  L  V  Q  E  C  Q  I  -

aaaagaacaactcatctatatccgggataaacgcaacggagaggtgaaaaacagatgaaa

ataatacttctgttttt
```

Figure 34. Nucleotide sequence and deduced amino acid structure of plprot4

Figure 35. Nucleotide structure and deduced amino acid sequence of plprot5

Putative signal sequence is underlined.

3.3.4.2. Genebank Homology Searches

Each putative protein amino acid sequence was scanned for homology with other known proteins. No significant similarity with known protein sequences was found for plprot2, plprot3 and plprot4. The protein plprot1 showed limited similarity to a region of the *Neisseria gonorrhoeae* opacity protein (133) which has been implicated in the virulence of these organisms, and other *Neisseria gonorrhoeae* outer membrane proteins. The region of strongest identity is shown in Figure 36.

The putative protein plprot5 showed 50 % identity and 74 % similarity with the precursor form of the major *E.coli* outer membrane lipoprotein, a 77 amino acid protein that is covalently attached to the murein component of the inner side of the bacterial cell wall (134). The alignment of the two sequences is shown in figure 37.

```
          140                                                   168
           |                                                     |
plprot1    S V N T L M L N A Y Y D F R N D S A F T P W V S A G I G Y
           + V + + L   L + A   Y D F +   +     F   P + +   A   +   Y
opacity    A V S S L G L S A V Y D F K L N D K F K P Y I G A R V A Y
           |                                                     |
          661                                                   747
```

Identity = 37 % Similarity = 68 %

\+ indicates functional similarity

Figure 36. Identity between plprot1 and *N.gonorrhoeae* opacity protein Identical and functionally similar residues are indicated in middle row.

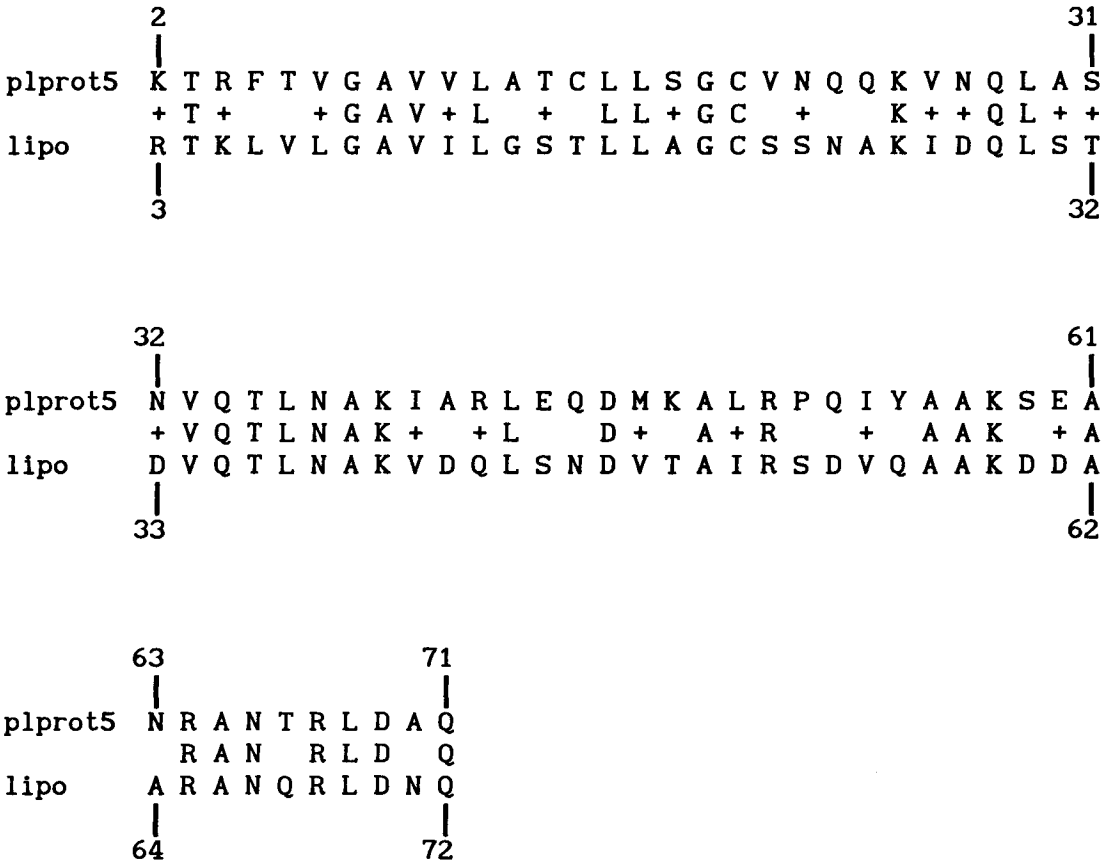


Figure 37. Identity between plprot5 and *E.coli* Major outer membrane lipoprotein precursor

Identical and functionally similar (+) residues are indicated in the middle row.

50 % identity, 72 % similarity

3.4. Discussion

The region of DNA that was cloned contained open reading frames coding for four putative periplasmic, membrane or secreted proteins. These proteins mostly showed no significant similarity to amino acid sequences present in protein databases, although plprot5 was found to be 50% identical to a known *E.coli* outer membrane lipoprotein. This lipoprotein is covalently attached to the murein layer in the periplasm and the gene for this protein is found in single copy on the chromosome (134). These findings suggest that an area of the F41 genome determining membrane structure and function was cloned into pBR322.

The nucleotide sequence of the plprot1 ORF was determined for both DNA strands as shown in Appendix 4. The nucleotide sequences of the other open reading frames were generated predominantly from one strand only, although in the case of plprot2 there is significant dual strand information. The nucleotide sequences of plprot2,3,4 and 5 may therefore contain errors although the presence of putative termination signals in the cases of plprot2 and plprot5 suggest that these possible errors may be relatively minor base substitutions rather than frameshift errors. The consistency of identity that plprot5 shows with lipoprotein throughout its sequence also indicates that the sequence information for this ORF is correct.

Subcloning results identify the 29,024 Da protein plprot1 as the agglutinating agent. Based on physical hemagglutination properties discussed in Chapter 4, plprot1 was named Heat Resistant Agglutinin 1 (HRA1). This protein has a putative secretory signal sequence which,

when cleaved, would produce a mature protein of 24,896 Da.

Antigenic determinants of the adhesin (HRA1) were predicted by computer using the method of Hopp and Woods (136), which searches for regions of highest hydrophilicity. This method performed very well on a set of control proteins, with the highest predicted antigenic determinant correlating to a known determinant in 100 % of the cases. Lower scoring predictions were correct 66 % of the time. Antigenic determinants are likely to be exposed at the surface of the protein and can, in some instances, be correlated to receptor binding areas. The highest scoring predicted antigenic determinant on HRA1 is shown below.

```
  132                                   138
- Arg - Asp - Asp - Leu - Lys - Asn - Glu -
   +     -     -           +           -
```

Figure 38. Predicted antigenic determinant on HRA1

The receptor binding site of the adhesin K99 has been identified using site-directed mutagenesis (105) to be the region shown in Figure 39 below. This region also corresponds to the highest predicted antigenic determinant in the protein. This motif appears to be implicated in the binding of three other sialic acid binding lectins, CFA1 (Colonization factor antigen 1), CT-B (Cholera toxin B) and LT-B (*E.coli* heat-labile toxin B) (106, Figure 39).

CFA1	Lys - Lys - Val - Ile - Val - Lys
CT-B	Lys - Lys - Ala - Ile - Glu - Arg
LT-B	Lys - Lys - Ala - Ile - Glu - Arg
K99	Lys - Lys - Asp - Asp - Arg
(HRA1	Arg - Asp - Asp - Leu - Lys - Asn - Arg)

Figure 39. Comparison of implicated and identified binding site amino acid sequences (adapted from 106).

Although the HRA1 antigenic determinant is quite different in sequence to the determined binding site regions, it has a similar residue composition and it is interesting to speculate on the possible receptor binding importance of this area. This region would be an excellent candidate for site directed mutagenesis experiments to assess any adhesive function.

Another interesting point about the deduced amino acid structure of HRA1 is that there are no cysteine residues present. The K88 and CFA1 adhesins (60,38) and a number of other outer membrane proteins contain no cysteine residues and it has been suggested (68) that this is necessary to protect the structure from conformational changes induced by differences in the redox potential of the external environment.

4. Biochemical Studies on Proteins Encoded by pETE1

4.1. Background

Once it was established that the recombinant plasmid pETE1 and some of its subclones mediated a mannose-resistant agglutination of human erythrocytes, attempts were made to isolate the adhesive protein from the surface of the cell. When physical, chemical and genetic methods of doing this met with limited success, experiments were designed to identify the receptor on the red cell membrane. Maxicell analysis on the clone was undertaken in an attempt to elucidate plasmid-encoded proteins. When a partial purification of the adhesive protein was achieved, its N-terminal amino acid sequence was determined and compared to information derived from the nucleotide sequence.

4.2. Materials and Methods

4.2.1. Bacterial Strains and Growth Conditions

The bacteria used were as in Section 2.2.1., with the addition of *Escherichia coli* B23 and C600 (117) which were used as plasmid hosts in the dissociation studies. *Escherichia coli* CSR603 (138) was obtained from the American Type Tissue Collection (ATCC), and used in the maxicell experiments.

For adhesin production studies, bacteria were grown on Minca medium (139, 1.36 g/l KH_2PO_4, 7.56 g/l Na_2HPO_4, 1 g/l glucose, 1 g/l casamino acids (Difco) and 1ml/l trace salts solution (40 mM Mg^{2+}, 5 mM

Mn^{2+}, 0.6 mM Fe^{2+} and 2.7 mM Ca^{2+}), autoclaved 20 min) or LB (Sect. 2.2.1.). In maxicell studies, bacteria were grown in M9 minimal media (117) (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1.0 g/l NH_4Cl, pH7.4. After autoclaving 20 min, $MgSO_4$ was added to 20 mM, glucose to 0.2 % and $CaCl_2$ to 0.1 mM. These solutions were previously filter sterilized.). For the ^{35}S-labelling of bacteria, the $MgSO_4$ was omitted from the media. Media was solidified by the addition of 1.5 % agar and supplemented with ampicillin, IPTG and cycloserine (200 μg/ml) (Sigma) where necessary.

4.2.2. Physical Methods for the Isolation of the Agglutinin

4.2.2.1. Temperature/vortex Experiments

The initial series of isolation attempts was based on observations that large amounts of F41 adhesin were removed from the cell surface by simple vortexing (69). *Escherichia coli* F41 and *E.coli* JM101, B23, B/R and C600 harbouring recombinant plasmids were grown on solid media overnight and harvested by scraping the cells into PBS. The cells were washed once with PBS and resuspended in PBS to an approximate concentration of $5x10^9$ cells/ml as measured by filling a microhematocrit tube with bacterial suspension, centrifugation for 10 min and measuring the percentage of the tube filled with packed cells (1 % ≅ $2.5x10^9$ cells/ml). The cells were then subjected to cycles of heating, vortexing and blending in a Waring blendor as detailed in Table 5.

Table 5. Disruption experiments

Expt.	Experimental Details
1.	vortex 5 min
2.	heat 60 ℃ 30 min, vortex 5 min
3.	heat 60 ℃ 30 min, vortex w/glass beads 5 min
4.	Waring blend 5 min
5.	heat 60 ℃ 1 h, Waring blend 5 min
6.	heat 60 ℃ 3 h, Waring blend 10 min
7.	heat 60 ℃ 1 h, Waring blend 20 min
8.	add 10 mM EGTA, heat 60 ℃ 1 h, Waring blend 20 min
9.	heat 70 ℃ 1 h, vortex 5 min
10.	heat 100 ℃ 5 min, vortex 5 min
11.	heat 100 ℃ 5 min, Waring blend 20 mins

After these treatments, the bacteria were centrifuged for 5 min at high speed in a microfuge, the supernatant was transferred to a new tube and the pellet was resuspended in the same volume PBS. The supernatant was usually concentrated 40x by centrifugation in Amicon microconcentrators with a 10,000 Da cutoff. Both the supernatant and the pellet were then tested for hemagglutinating activity in a microtitre serial-dilution assay (see Section 2.2.7.1.), electrophoresed on a 12.5 % denaturing polyacrylamide gel, and sometimes blotted onto nitrocellulose for receptor studies with biotinylated red cell ghosts (see Section 2.2.8.). Often several of the above experiments would be performed in sequence to the same bacteria resuspended in PBS.

4.2.2.2. Detergent Extraction Experiments

The bacteria were grown and washed as above and incubated with various detergents for 1 h, 60 °C, at concentrations below the critical micelle concentration (CMC). The detergents used are shown in table 6.

Table 6. Detergents utilized for solubilization of membrane proteins

Detergent	CMC[1]	Properties
Octyl Glucoside	25 mM	non-ionic
CHAPS[2]	0.49 %	zwitterionic
Deoxycholate	4-6 mM	anionic
SDS	84 mM	anionic

[1]CMC = Critical Micelle Concentration
[2]CHAPS = 3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate

The bacteria were then centrifuged and the supernatant was separated from the pellet, which was resuspended in the same volume. For hemagglutinations, both the resuspended pellet and the supernatant were extensively dialysed (2 days) against 50 mM phosphate buffer pH 7.4. For SDS-PAGE, the supernatants were concentrated for 90 min in Centricon filters with a 10,000 Da cutoff.

4.2.2.3 Osmotic Shock

Escherichia coli F41, JM101 and JM101(pETE1) were grown overnight and suspended to 12.5×10^9 cells/ml in 10 ml 20 % sucrose-PBS. The cells

were incubated overnight at 4 °C then poured rapidly into 75 ml 5 mM phosphate pH 8.0 and mixed well. The bacteria were centrifuged and the supernatants concentrated in Centricon filters with a 10,000 MWt cutoff. The supernatants and resuspended pellets were tested for hemagglutination and electrophoresed on SDS-PAGE.

4.2.2.4. Sonication

Crude membrane preparations were made by sonic disruption of *E.coli* F41, JM101, JM101(pETE1) and a number of subclones in a Sonifier 350 cell disrupter (Branson). The bacteria were grown overnight in 5 ml LB, harvested by centrifugation and resuspended in 0.5 ml 50 mM phosphate buffer pH 7.4. The cells were then sonicated for 30 s, 50 % pulse, setting 3, on ice. The resulting suspension was centrifuged and the supernatant (containing the cytosolic proteins) was separated from the pellet (crude outer and inner membranes), which was resuspended in the same volume. Hemagglutinations and SDS-PAGE were performed on both preparations.

4.2.3. Erythrocyte Receptor Studies

4.2.3.1. Species Hemagglutination Profile

The bacterium containing the recombinant plasmid, *E.coli* JM101(pETE1), was tested for hemagglutination against a variety of erythrocytes from different species. The host cell, JM101, and F41 were used as controls. The bacteria were suspended to 1.75×10^{10} cells/ml, and the erythrocytes were washed 3x in PBS and used at a 1 % hematocrit.

The erythrocytes came from the following species - goat, rat, mouse, guinea pig, cow, rabbit, chicken, cat, pig, dog and human.

4.2.3.2. Binding Studies with Erythrocyte Ghosts

(a) Erythrocyte Ghost Preparation

The results from the species' hemagglutination profile showed that there were some interesting differences between *E.coli* JM101, JM101(pETE1) and F41. Erythrocytes from two species, pig and dog, were chosen for further study as they interacted very differently in hemagglutination tests against these bacteria (see Section 4.3.2.1.). Red cell ghosts were prepared by washing the cells (5 ml) twice in PBS, then making the cells back up to 5 ml with PBS. The cells were lysed by pouring this suspension into 20 volumes ice-cold 10 mM phosphate pH 8.0 and incubating on ice for 30 min. The solution was centrifuged for 30 min at 6,000 g and the supernatant carefully pipetted off to leave a loose pellet of erythrocyte membranes (ghosts). The ghosts were washed by suspension in 10 mM phosphate pH 8.0 followed by centrifugation, until they lost most of their red colour (4-6 washes). The last wash was in water, and the pellet after centrifugation was frozen at -20 °C until needed.

(b). Biotinylation of Ghosts and Bacteria

The erythrocyte ghosts were biotinylated by suspension of 200 µl ghosts in 400 µl carbonate-bicarbonate buffer pH 8.5 and addition of 2 µl NHS-LC biotin (Pierce, 200 mg/ml in H_2O). The reaction was left on

ice for 1 h, centrifuged and resuspended in tris-buffered saline (TBS, 20 mM tris,500 mM NaCl pH 7.5). The NHS-LC biotin is a water soluble analogue of biotin with an extended spacer arm that reacts with amine groups. For this reason the reaction was performed in carbonate-bicarbonate buffer, and any excess biotin quenched with tris. Bacteria were biotin-labelled in an identical manner, with cells suspended in 400 µl carbonate-bicarbonate to a concentration of $12.5x10^{10}$ cells/ml. After biotinylation the cells were centrifuged, resuspended in 50 µl TBS and heated for 1 h at 60 °C. The cells were then vortexed for 5 min and centrifuged. The supernatant (containing any biotinylated proteins that were removed from the cell surface) was transferred to a new tube and the pellet (biotinylated bacteria) resuspended in 200 µl TBS. Biotinylated protein molecular weight standards for SDS-PAGE were prepared in a similar fashion.

(c). Erythrocyte-bacteria Binding Experiment

This experiment entailed electrophoresing non-labelled erythrocyte ghosts or bacteria whole cell lysates on a 12.5 % SDS-polyacrylamide gel then western blotting the proteins onto nitrocellulose. The nitrocellulose was rinsed in TBS, blocked for 1 h with TBS / 0.05 % tween / 3 % BSA, washed twice in TBS-tween and incubated for 3 h with samples of either biotinylated erythrocyte ghosts (50 µl in 5 ml TBS), biotinylated bacteria (50 µl / 5 ml) or biotinylated bacterial vortex supernatants (50 µl / 5 ml). Table 7 details this procedure.

Table 7. Erythrocyte ghost-bacteria binding experiment

gel 1			gel 2		
lane	sample	blotted w/	lane	sample	blotted α
1 2 3	standard pig dog	biotinylated F41 vortex sn	1 2 3	standard pig dog	biotinylated F41 bacteria
4 5 6	standard pig dog	biotinylated JM101 vortex sn	4 5 6	standard pig dog	biotinylated JM101 bacteria
7 8 9 10	standard pig dog	biotinylated JM101(pETE1) vortex sn	7 8 9 10	standard pig dog	biotinylated JM101(pETE1) bacteria
gel 3			gel 4		
lane	sample	blotted α	lane	sample	blotted α
1 2 3 4	standard F41sn JM101sn JM(p1)sn	biotinylated dog ghosts	1 2 3 4	standard F41sn JM101sn JM(p1)sn	biotinylated pig ghosts
6 7 8 9	standard F41pt JM101pt JM(p1)pt	biotinylated dog ghosts	6 7 8 9	standard F41pt JM101pt JM(p1)pt	biotinylated pig ghosts

sn = supernatant; pt = resuspended bacterial cell pellet after vortexing

The nitrocellulose filters were then washed twice in TBS-tween and incubated for 2 h with streptavidin-conjugated alkaline phosphatase (Pierce, 5 μl in 5 ml TBS). The filters were washed again and incubated in 0.1 M tris pH 9.6 containing X-phosphate (50 μg/ml) and nitroblue tetrazolium (10 μg/ml) for 5-10 min for the colour to develop. Blots were rinsed in water and air-dried.

4.2.3.3. Glycophorin Experiments

The human erythrocyte receptor for the F41 agglutinin is known to be glycophorin, a major red blood cell membrane glycoprotein. To determine whether the agglutinin encoded by pETE1 also recognized glycophorin, two experiments were performed. The first experiment was a glycophorin inhibition assay involving incubating 50 µl bacteria ($12.5x10^9$ cells/ml) with 50 µl glycophorin (5.0 mg/ml) (prepared by J. Cavanagh using the method of Marchesi and Andrews, 140) for 10 min at 23 °C, then performing a standard hemagglutination microtitre assay with serial dilutions of the bacteria against washed 1 % human or pig erythrocytes in PBS. The second experiment involved incubating erythrocytes (1 ml 10 % hematocrit) with *Vibrio cholerae* neuraminidase (Sigma, 1 U), for 2 h at 37 °C, followed by washing of the cells and microtitre hemagglutination with serial dilutions of bacteria. Neuraminidase is an enzyme that removes the terminal sialic acid residue from oligosaccharides present on erythrocyte surface glycoproteins (141).

4.2.4. Maxicell Analysis of Proteins Encoded by pETE1

Escherichia coli CSR603 was obtained from the American Type Culture Collection. This bacterium has mutations in its *rec*AI and *uvr*A6 genes which halt the functions of the gene products, proteins that allow recombination and repair DNA that has been damaged by UV light (138). When irradiated with UV therefore, the bacterial DNA is extensively degraded and replication ceases. If the bacterium contains a plasmid,

then that too will be degraded, but as there are multiple copies in the cell any that escaped a UV hit will continue to replicate with plasmid levels increasing tenfold by 6 h after illumination. When DNA damage is at a maximum, the non-dividing cell (maxicell) will contain mostly plasmid encoded proteins, as the cells transcription/translation systems are still functional. If ^{35}S-methionine is introduced as the only sulphur source, then the plasmid proteins will be radioactively labelled during synthesis (Sancar and Rupp (142)).

Plasmids pETE1 and pBR322 were transformed into $CaCl_2$ competent CSR603 (see Section 2.2.6.). The plasmid-containing bacteria were then grown overnight in 5 ml LB amp. A 500 µl aliquot of the culture was added to 25 ml M9 minimal media containing ampicillin and the cells were grown at 37 °C to exponential phase. This took approximately 12 h, as the *rec*A1 mutation causes the bacteria to grow very slowly (117). The cells were then exposed to UV light (a germicidal lamp held 30 cm from the cultures) for 30 s and incubation continued for 1 h at 37 °C. Cycloserine (200 µg/ml) was added to the cultures and incubation continued for 16 h. After this time the cells were centrifuged (6,000 rpm, 15 min) and washed twice with M9 salts. The bacteria were resuspended in 50 µl M9 media minus sulphate and ^{35}S-methionine (10 mCi/ml) was added to 5 µCi/ml. The cells were incubated for 2 h at 37 °C then washed twice with M9 salts and resuspended in SDS-PAGE loading buffer (40 µl). Samples (15 µl) were run on two 12.5 % gels, one of which was stained with Coomassie blue, the other of which was blotted onto nitrocellulose for 2 h at 1 A. The nitrocellulose was rinsed in water, air dried and exposed to X-ray film for as long as 4 days.

4.2.5. N-terminal Sequencing of Hemagglutinin

Gel electrophoresis of crude membrane fractions of JM101(pETE1) and JM101(pPL7) prepared by sonication showed the presence of a distinct band at ≅ 25 kDa compared to JM101 (Section 4.3.1.). These samples were blotted to PVDF (Immobilon) membrane and lightly stained with Coomassie blue. When the membrane was dry, the band was cut from the membrane and sequenced by automated Edman degradation at the University of Victoria protein sequencing facility.

4.3. Results

4.3.1. Membrane Association of Hemagglutinin

Temperature/vortex experiments on *E.coli* strains harbouring pETE1 showed that the agglutination effect was strongly associated with the cell surface and could not easily be removed. Figure 40 shows the hemagglutination patterns of some supernatants from the disruption experiments, along with SDS-PAGE results from these samples. A wide variety of disruption experiments were attempted and the samples shown in Figure 40 represent the best results achieved. The supernatant samples even when concentrated showed very little hemagglutination, but in the samples that produced a small amount of activity, SDS-PAGE showed the existence of a unique band of approximately 25,000 Da. The pETE1 plasmid was introduced into the different *E.coli* strains described in Section 4.2.1. and always conferred on the bacteria the agglutinating ability, but was always strongly associated with the cell surface. In the centrifuged pellets of cells containing pETE1 after these disruption treatments there remained a strong agglutinating presence which was very resistant to inactivation by the heat and mechanical forces used. In fact, it was found that the bacteria containing pETE1 were more resistant to these forces than the original bacteria, either JM101 or other strains. After identical treatment, the control cells would experience some degree of lysis as judged by the turbidity of centrifugation supernatants, whereas plasmid-containing cells would remain intact. This stabilization effect was also seen in sonication experiments.

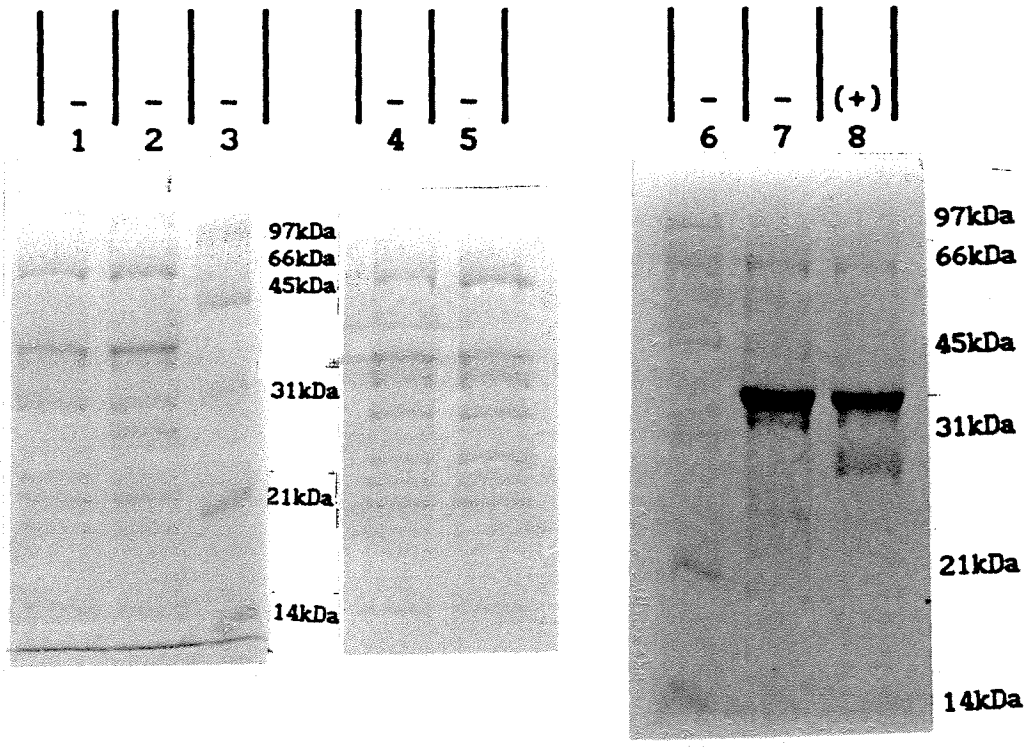


Figure 40. SDS-PAGE and hemagglutination profiles of samples from temperature/vortex experiments

(see Table 8, next page, for hemagglutination strength index)

lane 3,6 = molecular weight standards
lane 1 = B/R concentrated supernatant after experiment 6 (Section 4.2.2.1)
lane 2 = B/R(pETE1) conc. sn. after expt. 6
lane 4 = B/R conc. sn. after expt. 4
lane 5 = B/R(pETE1) conc. sn. after expt. 4
lane 7 = B/R conc. sn. after expt. 10
lane 8 = B/R(pETE1) conc. sn. after expt. 10

Table 8. Agglutination scale (used throughout this document)

+ + + +	= agglutination to approximately 6.0 x10^7 bacteria/ml (or an erythrocyte:bacteria ratio of 2:1).
+ + +	= agglutination to approximately 2.1 x10^8 bacteria/ml
+ +	= agglutination to approximately 1.0 x10^9 bacteria/ml
+	= agglutination to approximately 4.0 x10^9 Bacteria/ml
(+)	= trace partial agglutination
-	= no visible agglutination

Microtitre Hemagglutination assays carried out as described in section 2.2.10.1

Experiments with detergents met with little success. The detergents removed considerable amounts of material from the cell wall (SDS-PAGE Fig. 41), but there were no discernible differences between the control bacteria and the plasmid-containing cells. After dialysis and concentration, no supernatant sample contained agglutinating properties. Osmotic shock of the bacterial cells gave a large number of protein bands on SDS-PAGE, but again no differences were seen between the control and the test bacteria (Figure 42).

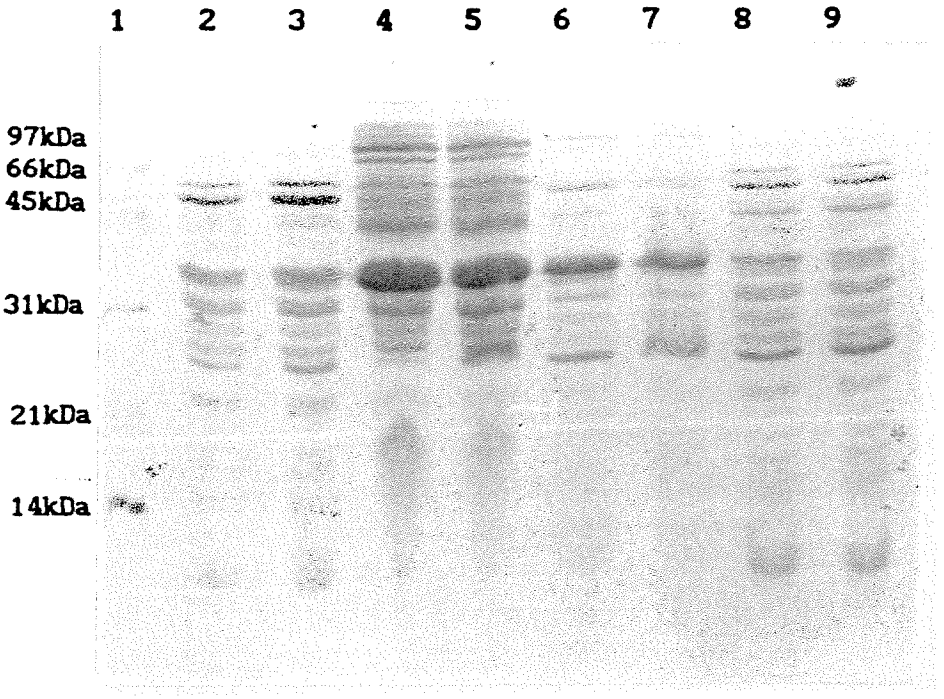


Figure 41. SDS-PAGE of detergent-solublized proteins

lane 1 = molecular weight standards
lane 2 = B/R supernatant after octyl glucoside treatment
lane 3 = B/R(pETE1) supernatant after octyl glucoside treatment
lane 4 = B/R supernatant after SDS treatment
lane 5 = B/R(pETE1) supernatant after SDS treatment
lane 6 = B/R supernatant after CHAPS treatment
lane 7 = B/R(pETE1) supernatant after CHAPS treatment
lane 8 = B/R after deoxycholate treatment
lane 9 = B/R(pETE1) after deoxycholate treatment

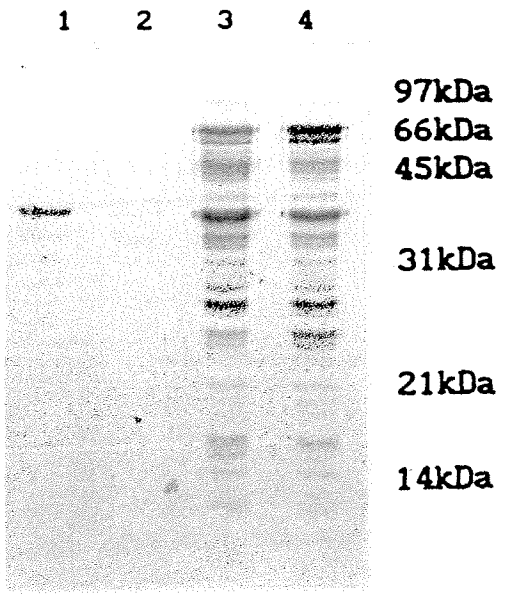


Figure 42. SDS-PAGE of proteins released by osmotic shock

Bacteria were subjected to osmotic shock as described in Section 4.2.2.3.

lane 1 = B/R supernatant
lane 2 = B/R(pETE1) supernatant
lane 3 = B/R concentrated supernatant
lane 4 = B/R(pETE1) concentrated supernatant

As the evidence suggested that the agglutinating factor was very tightly bound in the bacterial membrane, sonic disruption of the cells was tried to obtain crude membrane preparations. The crude membrane preparations from JM101(pETE1) still showed very strong red blood cell aggregation and SDS-PAGE of these membranes contained a distinct band over the cloning vehicle that ran at around 25,000 Da. The gel is shown in Figure 43. Subclones and controls were sonicated and the membrane pellets and supernatants tested for HA and electrophoresed. The results showed that hemagglutinating activity and the 25 kDa protein band always coexisted. Membranes prepared from subclones pPL2, 3 and 4, containing the 1.3, 1.0 and 2.3 kb *Hinc*II fragments respectively, were inactive in HA microtitre and did not contain an obvious band (Fig. 43). Bacteria containing pBR322 were also negative. The plasmid pPL7 however behaved similarly to pETE1 with both the agglutination and the band present. A subclone of pPL7 with a 395 bp *Nco*I deletion in the open reading frame of plprot1, called pPL7N, showed no band and no agglutination (Fig. 44). This result locates the hemagglutination effect to that open reading frame.

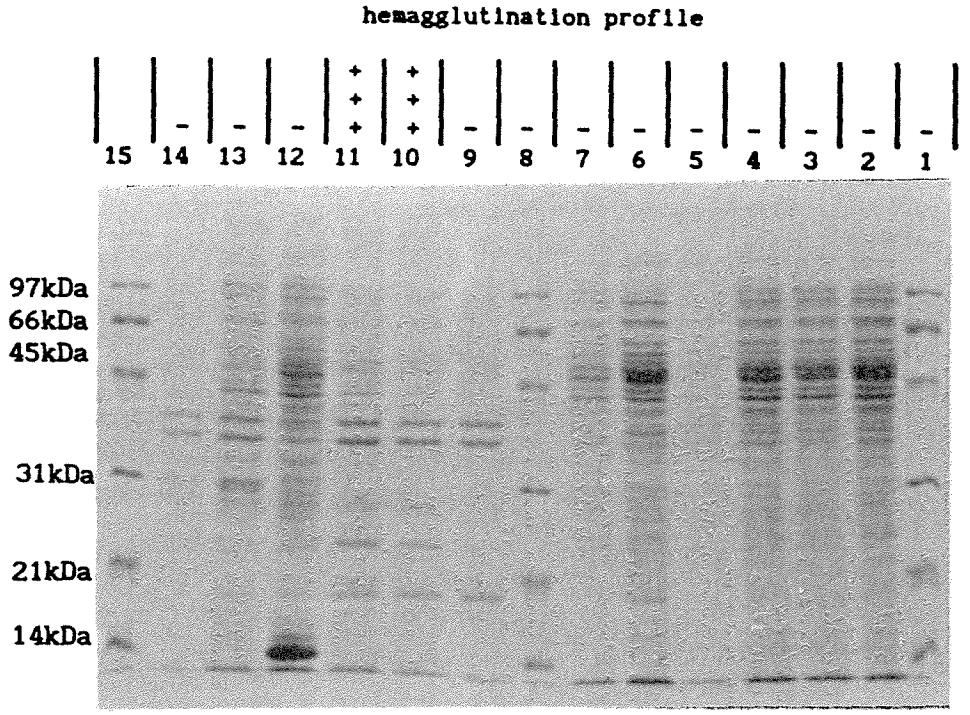


Figure 43. SDS-PAGE and hemagglutination profile of crude membrane fragments prepared by sonication.

lanes 2-7, bacterial supernatants after sonication and centrifugation
lanes 9-14, crude membrane pellets after sonication and centrifugation
lanes 1,8 and 15, protein molecular weight standards

2 and 9 = JM101
3 and 10 = JM101(pETE1)
4 and 11 = JM101(pPL7)
5 and 12 = JM101(pPL4)
6 and 13 = JM101(pPL3)
7 and 14 = JM101(pPL2)

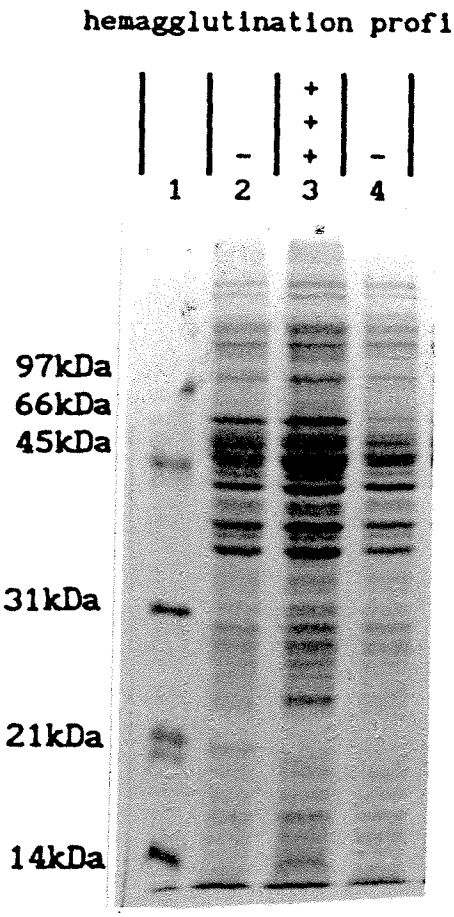


Figure 44. SDS-PAGE and hemagglutination profiles of crude membrane preperations harbouring pPL7N control plasmid.

lane 1 = molecular weight standards
lane 2 = JM101 crude membrane pellet
lane 3 = JM101(pPL7) crude membrane pellet
lane 4 = JM101(pPL7N) crude membrane pellet

4.3.2. Comparison with F41

4.3.2.1. Agglutination Strength and Specificity

The bacteria containing pETE1 agglutinated human red blood cells to the same degree in a microtitre assay as the parent, F41, but without the requirement of specialized media. The agglutination was of the same magnitude whether grown on plates or liquid media, and was unchanged in the presence of 10 mM mannose. Unlike F41 or other mannose resistant hemagglutinins characterized from enterotoxigenic *E.coli* strains, agglutinating activity was not destroyed by heating at 60 ^{o}C for thirty minutes, or even by transiently boiling the bacteria. For this reason the hemagglutinin was named Heat Resistant Agglutinin 1 (HRA1). Bacteria harbouring pETE1 did not appear to be fimbriated (see Section 5.3.6.). The hemagglutination was not inhibited by glycophorin, the F41 receptor, and neuraminidase treatment of erythrocytes prior to hemagglutination by JM101(pETE1) had no effect. For further characterization, the agglutinating activity of the clone against a range of species erythrocytes was investigated. The species agglutination profile is shown in Table 9.

These results are interesting for several reasons -

(1). The cloning vehicle, JM101, agglutinates certain species' erythrocytes very strongly, indicating the presence of some type of surface adhesin. This observation may be explained by later results indicating that this strain expresses the mannose sensitive type 1 adhesin, though the type 1 fimbriae are known to agglutinate erythrocytes from a broader range of species (12).

Table 9. Agglutination profile for washed erythrocytes of the species indicated; T = 21 °C; suspending medium was PBS, no mannose. Experiment performed as Section 4.2.3.1.

Species	Extent of Hemagglutination[1]			
	F41 Adhesin	F41 cell	JM101	JM101(pETE1)
Goat	+ + +	+ + +	–	+
Rat	+ + +	+ + +	+ + + +	+ + + +
Mouse	–	+	+	+ + +
Guinea Pig	+ + +	+ + +	+	+ + +
Cow	–	+	–	+
Rabbit	–	+	+	+ + +
Chicken	+ + +	+ + +	–	+ +
Cat	+ + + +	+ + + +	–	+
Pig	+ + + +	+ + + +	–	+ + +
Dog	–	+	–	+ + +
Human	+ + +	+ + + +	–	+ + +

Agglutination scale as described in Table 8.

(2). F41 cells express more than just the F41 adhesin. This bacterium is known to express the K99 adhesin also. Unfortunately a sample of $K99^{+}F41^{-}$ *E.coli* was not available at the time of the test, so it cannot be determined which species' agglutination is caused by this agent. Studies in the literature (65) have shown that K99 can cause agglutination of horse, sheep and cow erythrocytes, but not guinea pig or human. It can be concluded therefore that K99 is not responsible for the agglutinating behavior of JM101(pETE1) because of the strong hemagglutination of human red cells mediated by this strain.

(3). Dog erythrocytes are agglutinated weakly by F41 bacteria, but not at all by the purified adhesin or by JM101. This species however shows strong aggregative behavior with JM101(pETE1), which could indicate the amplification of a minor agglutinin on F41 by cloning into a plasmid vector.

(4). The cloned agglutinin does not appear to be a non-specific "sticky" protein due to the large differences in strength of adherence between species.

4.3.2.2. Erythrocyte receptor results

Experiments with erythrocyte ghosts failed to unequivocally elucidate either the receptor on the red cell or the bacterial protein responsible for the adherence. Of the set of experiments where the red cell membranes were electrophoresed and blotted against the biotinylated bacteria supernatants, only F41 showed binding to the immobilized membrane proteins (Fig 45). The binding was not specific to a single band, but several bands were obviously more favoured than others; for example, the band at approximately 55 kDa corresponding to glycophorin, the known receptor for F41. Some differences were seen between the binding patterns of F41 to pig and dog ghosts. No binding was observed for JM101 as expected, and no binding was seen when JM101(pETE1) supernatant was incubated with the membrane. This is not surprising as very little, if any, of the agglutinating agent is removed from the cell surface by vortexing. Unfortunately, when the same experiment was attempted with the whole bacteria, there was no binding from any of the strains indicating that the bacteria were too large to be tightly bound to the membrane proteins in the nitrocellulose.

Experiments in which the bacterial vortex supernatant proteins were blotted onto the membrane and then incubated with biotinylated erythrocyte ghosts showed that both pig and dog ghosts recognized a 29,000 Da protein from the F41 bacteria supernatant (Figure 46). This would correspond to the F41 adhesin and is interesting since this adhesin when purified shows strong affinity for pig erythrocytes but not dog. The experiments with whole cells however showed many non-specific diffuse bands for all samples which made interpretation impossible. This is unfortunate as the adhesin present on JM101 is strongly associated with the cell surface and could not be purified for cell-free binding experiments. Both JM101 and JM101(pETE1) supernatants show common ghost-recognized bands in Figure 46, which may be due to non-specific interactions, and in the bacterial supernatant blot against dog ghosts, a faint 25 kDa band is visible in the JM101(pETE1) lane.

These results are very inconclusive. The receptor for the JM101(pETE1) agglutinin on the erythrocyte was not determined, and the agglutinin itself was not identified with certainty, the only possibility being the weak 25 kDa band seen against a non-specific background. The weak band seen may have occurred because the interaction was not of sufficient strength to withstand the nitrocellulose membrane washing procedure or because denaturing the proteins on SDS-PAGE destroyed their specificity. Evidence for the former speculation was found during the viscometric analysis (Section 5.2.2.), and evidence for the latter is provided by the weak interaction seen between blotted F41 whole cell digests and biotinylated ghosts. Also, the biotinylation of amine groups on the bacterial proteins and on

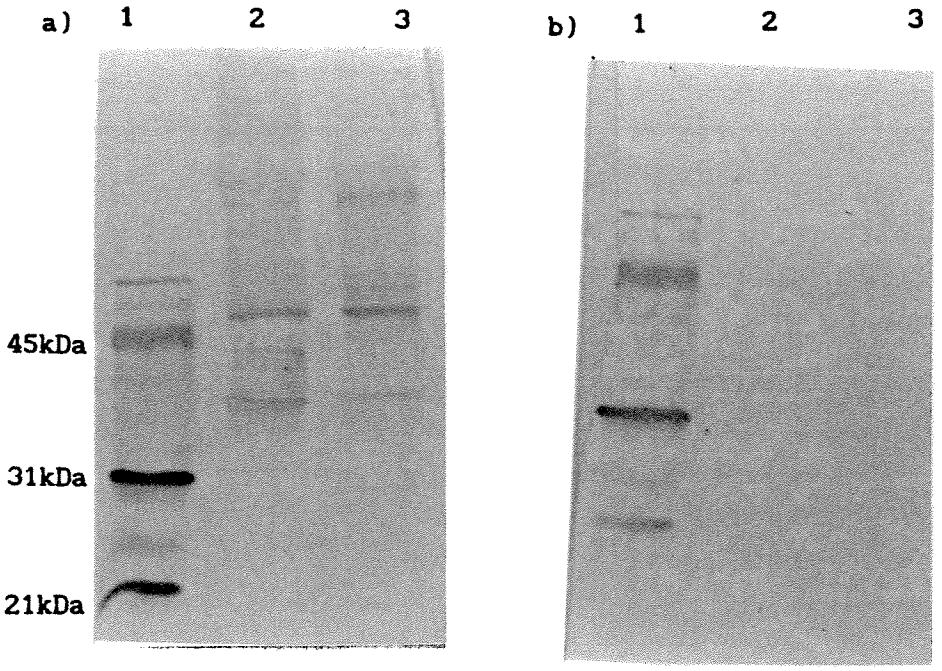


Figure 45. Nitrocellulose immobilized erythrocyte ghost proteins blotted against biotinylated bacterial proteins.

a) Erythrocyte ghosts blotted against biotinylated F41 supernatants.

b) Erythrocyte ghosts blotted against biotinylated JM101(pETE1) supernatants. All other samples were identical to this blot.

lane 1 = biotinylated molecular weight standards
lane 2 = pig erythrocyte ghosts
lane 3 = dog erythrocyte ghosts

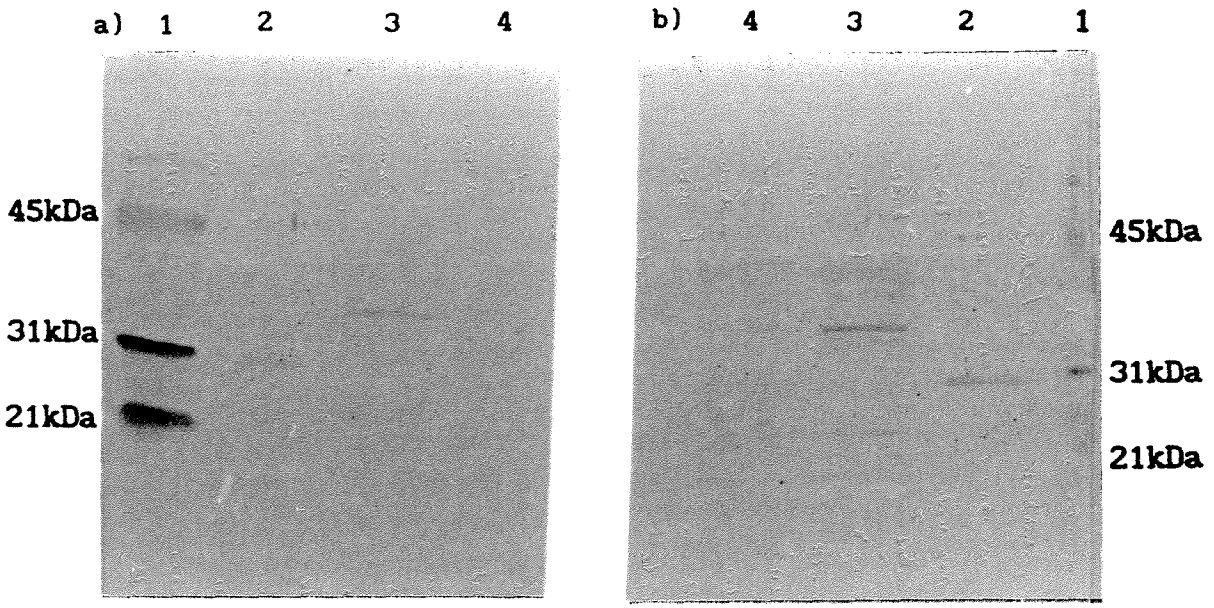


Figure 46. Nitrocellulose immobilized bacterial proteins blotted against biotinylated erythrocyte ghosts

Experiment performed as described in Section 4.2.3.2.

a) pig erythrocyte ghosts, b) dog erythrocyte ghosts

lane 1 = molecular weight standards
lane 2 = F41 supernatant
lane 3 = JM101(pETE1) supernatant
lane 4 = JM101 supernatant

the erythrocytes may have interfered with the binding reaction (the K99 adhesin erythrocyte binding site incorporates lysine residues and HRA1 contains a similar sequence; see Section 3.4.).

4.3.2.3. Maxicell Results

Maxicell results are shown in Figure 47. The autoradiograph shows production of a radiolabelled protein of 25 kDa by JM101(pETE1). The β-lactamase encoded by the ampicillin resistance gene of pBR322 is probably the band visible at approximately 30 kDa. There is a high background suggesting that the UV exposure time was not optimal, but lengthening the exposure time led to a drastic loss of labelling. No bands are seen in the JM101(pBR322) control lane indicating that labelling did not occur efficiently in this sample for unknown reasons. The Coomassie blue stain of these bacteria showed enhancement of production of these proteins as expected.

4.3.3. N-terminal Sequence

The N-terminal sequence of the 25k band visible in the SDS-PAGE of the crude membrane preparations of JM101(pETE1) and JM101(pPL7) was determined. The band sent for analysis was impure, as shown on SDS-PAGE (Fig 43). There is a faint contaminating band in the JM101 membrane fraction lane running at approximately the same molecular weight.

The N-terminal sequence was determined to be -

H_2N-Asp-Glu-Ala-Gly-Thr-Phe-Phe-Tyr-Arg(?)-Thr-Gly-Gly(?)-Ala

Question marks indicate inconclusive sequence data.

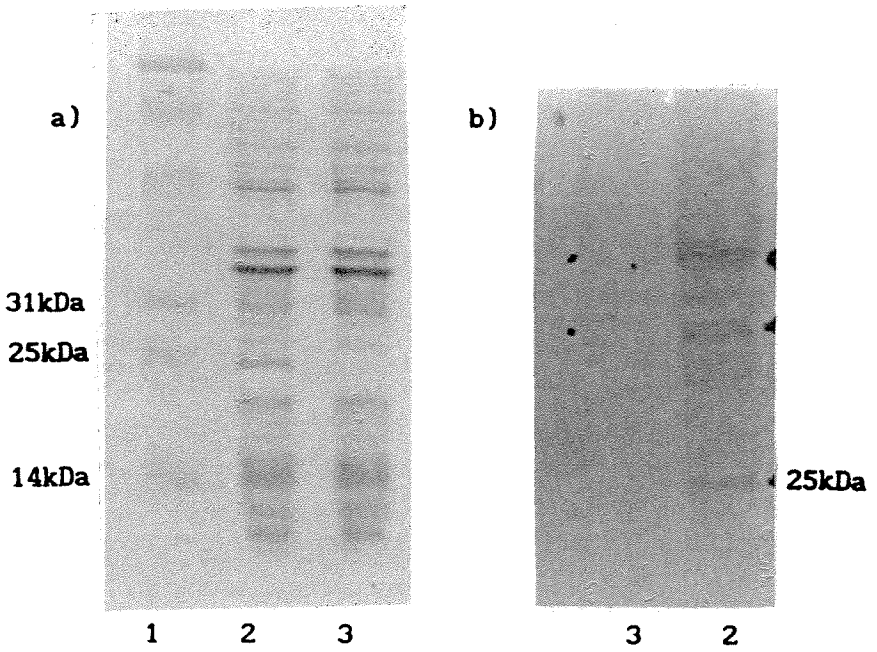


Figure 47. Coomassie stain, (a), and autoradiograph, (b), of SDS-PAGE of maxicells.

lane 1 = molecular weight standard

lane 2 = CSR603(pETE1)

lane 3 = CSR603(pBR322)

The predicted N-terminal sequence of the mature plprot1 protein (that is, after cleavage of the highest-scoring predicted signal peptide) from the nucleotide sequence is show below, aligned with the determined sequence.

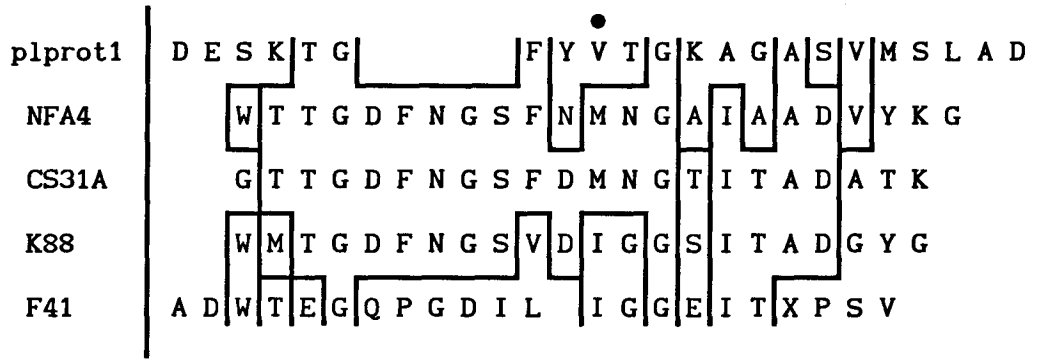
PREDICT: Asp-Glu-Ser-Lys-Thr-Gly-Phe-Tyr-Val-Thr-Gly-Lys-Ala

DETERM: Asp-Glu-Ala-Gly-Thr-Phe-Phe-Tyr-Arg-Thr-Gly-Gly-Ala

The predicted sequence shows 62 % sequence identity with the determined sequence which indicates strongly that plprot1 is present as a 25,000 Da protein in the crude membrane preparation and is the causative agent of agglutination. The sequence information derived from the N-terminal analysis is not identical to the sequence predicted from the determined nucleotide sequence however, and there are several possible reasons for this. Firstly, there could be errors in the nucleotide sequence data. The nucleotide sequence information in this region is very clear and was determined independently for both strands (see Appendix 4), which eliminates the possibility of codon reading errors. Secondly, the inconsistencies in the sequences could arise from a contaminating protein present in the sample sent for analysis. This appears to be a likely explanation as SDS-PAGE of JM101 control crude membrane preparations (p.115) shows the presence of a faint band with approximately the same molecular weight. The analysts report stated that the sequence data contained contaminating signals. Lastly, the pronounced 25 kDa band present in SDS-PAGE of crude membrane preparations of JM101(pETE1) and JM101(pPL7) could be a different protein, although evidence that this band is not pronounced in membrane preparations of JM101(pPL7N) (which contains a deletion in the plprot1

ORF) makes this an unlikely possibility.

Using the PCgene program ALIGN (which utilizes the method of Myers and Miller (143)), possible homology with other adhesin N-terminal sequences was investigated. The results are shown in figure 48.



By inserting one space into the plprot1 sequence, six identical residues can be aligned with the NFA4 N-terminal sequence (27.3 %), with one residue showing a functional similarity.

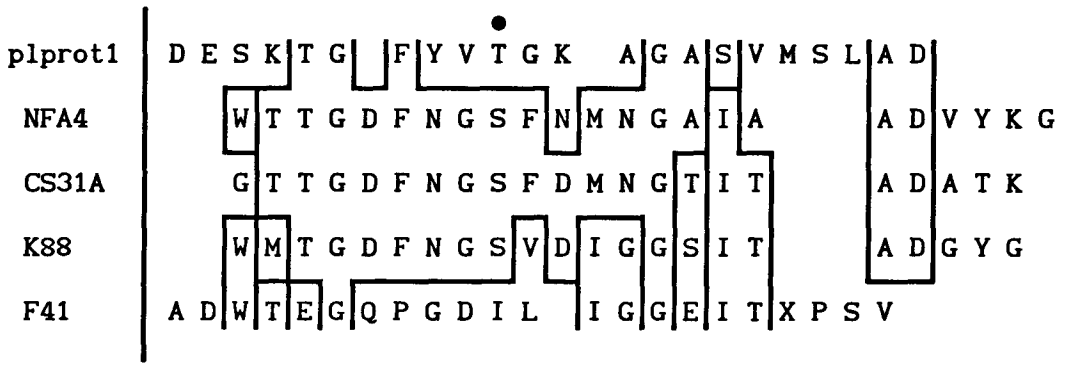


Figure 48. N-terminal sequence homology of plprot1

Inserting two gaps into plprot1 and one into NFA4, seven residues can be aligned with NFA4 (32 %) with one showing functional similarity. This data suggests that plprot1 could be distantly related to the non-fimbrial adhesin 4 family of adhesins, although the degree of similarity is not high.

4.4. Discussion

This section determines the pETE1-coded agglutinin to be very strongly associated with the bacterial membrane. N-terminal amino acid sequencing of a distinct 25 kDa band present in SDS-PAGE of crude membrane preparations of JM101(pETE1) showed that this band probably contained the protein product plprot1, coded on ORF1 of the pETE1 plasmid. This protein was shown by deletion subcloning (Section 3.2.4.) to be responsible for the hemagglutination effect. The agglutinin is very different from the F41 adhesin and most other adhesins in that heating to 60 ^{0}C does not inactivate the protein. For this reason the agglutinin was named Heat Resistant Agglutinin 1 (HRA1). Heat Resistant Agglutinin 1 showed a small degree of N-terminal sequence homology with the NFA4 family of adhesins.

The species hemagglutination profile showed that HRA1-mediated agglutination is somewhat species specific, but an attempt to identify the erythrocyte receptor gave equivocal results.

Subclone pPL4 was very much more resistant to cellular disruption by sonication than the other bacteria, requiring sonication times three-fold higher than other clones to achieve the same degree of disruption, measured visually as a clearing of the suspension. This stabilization effect is likely due to the gene products of the 2.3 kb *Hinc*II fragment that this subclone contains. These products (detailed in Section 3.3.3.) include the plprot5 protein which is highly homologous to the major *E.coli* lipoprotein, a protein that is known to stabilize the outer membrane (137). The strain containing this plasmid

may express more of the plprot5 protein than pETE1, since the fragment is in a high copy number pTZ vector. The expression of a membrane-stabilizing protein could also explain the greater resistance to lysis under severe physical conditions (temperature/vortex) that JM101(pETE1) exhibited compared to JM101.

5. Physicochemical Characterization of the Agglutinin

5.1. Background

Two non-traditional physical chemical techniques, namely aqueous two-phase partitioning and low-shear viscometry, were employed for study of the recombinant strains expressing the agglutinin. Both systems are very sensitive to cell surface differences between closely related bacteria, and can give qualitative and quantitative information on the degree of difference. Cell electrophoresis was used to calculate the electrophoretic mobility of the clone, and phase-contrast microscopy was employed to visualize the adherence event. Electron microscopy was used to search for surface appendages on both F41 and the recombinant organism.

5.2. Materials and Methods

5.2.1. Aqueous Two-Phase Partitioning

The principles of partitioning cells in aqueous two-phase polymer systems are explained in Section 1.6. In this work, a variety of polymer systems with varying properties were utilized to assess cell surface differences between the bacterial strains.

5.2.1.1. Preparation of the Phase Systems

Phase systems were made up by mixing appropriate weights of the following stock solutions.

(a). dextran T500 (M_w= 461,700, Pharmacia, Uppsala, Sweden, lot no. FD 160-27), 20 g in 100 g PBS. Dextran stock solution concentrations were determined polarimetrically (108).

(b). dextran T40 (M_w= 40,000 Pharmacia, lot no. 40601), 30 g in 100 g PBS.

(c). poly(ethylene glycol) (PEG) 8000 ($M_w \cong$ 8,000, Union Carbide, New York, NY, lot no. B-688-0232-2), 30 g in 100 g PBS. PEG stock solution concentrations were measured by refractive index measurements (108).

(d). ficoll 400 (M_w= 400,000 (by light scattering), Pharmacia, lot no. IL-33505), 40 % w/w.

The phase systems used in this work were then prepared.

(a). [(5,4)5] : 5 % w/w dextran T500; 4 % w/w PEG 8000; 150 mM NaCl, 6.84 mM Na_2HPO_4, 3.16 mM NaH_2PO_4, pH 7.20

(b). [(5,4)I] : 5% w/w dextran T500; 4 % w/w PEG 8000; 75.2 mM Na_2HPO_4, 34.8 mM NaH_2PO_4, pH 7.20

(c). [(7.4,4.7)55] : 7.4 % w/w dextran T40; 4.7 % w/w PEG 8000; 37.6 mM Na_2HPO_4, 17.4 mM NaH_2PO_4, pH 7.20

(d). [(10,7.5)5] : 10 % w/w dextran T500; 7.5 % w/w PEG 8000; 150 mM NaCl, 6.84 mM Na_2HPO_4, 3.16 mM NaH_2PO_4, pH 7.20

(e). [(6,10)5] : 6 % w/w dextran T500; 10 % Ficoll 400; 150 mM NaCl, 6.84 mM Na_2HPO_4, 3.16 mM NaH_2PO_4, pH 7.20

(f). [(7.4,4.7)5] : 7.4 % w/w dextran T40; 4.7 % w/w PEG 8000; 150 mM NaCl, 6.84 mM Na_2HPO_4, 3.16 mM NaH_2PO_4, pH 7.20

(g). [(5,3.5)I] : 5 % w/w dextran T500; 3.5 % w/w PEG 8000; 75.2 mM Na_2HPO_4, 34.8 mM NaH_2PO_4, pH 7.20

(h). [(6,10)I] : 6% w/w dextran T500; 10 % w/w Ficoll 400; 75.2 mM Na_2HPO_4, 34.8 mM NaH_2PO_4, pH 7.20

(i). [(7.5,10)55] : 7.5 % w/w dextran T500; 10 % w/w Ficoll 400; 37.5 mM Na_2HPO_4, 17.4 mM NaH_2PO_4, pH 7.20

(j). [(7.5,10)5] : 7.5 % w/w dextran T500; 10 % w/w Ficoll 400; 150 mM NaCl, 6.84 mM Na_2HPO_4, 3.16 mM NaH_2PO_4, pH 7.20

5.2.1.2. Radiolabelling the Bacteria

The bacterial strains used were *E.coli* F41, JM101, B/R, JM101(pBR322),JM101(pETE1), JM101(pPL2), JM101(pPL3), JM101(pPL4), JM101(pPL7), B/R(pETE1) and B/R(pPL7). Another control subclone was constructed by excising a 17 base pair DNA fragment from pBR322, recircularization of the plasmid and electroporation (see Table 2 Section 3.2.5) back into JM101. This was designed to inactivate the tetracycline resistance gene to create a plasmid that differed from pETE1 only in the foreign DNA content. The clone was selected by its ability to grow on LB amp and inability to grow on LB tetracycline plates.

The bacteria were all radiolabelled in the same way. Carbon-14 labelled amino acid mix (50 µl, 10 µCi/ml, Amersham) was spread on the central portion of minca or LB plates containing the appropriate antibiotics and inducers. The radiolabel was allowed to soak into the plates for 5 min, then 25 µl of a -70 °C DMSO stock bacteria suspension

was spread in the same area. The bacteria were then grown at 37 °C overnight and were harvested by swabbing into PBS and washed thrice. Bacteria concentration was estimated by centrifugation in microhematocrit tubes (see Section 4.2.2.1).

5.2.1.3. The Partitioning Experiment

The phase systems were made up in 50 ml polyallomer tubes and allowed to equilibrate for 1 h at room temperature. The top phase was carefully drawn off into a separate tube leaving approximately 1 ml near the interface. The lower phase was drawn off by puncturing the tube with a needle and collecting. Lower phase (1 ml) was aliquotted into a 5 ml polycarbonate test tube and 1.5 ml of upper phase (the load mix) placed in a separate 5 ml tube. Bacteria (200 µl, $2x10^9$ cells/ml) or erythrocytes (2 % hematocrit, 3x washed, in PBS) were added to the load mix, mixed and 1 ml of the suspension was then added to the lower phase. The experiment was always done in triplicate. The tubes were capped and inverted rapidly 20 times, the caps quickly removed to avoid dripping and the systems were left to equilibrate for 20 min at room temperature. After this time, 200 µl of each phase was sampled from the upper, lower and load mixes. For ^{14}C-labelled bacteria, the sample was transferred into a scintillation vial, to which was added 7 ml scintillation fluid (Atomlight, Du Pont). The vials were vortexed and counted in a Phillips FW3400 scintillation counter. For erythrocytes, the samples were added to 12 ml diluent (Hematall azide-free isotonic, Fisher) and the cells were counted by impedance (coulter) counting with an Electrozone cell counter. The partition ratio was expressed as the quantity of cells present in the upper or lower phase as a percentage of the total cells

added to the system.

5.2.1.4. Bacteria-Erythrocyte Binding Partition Experiments

When optimum phase systems had been found (i.e. those that showed the greatest difference between the bacteria and the red cells) a binding experiment was performed. Red blood cells (350 µl, 2 % hematocrit) were preincubated with 350 µl radiolabelled bacteria ($2x10^9$ cells/ml) for 30 min at 37 °C. The mixture (200 µl) was then added to the phase system load mix as before and the partition carried out. When the phase systems had reached equilibrium, two 500 µl aliquots were taken from each phase, one of which was counted for red cells in the cell counter, and the other of which was counted for bacteria in the scintillation counter. Controls were performed with erythrocytes alone and bacteria alone under the same conditions.

5.2.2. Viscometry of bacterial strains

The principles and rational of the viscometric assay for studying bacterial adhesion to erythrocytes are explained in Section 1.5. The adhesion of bacteria to human colon adenocarcinoma 201 (colo 201) cells was also examined by this technique.

5.2.2.1. Bacterial strains

The bacteria used were *Escherichia coli* of the following strains.

(a). JM101 and recombinant clones JM101(pETE1) and JM101(pPL7)

(b). F41

(c). K12W, R1316, B/R and K802, strains obtained from R. Graham that were scanned in the viscometer for agglutination in the absence of mannose to find a plasmid host with no detectable agglutination background.

(d). recombinant clones B/R(pETE1) and B/R(pPL7)

The bacteria were grown overnight on solid media, either minca or LB with the appropriate antibiotics and harvested into HBSS-hepes (Hanks balanced salt solution, 5 mM KCl, 0.3 mM KH_2PO_4, 138 mM NaCl, 4 mM $NaHCO_3$ and 0.3 mM Na_2HPO_4, with 5 mM hepes (4-(2-hydroxethyl)-1-piperazineethanesulphonic acid); pH 7.2, 278 mOs).

5.2.2.2. Growth of Human Colon Adenocarcinoma Cells.

Human colon adenocarcinoma cells, line 201 (145), were obtained from the ATCC. The cells were grown in Dulbecco's Modified Eagle medium (DME, Gibco, for composition see Appendix) with 20 % fetal bovine serum (Flow Laboratories at 37 ^{o}C in a 10 % CO_2 atmosphere. The cells were harvested by centrifugation and suspended in HBSS-hepes to a density of $4.4x10^6$ cells/ml.

5.2.2.3. Viscometric Hemagglutination Assay

The viscometry was performed in a Contraves LS-2 couette viscometer (Contraves, Zürich, Switzerland). Erythrocyte suspensions were prepared by centrifugation of whole blood to remove plasma, followed by washing of the red cells 3x in HBSS-hepes. The erythrocytes were used at a hematocrit of 47 %.

The bacteria studied were washed 3x in HBSS-hepes and suspended in

HBSS-hepes to approximately $10x10^{10}$ cells/ml (45% "bugcrit"). Red cells (800 µl) were put into the well of the viscometer and allowed to equilibrate at 37 °C for 20 min then sheared for one minute (for shear rates, see Results section) before addition of 100 µl bacteria. A control was carried out by adding instead 100 µl of buffer. The reaction was allowed to proceed for 5-20 min and the shear stress increase was plotted as a function of time with a Fisher Recordall 500 chart recorder.

5.2.3. Cell Electrophoresis

Cell electrophoresis was carried out to estimate the charge on *E.coli* F41, JM101 and JM101(pETE1). The electrophoretic mobility of human erythrocytes was also determined as a control. Cells were suspended in 0.15 M NaCl buffered to pH 7.3 with $NaHCO_3$ in the cylindrical chamber of a Rank Mark I (Cambridge, U.K.) cell electrophoresis apparatus. The cells were then viewed at 200x magnification with a horizontal microscope lens equipped with a water immersion objective focused at the stationary layer (146). A diagram of this apparatus is shown in Figure 49. A voltage (40V, electric field 4V/cm) was applied across the sample and movement of the cells against a fixed grid background of known dimensions was observed and timed with a hand timer to ± 0.5 s. Mobility measurements were made (at least 20 for each sample) and electrophoretic mobilities (velocity/electric field) determined for each cell using the formula-

$$\mu = \frac{1}{t} \cdot \frac{D.Le}{V}$$

where t = averaged time for one transit (across calibration grid)

D = size of grid division (in μm)

Le = electrical distance between electrodes (cm), determined as described (146).

V = voltage

μ = electrophoretic mobility (in $\mu m.sec^{-1}\ V^{-1}cm$)

5.2.4. Microscopy

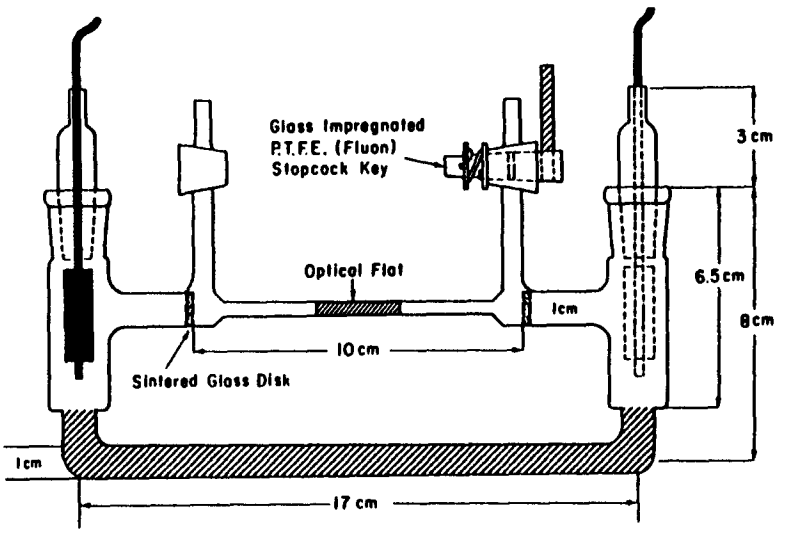
5.2.4.1. Electron Microscopy

Escherichia coli F41, JM101 and JM101(pETE1) were stained with uranyl acetate and viewed with a Phillips electron microscope at magnifications of up to 40,000x for evidence of piliation.

5.2.4.2. Optical Photomicroscopy

Phase contrast photomicroscopy was used to study the aggregation of human erythrocyte ghosts and cultured colo 201 cells with *E.coli* F41, JM101 and JM101(pETE1) in the presence and absence of mannose. The cells were spotted onto glass slides and viewed without coverslips using a Carl Zeiss 67547 photomicroscope with a 400x objective.

a)



b)

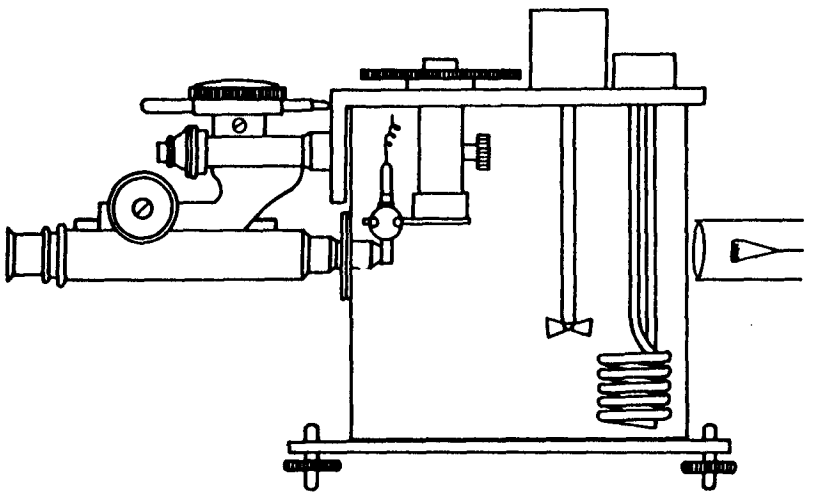


Figure 49. Diagrammatic representation of cell electrophoresis apparatus.

a) Front view - movement of cells is measured at the stationary layer in the region of the optical flat.

b) Side view showing microscope lens.

5.3. Results

5.3.1. Influence of pETE1 on Bacterial Partition

The results from the bacterial partition in two-phase polymer systems are shown graphically in Figures 50-56. As the uncertainty in the averaged partition of cells in these experiments (expressed as a percentage) was usually very low, of the order of 1-2 %, error bars have been omitted from the figures unless determined to be unusually high.

There are many notable features of these experiments -

(1). F41 grown on minca shows a very different partition in the [(10,7.5)]5 system than F41 grown on LB. This shows that this system is a very sensitive indicator of the levels of F41 antigen expression induced by the different media (Figure 50). The sensitivity of this system can be appreciated by reference to Western blot results for F41 antigen production on the different media in Section 2.3.2.3.)

(2). JM101(pETE1) partitions very differently from both JM101 and F41 in several PEG\dextran systems indicating that the cell surface of JM101(pETE1) is very different from that of JM101 (Figure 51). This difference could only have arisen from the introduction of the pETE1 plasmid.

(3). JM101(pBR322) and JM101 harbouring the constructed control plasmid pBR322-*Ban*II deletion partition in a very similar manner to JM101 in the phase systems (Figure 52). This provides good evidence

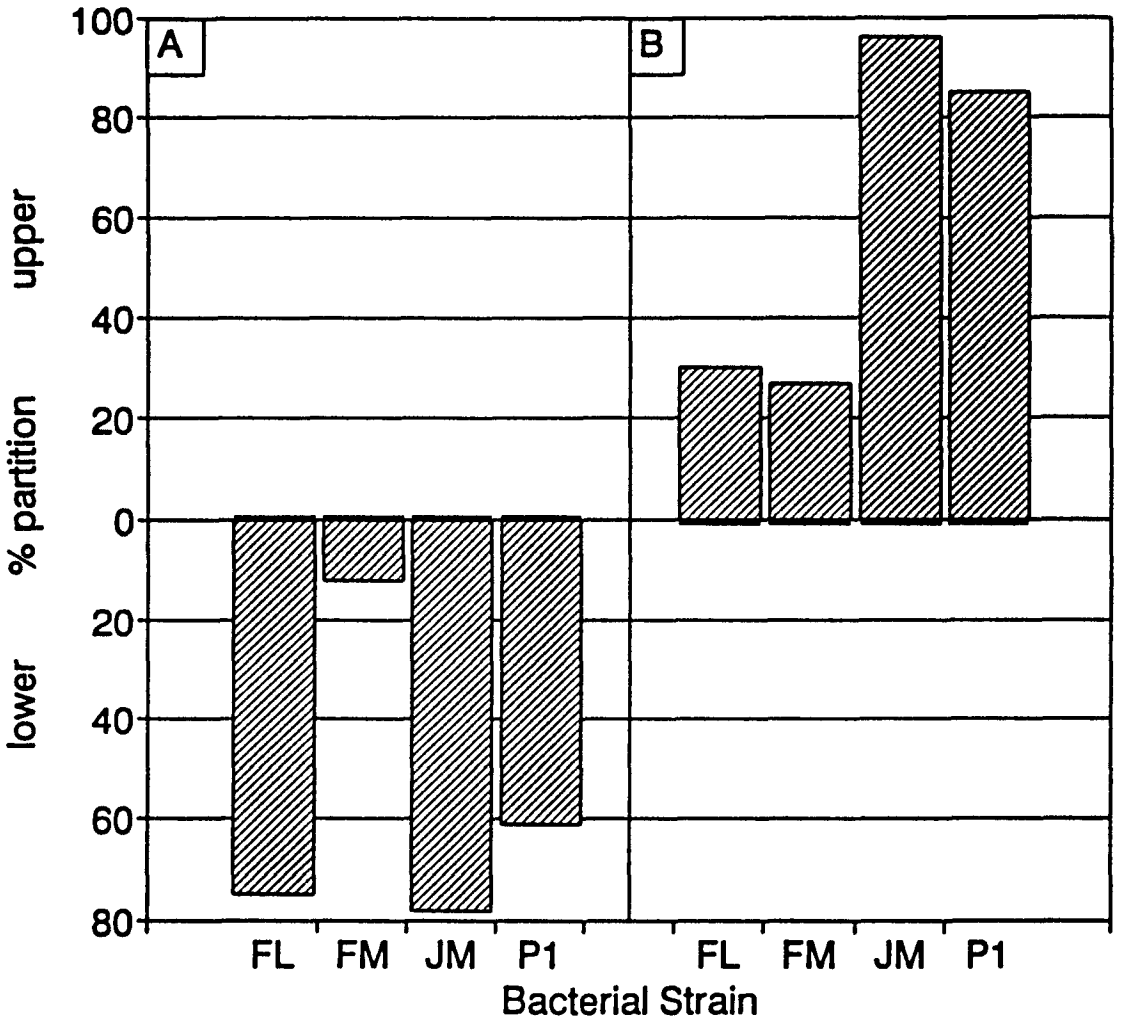


Figure 50. Bacterial partition in A) [(10,7.5)5] and B) [(6,10)5] phase systems.

FL = F41 grown on LB; FM = F41 grown on minca

JM = JM101; P1 = JM101(pETE1)

Cells are partitioned between the upper and lower phases and the interface.

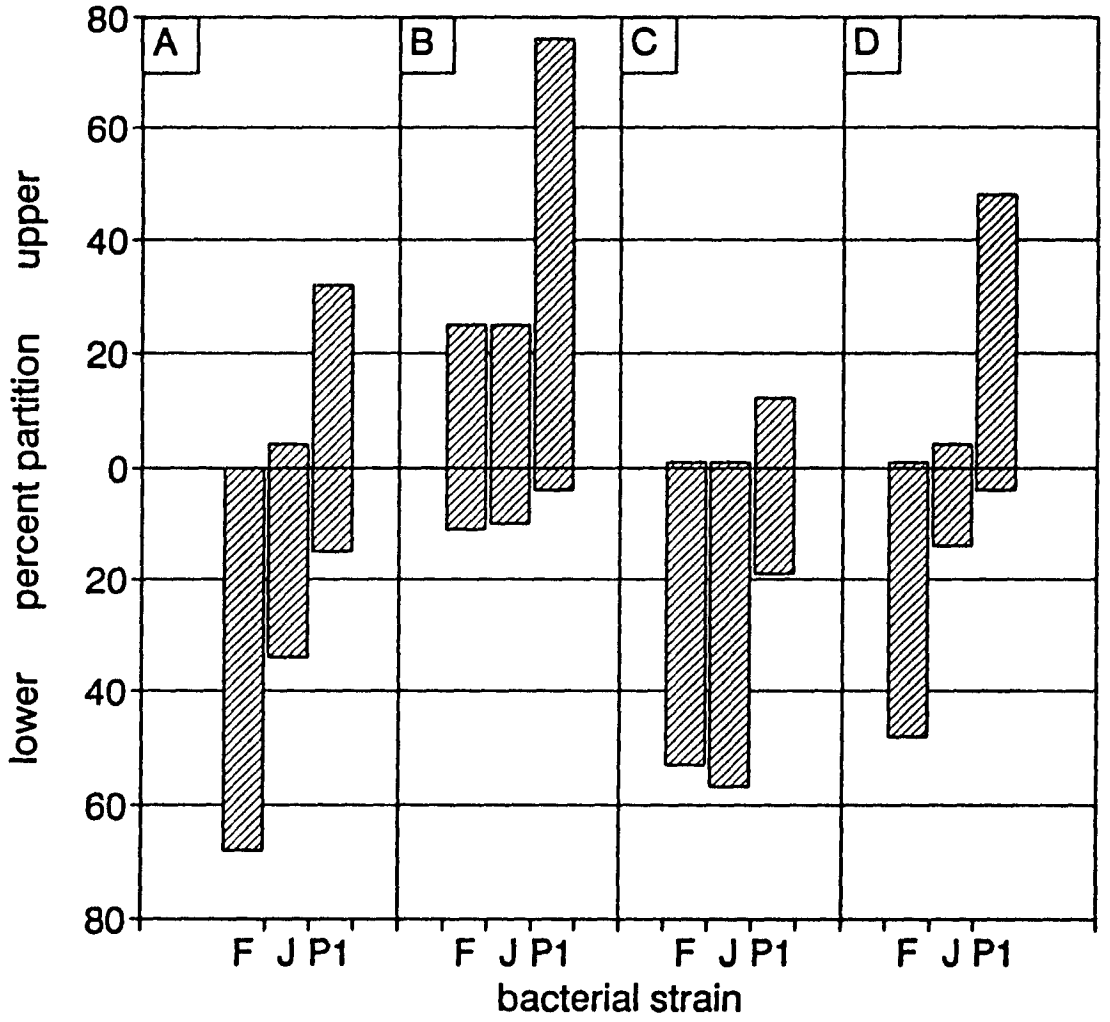


Figure 51. Partition of JM101(pETE1) in various PEG/dextran systems.

A = [(5,4)5]

B = [(5,4)I]

C = [(7.4,4.7)5]

D = [(7.4,4.7)55]

F = F41 (minca grown)

J = JM101

P1 = JM101(pETE1)

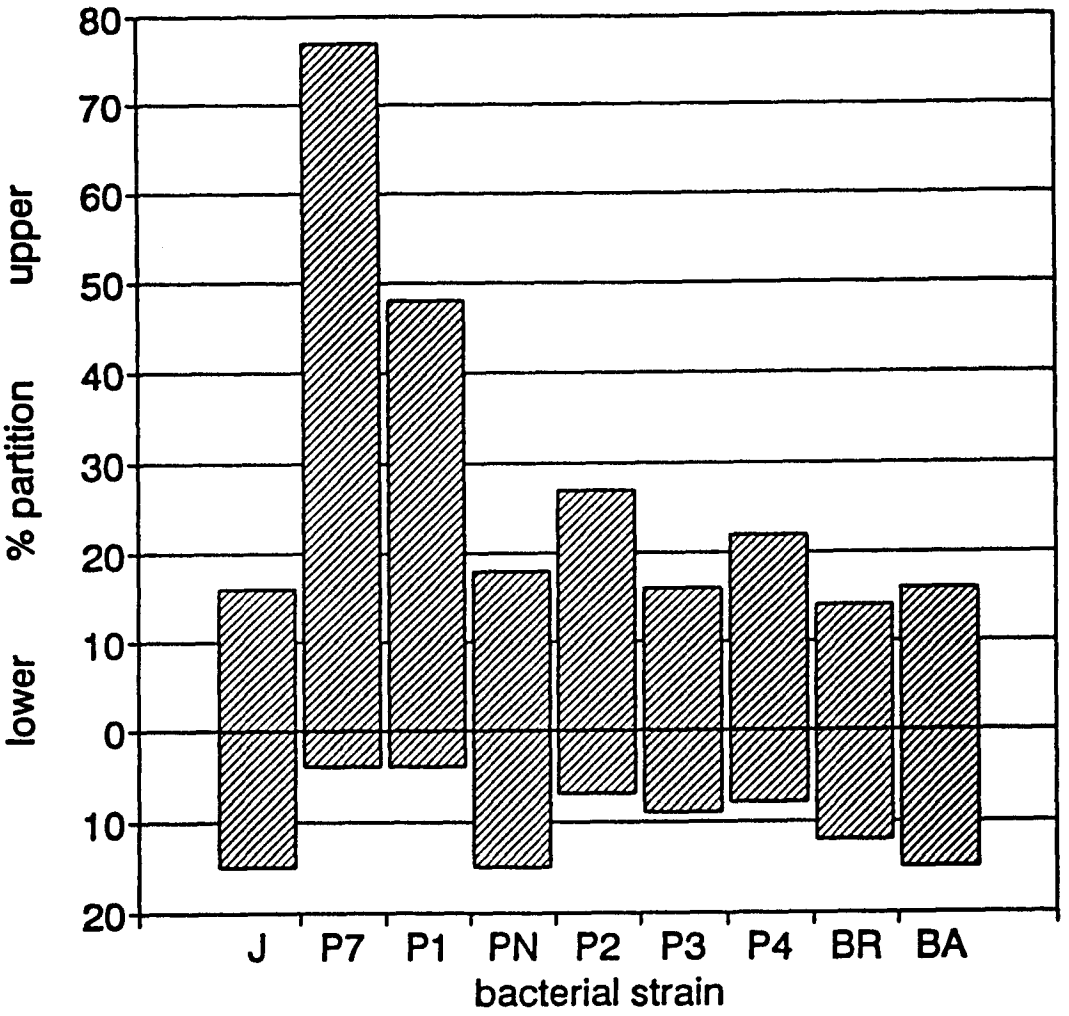


Figure 52. Partition of various pETE1 subclones in [(7.4,4.7)55]

J = JM101

P7 = JM101(pPL7)

P1 = JM101(pETE1)

PN = JM101(pPL7N)

P2 = JM101(pPL2)

P3 = JM101(pPL3)

P4 = JM101(pPL4)

BR = JM101(pBR322)

BA = JM101(pBR322-*BanII* deletion)

that the partition difference seen for JM101(pETE1) is due to the products of the F41 DNA insert in that plasmid.

(4). *E.coli* JM101 harbouring pPL7 behaves in a very similar manner to JM101(pETE1) in all systems studied (Figure 53). Often the effect of pPL7 is greater than that of pETE1. This indicates that the partition difference seen with pETE1 originates in the 1.3 + 1.0 kb *Hin*cII fragments, and may also show a higher level of expression caused by the vector pTZ19R, which has a higher copy number than pBR322.

(5). The subclones pPL2, pPL3 and pPL4 containing the single *Hin*cII fragments partition very similarly to JM101 in [(7.4,4.7)55] (Figure 52). This, together with previous points, is good evidence for the localization of the partition difference to the open reading frame spanning the *Hin*cII site between the 1.3 and 1.0 fragments. This data is consistent with hemagglutination data and implicates the agglutinating agent as the cause of the partition difference seen. In the subclone pPL7N, which contains an *Nco*II deletion in the open reading frame of plprot1, the partition difference is abolished (as is the hemagglutination). This further supports identification of the protein HRA1 as the agent of the partition difference.

(6). In dextran\Ficoll systems, the F41 bacteria partition is highly sensitive to phosphate concentration, partitioning strongly in the upper (dextran rich) phase at low phosphate concentrations (system [(7.5,10)5], Figure 54). When introduced to a system containing higher concentrations of phosphate, [(7.5,10)55], the bacteria partition strongly into the lower layer. The JM101 and JM101(pETE1) bacteria show preference for the lower layer in both systems.

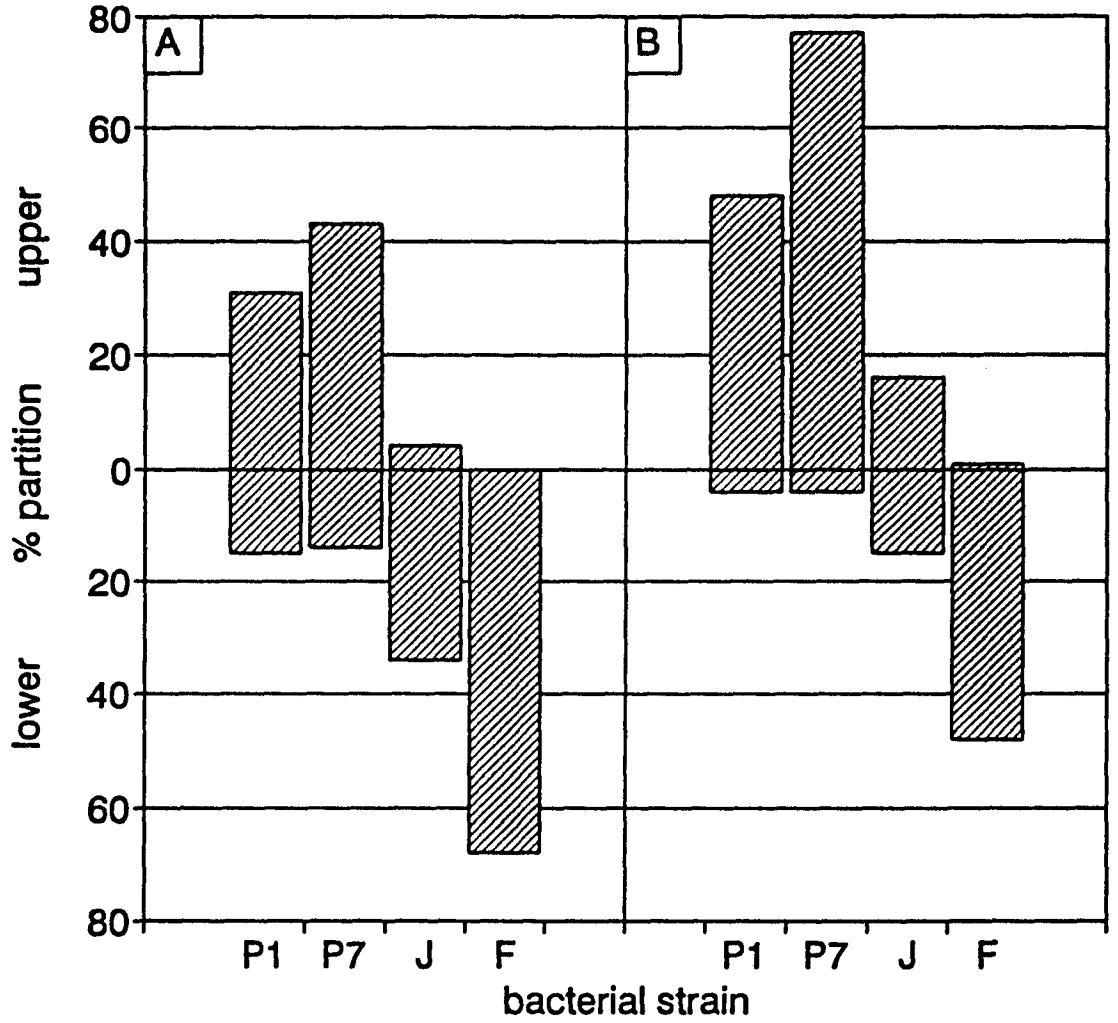


Figure 53. Partition of JM101(pPL7) in A) [(5,4)5] and B) [(7.4,4.7)55]

J = JM101

F = F41 (minca grown)

P1 = JM101(pETE1)

P7 = JM101(pPL7)

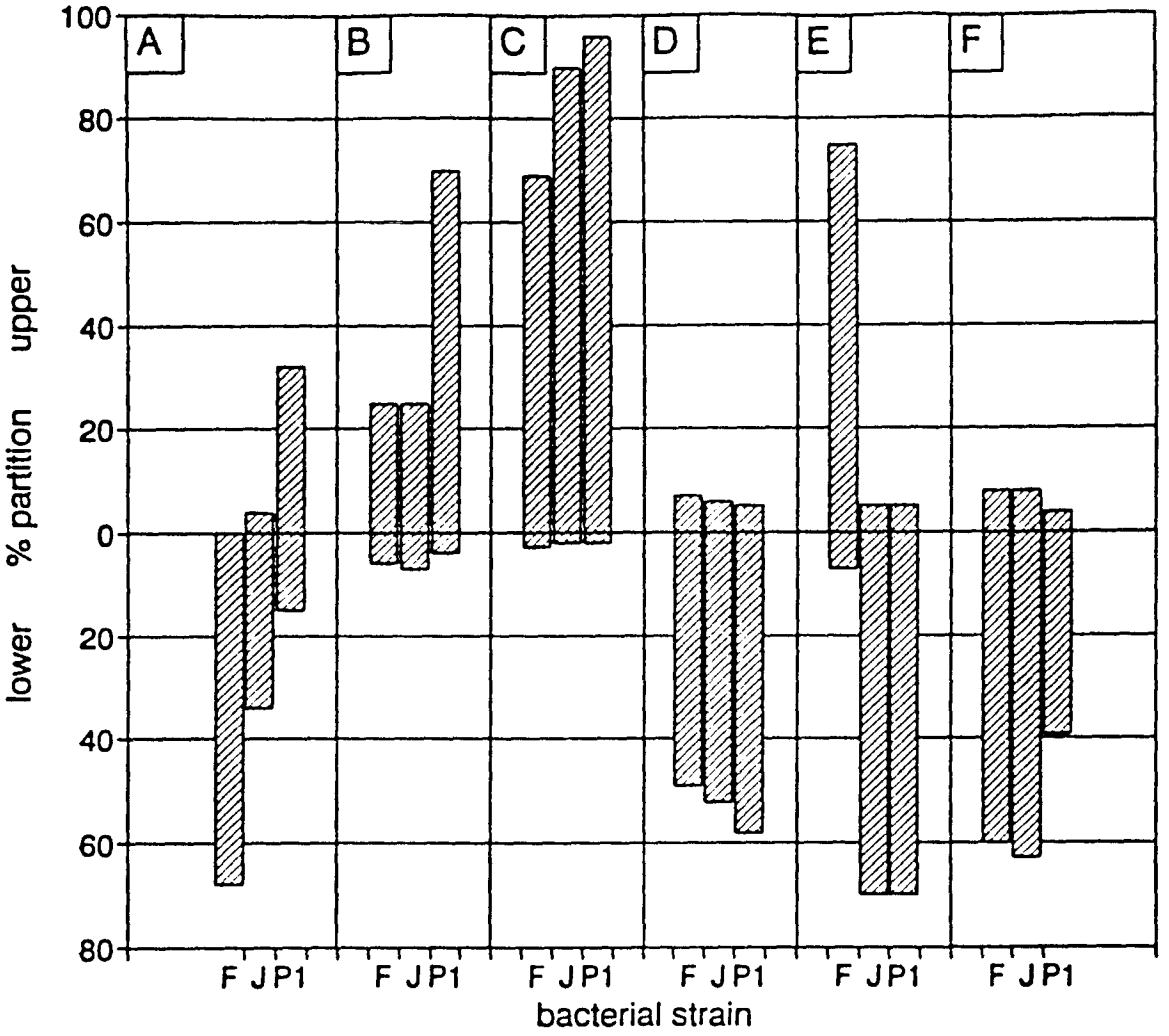


Figure 54. Bacterial partition in charge-sensitive systems and dextran/ficoll systems.

A = [(5,4)5] (non charge-sensitive)
B = [(5.4)I]
C = [(5,3.5)I]
D = [(6,10)I]
E = [(7.5,10)5]
F = [(7.5,10)55]

F = F41
J = JM101
P1 = JM101(pETE1)

(7). Partition in the charge sensitive systems used, [(5,4)I] and [(5,3.5)I] (Figure 54), indicates that although all bacterial strains are drawn up into the relatively positive upper PEG-rich phase (very strongly in the case of the [(5,3.5)I] system), the inherent negative charge on JM101(pETE1) is not disproportionately large or small compared to the JM101 control strain. The F41 strain shows the greatest change in partition when its partition in the charge sensitive system is compared to that in the non charge-sensitive analogue, [(5,4)5]. The F41 bacteria move from the lower phase through the interface to partition strongly in the upper. This movement suggests the presence of a large accessible negative charge on this strain. There is no suggestion from this data (nor from cell electrophoresis measurements, Sect. 5.3.3.) that the hemagglutination and partition effects seen in JM101(pETE1)are due to a non-specific highly positively charged protein on the cell surface. Rather, they likely arise from the overexpression of an active bacterial membrane adhesive protein.

(8). *E.coli* B/R, which was shown to have no agglutinating activity in the viscometer and hence is postulated not to express type 1 fimbriae, has a different partition from JM101 in several systems. The difference between B/R and B/R(pETE1) is even more pronounced than that seen for JM101 in [(5,4)5] and [(5,4)I] and is in the same direction (Figure 55).

(9). Bacteria-erythrocyte binding experiments in two-phase systems produced equivocal results, generally because the three strains under test (F41, JM101 and JM101(pETE1)) partitioned so differently that it was impossible to choose a test system in which all strains partitioned differently from the erythrocytes. This difference was necessary in

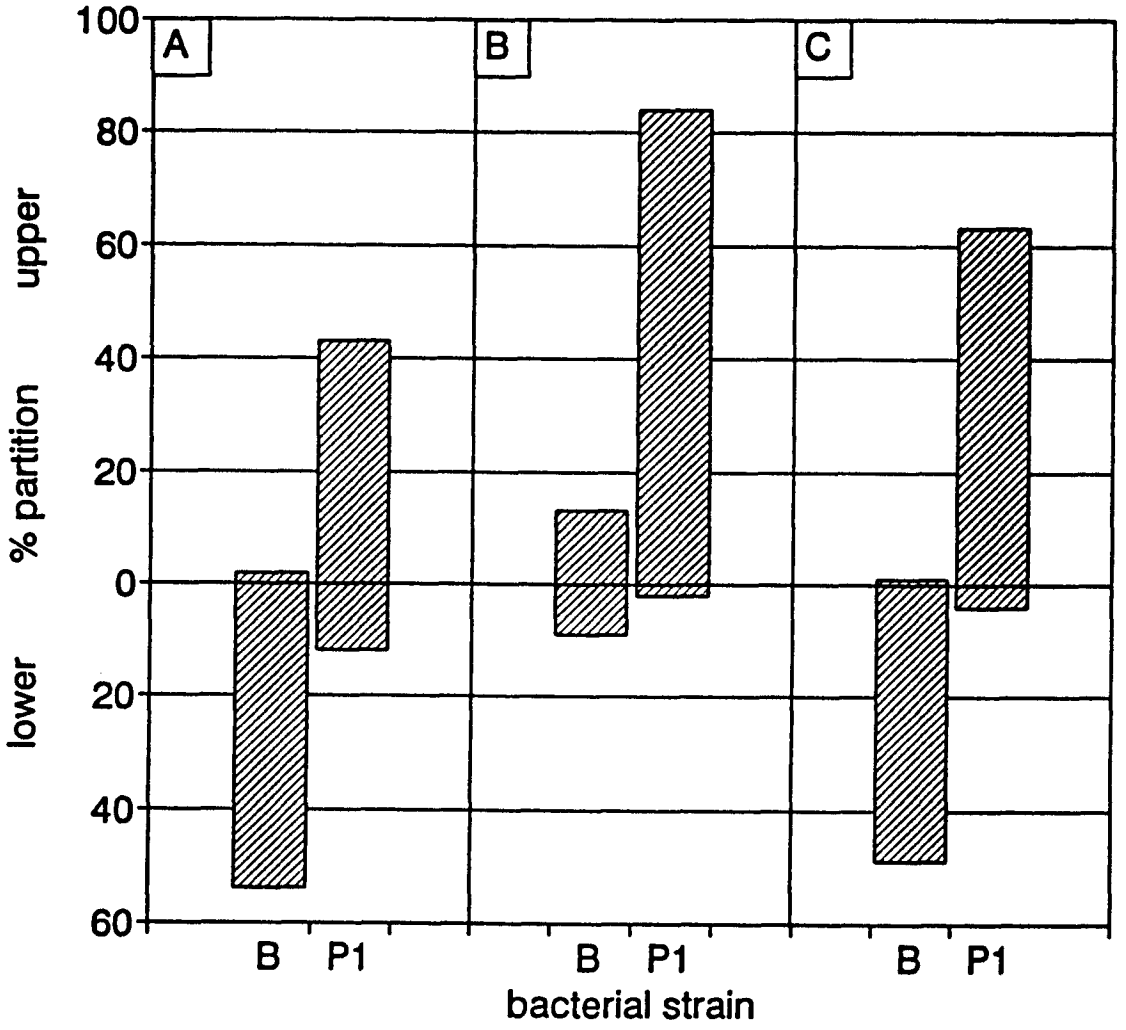


Figure 55. Partition of B/R (B) and B/R(pETE1) (P1)

in PEG/dextran systems

A = [(5,4)5]

B = [(5,4)I]

C = [(7.4,4.7)55]

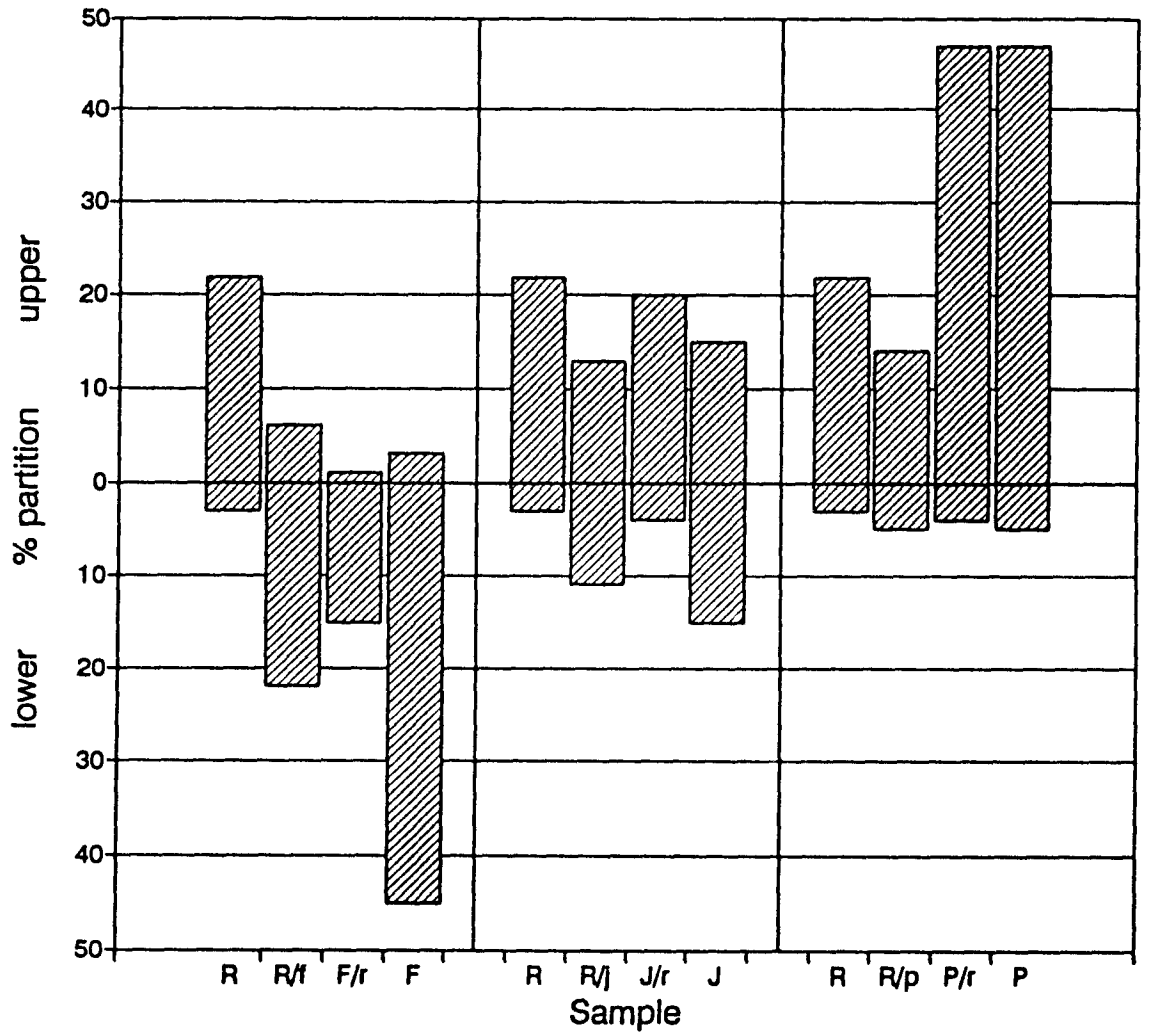


Figure 56. Bacteria-Erythrocyte Partition Binding Experiment

R = Erythrocyte; F or f = F41; J or j = JM101; P or p = JM101(pETE1)

R/x = Partition of erythrocyte/bacteria mix measured by counting erythrocytes (where x = f,j or p)

X/r = Partition of erythrocyte/bacteria mix measured by counting bacteria (where X = F,J or P)

System used = [(7.4,4.7)55]

order to observe a change in the bacteria or erythrocyte partition coefficient, which would imply that an interaction between cell types was occurring. In the systems chosen, [(5,4)5], [(5,3.5)I], [(7.4,4.7)5] and [(7.4,4.7)55], some effects can be seen, with the bacterial partition coefficient moving when partitioned with erythrocytes. All strains seemed to show effects, F41 being the strongest, often agglutinating the red cells too much to allow counting in the coulter counter. The results are difficult to compare between strains because of the differences in partition. The JM101 aggregation seen was inhibited when the partition was performed in 10mM mannose.

5.3.2. Viscometric Analysis of Hemagglutination Induced by *E.coli* Strains

Viscometric data from experiments studying the effect of shear on the red cell aggregation induced by JM101(pETE1) showed a surprisingly large rise in shear stress when the control JM101 was examined (Figure 57). This agglutination was inhibited by mannose. Although type 1 agglutinins are known to agglutinate human erythrocytes, the observation was surprising because no such aggregation was ever seen in microtitre assays, even with very large initial bacteria concentrations and at 37 ^{o}C. Viscometry of JM101 and JM101(pETE1) in the presence of 10 mM mannose at a shear rate of 43 s^{-1}, 37 ^{o}C (Fig. 57) shows that the bacteria harbouring the pETE1 plasmid produce a modest but readily measurable increase in the viscosity of the erythrocyte suspension over time compared to the cloning vehicle, JM101. This observation can be

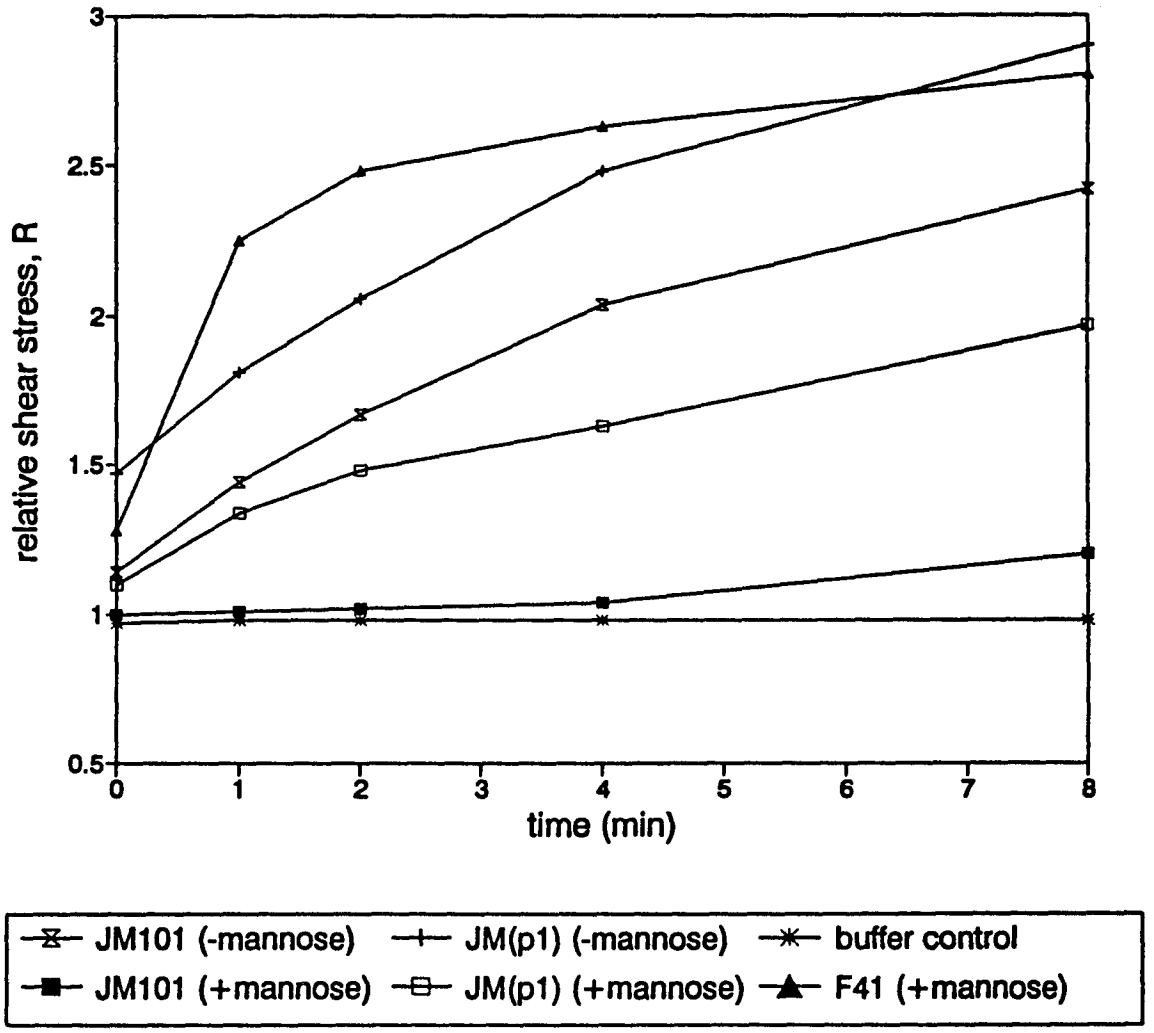


Figure 57. Viscometric analysis of hemagglutination mediated by *E.coli* strains in the absence and presence of 10 mM mannose

Shear rate = 43 s^{-1}, 47 % Hct RBC in HBSS, 37 °C.

rationalized, based on previous observations, as indicating that the bacteria-erythrocyte interaction caused by HRA1 is weaker than the aggregation caused by *E.coli* F41 or *Aeromonas salmonicida* 438, with the bonds between the cells being either lower in number, slower to form, or more easily broken. This shows that viscometry measures different properties of the aggregative event from the microtitre hemagglutination assay. This point is discussed further in the discussion at the end of this chapter (Section 5.3.7.).

The observation that the mannose-sensitive aggregation associated with type 1 pili induced by JM101 seen in the viscometer is entirely absent in microtitre assays could indicate a shear-enhancement (100) of the agglutination. To investigate this point further, five *E.coli* strains were examined for mannose-sensitive hemagglutination in the viscometer (Fig. 58) and *E.coli* B/R was chosen as a strain with no background agglutination. The plasmid pETE1 was introduced into this strain by electroporation and the same mannose-resistant hemagglutination effect was seen (Fig. 59). The adhesive effect of B/R(pETE1) was more obvious at low shear rates ($2.6s^{-1}$, Figure 59). The B/R(pETE1) bacteria was also shown to agglutinate colo 201 cells quite strongly at low shear rates (Figure 60). This experiment was not carried out at higher shear rates because the colo cells were not mechanically stable.

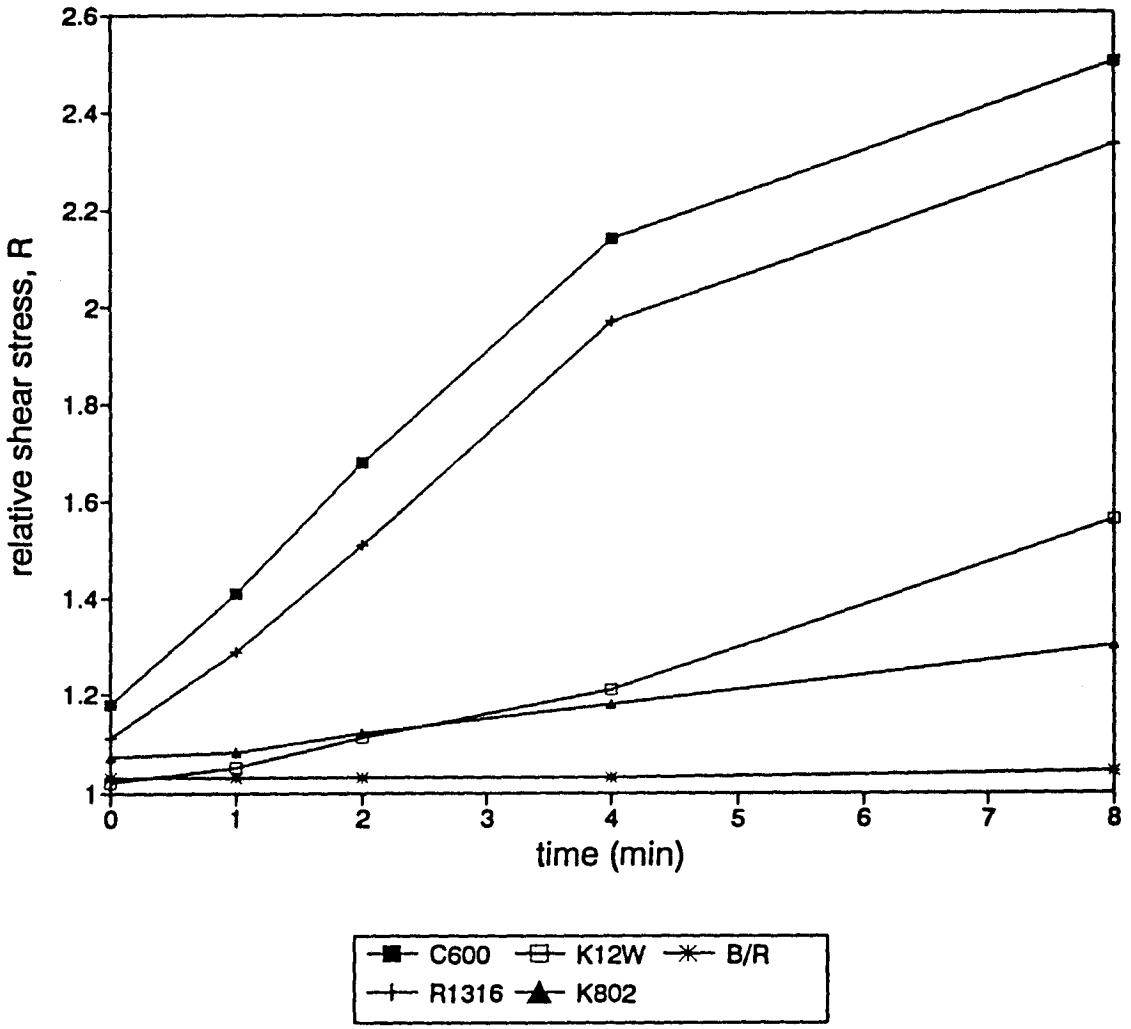


Figure 58. Hemagglutinating behavior of various *E.coli* strains

Shear rate = 43 s^{-1}, 47 % Hct RBC in HBSS, 37 °C.

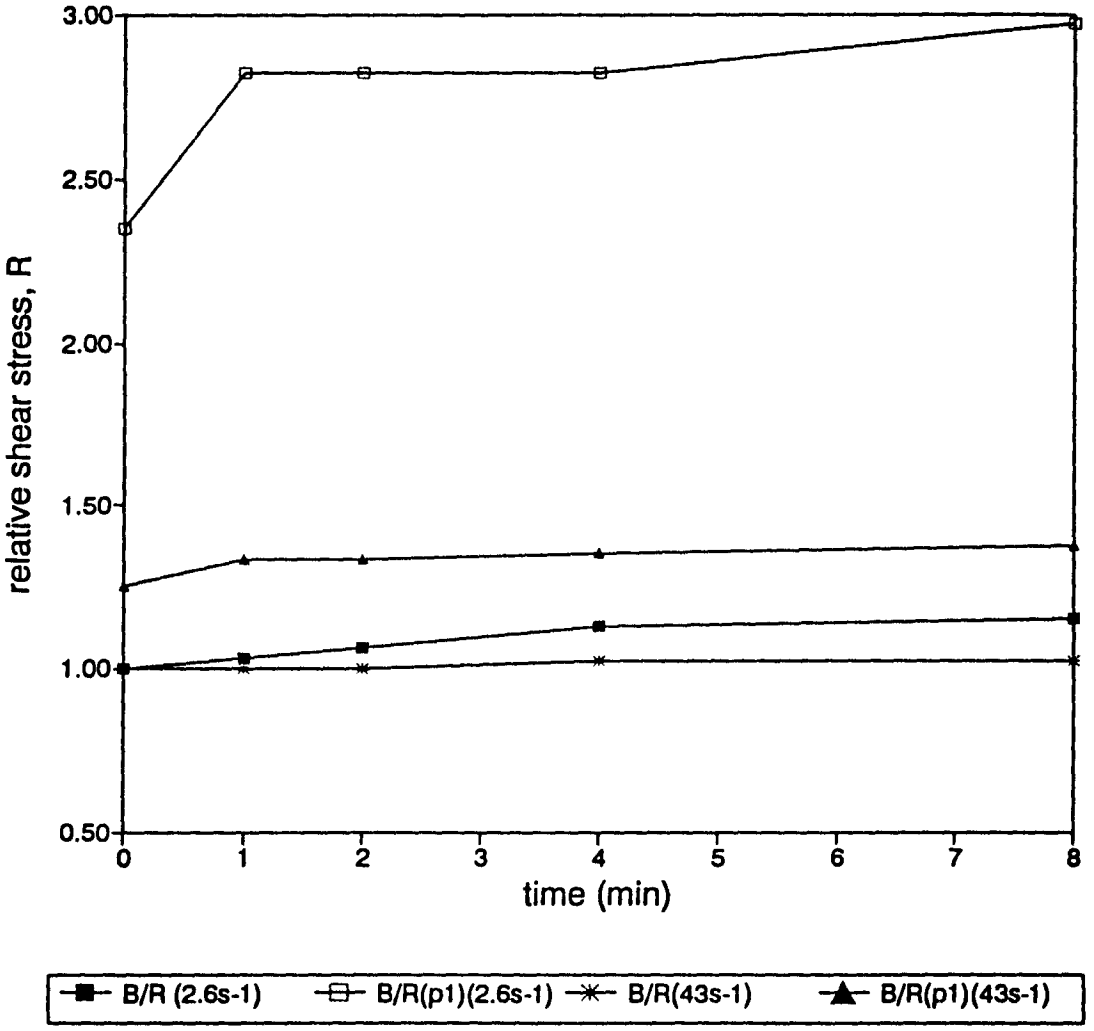


Figure 59. Increased hemagglutination of B/R(pETE1) at lower shear rates.

Shear rates as legend, 47 % RBC in HBSS, 37 °C.

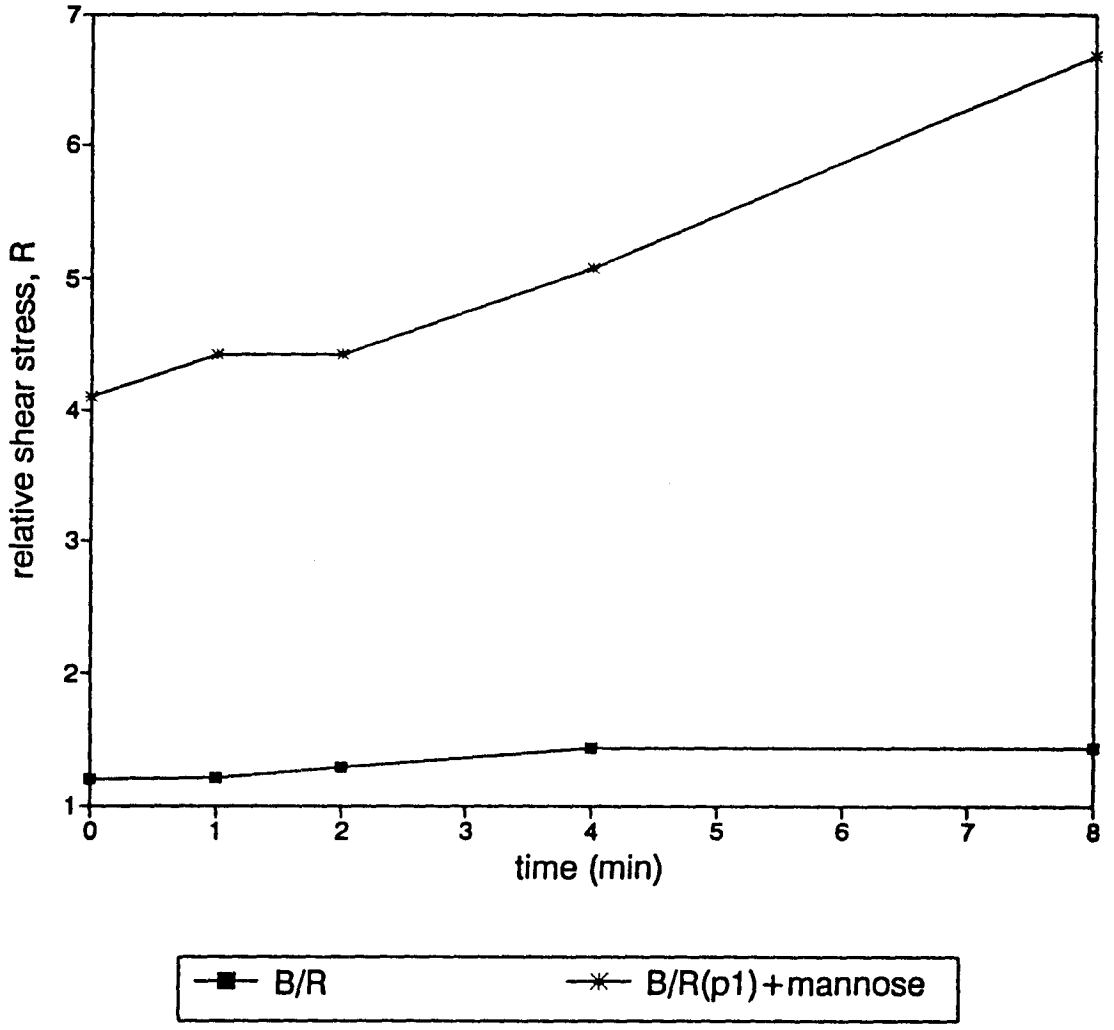


Figure 60. Aggregation of colo 201 cells mediated by B/R(pETE1) at a shear rate of 2.6 s^{-1}, 47 % Hct RBC in HBSS, 37 °C.

5.3.3. Bacterial Electrophoretic Mobility

The electrophoretic mobilities determined by cell electrophoresis in 150mM NaCl were are shown below.

Erythrocytes.	μ = -1.08 +/- 0.01 $\mu m.sec^{-1}.V^{-1}.cm$
E.coli F41.	μ = -0.78 +/- 0.05 $\mu m.sec^{-1}.V^{-1}.cm$
E.coli JM101	μ = -1.28 +/- 0.05$\mu m.sec^{-1}.V^{-1}.cm$
E.coli JM101(pETE1)	μ = -1.38 +/- 0.05 $\mu m.sec^{-1}.V^{-1}.cm$

The erythrocyte measurement produced the accepted value (146). The data shows that there is very little difference in electrophoretic mobility between JM101 and JM101(pETE1). This is further evidence that the agglutination and partition effects seen in JM101(pETE1) are not due to a non-specific charge effect since it would be expected that the adhesin would exhibit a net positive charge to cause adhesion to a negatively charged red cell surface. Exposure of such a protein would reduce the negative mobility, which was not observed. The mobility value derived for F41 is appreciably lower than those derived for JM101 and JM101(pETE1), perhaps indicating a lower surface charge, a finding that appears at odds with data from the partitioning experiments (Section 5.3.1). It should be noted that the above measurement is simply a measure of the rate of movement of the cells in an electric field however. To calculate the surface charge distribution other parameters related to cell surface structure have to be taken into account (146), parameters which were not available in this limited study.

5.3.4. Photomicrographs of the agglutination event

Photomicrographs show that JM101(pETE1) does show a binding affinity for erythrocytes but that the effect appears very weak compared to F41. Figure 61 shows red cell ghosts agglutinated by F41. The cells are heavily clumped and distorted. In contrast Figure 62 shows the agglutination mediated by JM101(pETE1) in the presence of mannose. The erythrocyte ghosts show loose clumping and no distortion. Figure 62 also shows a close-up of JM101(pETE1) cells binding to a single erythrocyte ghost. The cell JM101 is shown with ghosts in the absence and presence (Fig. 63) of mannose. In the absence of mannose the ghosts are strongly agglutinated, whereas with mannose present there appears to be little or no interaction between the bacteria and the cell membranes. Figure 64 details the aggregation of colo 201 cells by JM101(pETE1) in the presence of mannose.

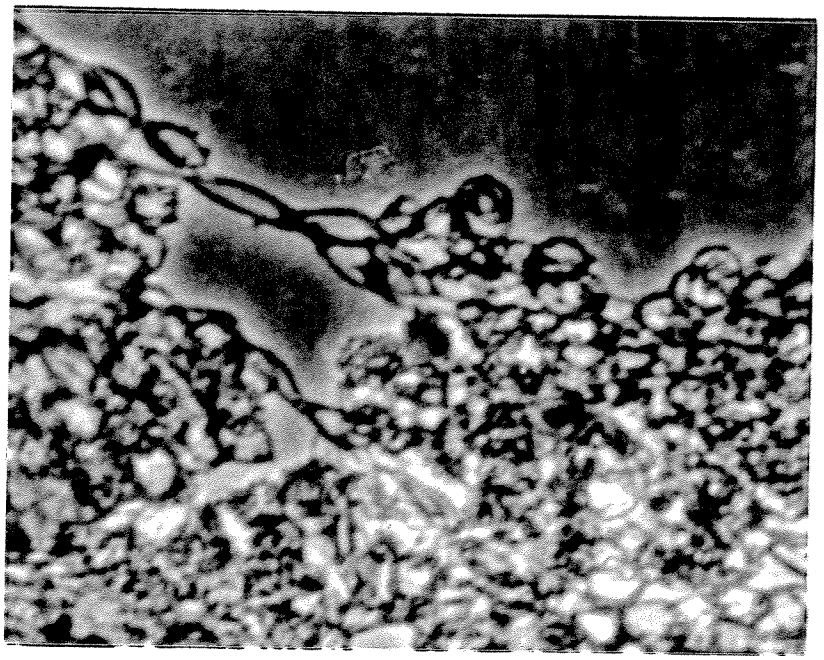


Figure 61. Photomicrograph of erythrocyte ghosts agglutinated and distorted by F41 bacteria.

(400x magnification)

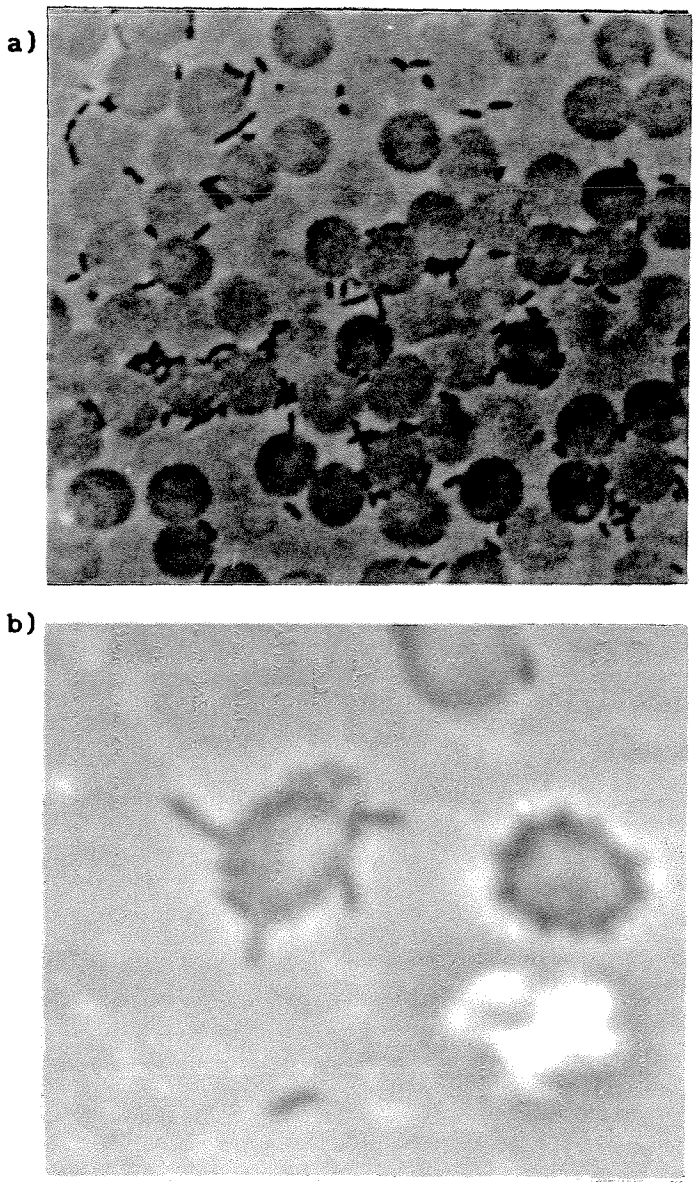


Figure 62. Photomicrographs of agglutination of human erythrocyte ghosts mediated by JM101(pETE1) (400x magnification, phase contrast, 10mM mannose).

a) + mannose, b) + mannose enlargement

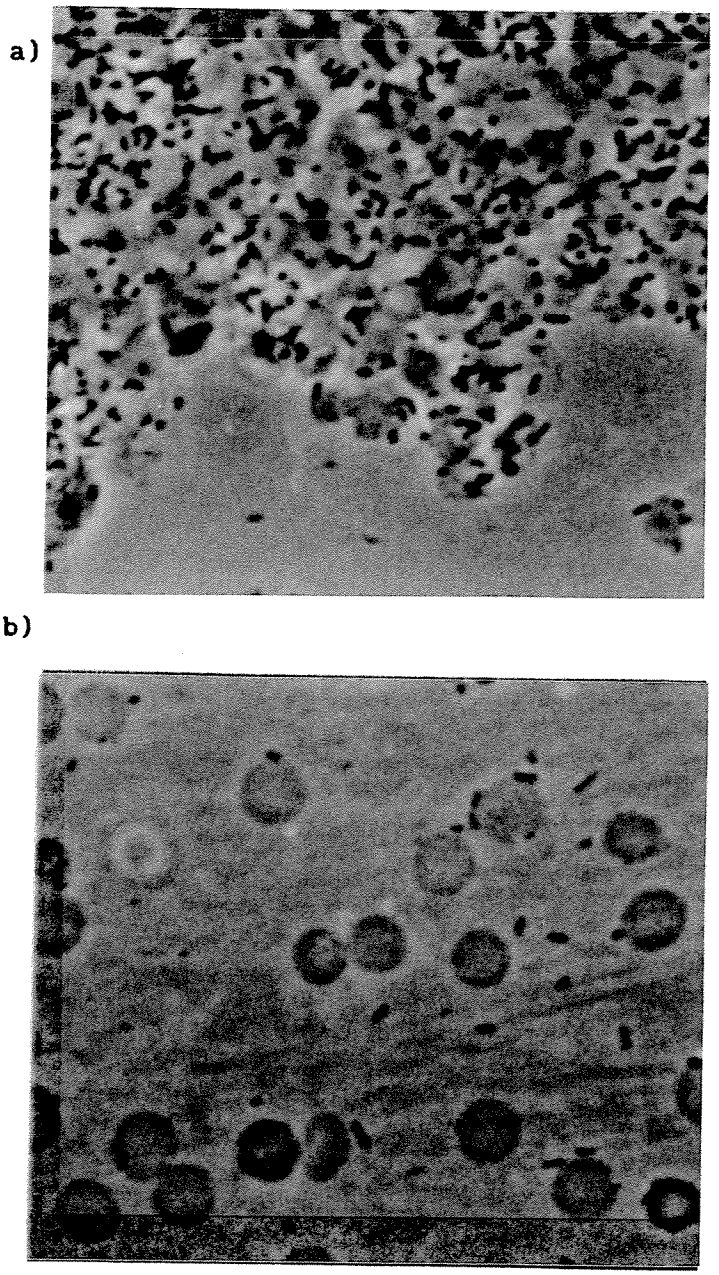


Figure 63. Photomicrographs of agglutination of human erythrocyte ghosts mediated by JM101. (400x magnification, phase contrast)

a) no mannose, b) 10 mM mannose

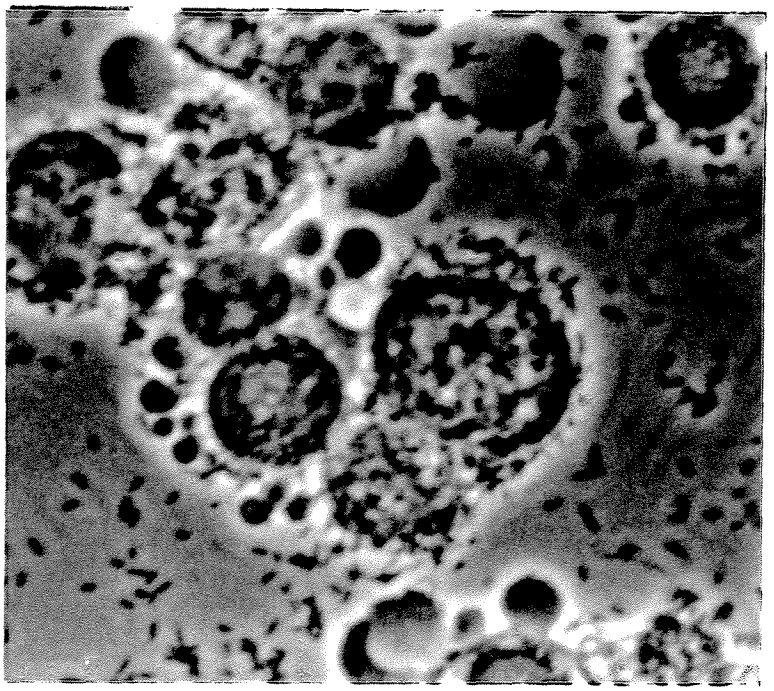


Figure 64. Photomicrograph of JM101(pETE1) agglutinating colo 201 in the presence of 10 mM mannose. (400x magnification, phase contrast)

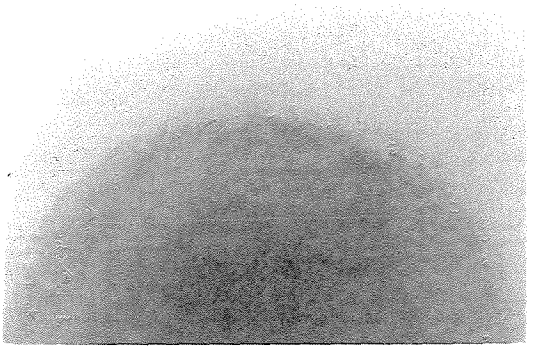
5.3.5. Electron microscope results

Electron micrographs of uranyl acetate stained F41, JM101 and JM101(pETE1) failed to show evidence of piliation (Figure 65). The results for F41 are consistent with previous findings in our laboratory (114), even though this strain is known to express the F41 and K99 agglutinins, both of which have been described as fimbrial. The JM101 strain also shows a smooth surface despite the type 1-like agglutination it shows in the viscometer.

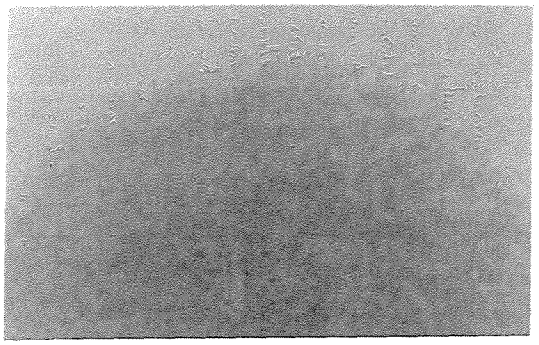
5.3.7. Discussion

Aqueous two-phase partitioning studies determined that the recombinant plasmid pETE1 induced pronounced cell surface changes in the host bacteria. Partitioning of subclones of this plasmid showed that these effects could be localized to the product of an open reading frame present on the foreign DNA insert in the plasmid, plprot1 (see Section 3.3.2.). This proteinaceous agent of the partition difference has also been identified as the hemagglutinating agent (Section 3.3.2.). The partitioning results show that this protein is expressed on the cell surface and is exposed to the surrounding environment, and that the difference it induces in the cell surface of JM101 is not solely due to a charge effect. This suggestion is strengthened by the electrophoretic mobility studies, and combined with earlier results regarding the specific agglutination by JM101(pETE1) of erythrocytes from different species (Section 4.3.2.1.), strongly indicates that the protein

a)



b)



c)

Figure 65. Electron micrographs of uranyl acetate stained bacterial cell surfaces.

a). F41 (30,000x) b). JM101 (30,000x) c). JM101(pETE1) (30,000x)

plprot1 (HRA1) is an *E.coli* surface lectin, with unknown receptor specificity.

Viscometry showed that the aggregation of erythrocytes mediated by JM101(pETE1) in the presence of mannose was weaker than that associated with F41 expression, producing small viscosity increments at moderate shear rates. At low shear rates B\R(pETE1) produced an appreciable agglutination of human colon adenocarcinoma cells, suggesting that the protein could have physiological relevance with regard to bacterial attachment to the intestinal epithelial cells. Photomicrograph results correlate with this hypothesis.

The bacterium JM101(pETE1) showed a very pronounced hemagglutination profile in the microtitre assay (Section 2.2.10.1), agglutinating erythrocytes down to bacterial concentrations of approximately one bacteria to two red cells, a very similar degree to the F41 strain. The fact that JM101(pETE1) shows a weaker aggregating effect in the viscometric assay reflects the different parameters measured by the two assays. The microtitre assay indicates the presence and accessibility of agglutinating agents on the bacterial cell surface. Since there is very little shear involved, even a weak interaction between the bacteria and the erythrocyte will be sufficient to cause agglutination providing that there is more than one adhesive molecule on each bacteria (and more than one receptor on each erythrocyte). This agglutination will be macroscopically indistinguishable from that of a bacterial strain with a much physically stronger adhesive system if the accessibility of the agglutinins are equivalent. By providing a variable destructive shear force, the viscometric assay can give a

semi-quantitative index of the physicochemical strength of the adhesive interaction.

Physical strength of adhesive interaction is not the only reason that the agglutination effect may appear weak in a viscometric assay compared to a microtitre assay. The kinetics of the binding interactions must be taken into account. In the microtitre assay the agglutinins have a relatively long time in which to recognize and adhere to their receptors on the erythrocyte as the only movement is a gentle downward settling of the red cells. By contrast, in the viscometric assay the shear rate determines that the adhesive interactions must occur quickly before the cells are separated by the fluid flow (the characteristic time constant is the inverse of the shear rate). The fast interaction necessary in the viscometric assay favours high molecular weight multivalent easily accessible adhesin systems such as the F41 agglutinin. The HRA1 protein may well be present as a monomer anchored in the bacterial outer membrane (see Chapter 6) and as such may have a much less accessible binding site.

The large mannose-sensitive agglutination of human erythrocytes under shear exhibited by JM101 was unexpected given the inactivity of the strain in a microtitre assay. Previous reports (12) have indicated that *E.coli* strains expressing type 1 fimbria do show an agglutination effect in microtitre assays. Other reports (17) indicate that the type 1 determinant can be activated by repeated culturing, with the effect being initially very weak. The difference between the extent of agglutination observed in the microtitre assay and that observed in the viscometric assay could arise from shear enhancement of the event (100). The JM101 strain showed no evidence of type 1 fimbriae expression when

examined by electron microscopy. Hultgren *et al* (147) have also described mannose-sensitive hemagglutination in the absence of piliation in *E.coli*. The other strains studied also showed no piliation and although the findings for the F41 strain are in agreement with previous results from our laboratory (114) it is possible that the experimental conditions precluded the observation of these structures.

Chapter 6. Concluding Discussion

This thesis documents the molecular cloning and nucleotide sequencing of a region of DNA isolated from *Escherichia coli* O9:H10:K99. The region contains five open reading frames, four of which code for putative proteins containing an N-terminal prokaryotic secretory signal peptide, indicating that these proteins are either exported into the intermembrane periplasm, inserted into the outer membrane or secreted. Most of these proteins show little homology with protein sequences available in the database. One, however, plprot5, shows 50 % identity along virtually its entire length with the amino acid sequence deduced from RNA of the *E.coli* B major outer membrane lipoprotein (134). These data suggest that the region of DNA cloned was involved in determination of the structure and function of the outer membrane. The high homology of plprot5 with lipoprotein is useful in mapping the cloned region on the bacterial chromosome. The plprot5 protein probably serves a related function to *E.coli* B lipoprotein, though its primary structure is sufficiently different to that protein to suggest that the molecule may give rise to differences in outer membrane properties.

Lipoprotein is an abundant protein in *E.coli*, and usually represents about 6 % of total cell protein (68). Approximately one third of the protein is covalently bound to the peptidoglycan on the inner side of the cell membrane by its C-terminal lysine residue, and the rest exists freely in region of the outer membrane. Mature lipoprotein bears a cysteine at the N-terminus which is is covalently linked to a diacyl glycerol molecule by a thioester bond.

Although a lipoprotein-like molecule would appear to be a candidate for modification of the surface of JM101 when the gene is introduced in the pETE1 plasmid, possibly leading to the partition difference and hemagglutination effect, subcloning has shown that this is not the case. Lipoprotein is not a receptor for any known bacteriophages (68), although antibodies prepared against purified lipoprotein have been shown to bind to intact bacterial cells that have defects in LPS structure (137). This data indicates that the majority of lipoprotein molecules are usually inaccessible to the surrounding environment, either on the periplasmic side of the outer membrane or in the membrane screened by the LPS. Aside from the major *E.coli* lipoprotein, there are many other diverse lipid-modified proteins in the vicinity of the outer membrane (157) and it seems likely that plprot5 is related to this family. The protein plprot5 does not have an C-terminal lysine for covalent attachment to peptidoglycan, but it does have an arginine residue close to the C-terminus that could fulfill the same function. The plprot5 protein does have a very similar signal sequence and an N-terminal cysteine residue which could be modified in the same manner as the previously described lipoprotein. The gene encoding plprot5 also has a double stop codon, as does lipoprotein, but the codons are in reverse order.

The location of a lipoprotein-like gene in this DNA fragment puts the other determined protein sequences into the context of membrane structure and function. The plprot2 gene product lies next to the lipoprotein determinant and the 18.5 kDa peptide not only has a signal sequence but also contains a cysteine at the N-terminus of the mature protein that has the correct consensus sequence for lipid modification. The protein is also predicted to have three transmembrane helices and is

classified as an integral membrane protein. It is therefore likely that this is a membrane-bound lipoprotein that plays a role in the construction or stability of the outer membrane. The 24.5 kDa plprot3 protein coded on the 2.3 kb *Hinc*II fragment also contains a signal sequence, and therefore probably has some function related to bacterial membrane maintenance.

The protein identified as the hemagglutinin and the source of the partition differences is the 25 kDa (mature) protein plprot1, named HRA1 (heat resistant agglutinin 1). Like the proteins described above, this protein contains a high scoring predicted signal sequence. The protein bears very little resemblance to other documented *E.coli* hemagglutinins, either in amino acid sequence or physical properties. The N-terminal region of the mature protein shows weak similarity to the NFA4 non fimbrial adhesin and some related agglutinins, which could indicate a relationship between the molecules, but other considerations make it seem likely that the HRA1 agglutinin is an entirely different system. These considerations include the fact that there is no requirement for an operon of genes for the correct expression of the hemagglutination effect (although it is conceivable that the expression of this protein in JM101 is mediated by the proteins involved in the biosynthesis and expression of the type 1 adhesin that this strain was found to possess).

There is also the observation that HRA1 is extremely difficult to remove from the outer membrane of the cell, even with detergent. Despite this experimental result, HRA1 was not predicted to have any membrane associated areas (See Section 3.3.3.). This phenomenon is well known in the field of *E.coli* outer membrane porins, where OmpF, OmpC and PhoE are known to be strongly associated with the outer membrane and extremely resistant to detergents and proteases, yet their sequences are

found to be predominantly polar, containing no long hydrophobic segments (158). This raises the possibility of HRA behaving in an analogous manner to these proteins, existing as a monomer or small oligomer in the membrane and interacting in a similarly strong manner with the membrane components and LPS (149). Porins generally contain large amounts of β-sheet secondary conformation (160,161,162). The secondary structure prediction of Garnier generated for HRA1 (Section 3.3.3.) showed a predominantly β-sheet form, but it should be remembered that this prediction method is best applied to globular proteins and reports have shown that even for these proteins the algorithms used are only accurate to approximately 50 % (163,164).

The experimental result that heating the bacteria to 60 °C or even briefly to 100 °C does not destroy the agglutination together with the strong membrane association and the lack of necessary expression systems also show that the molecule is different from known adhesins and that it probably does not exist in a fimbrial structure. Fimbriae were not apparent on JM101(pETE1) when the bacteria were viewed with an electron microscope, nor were any seen on the source strain, F41. Although some researchers have described the F41 adhesin as fimbrial (65,70), in our laboratory no evidence has ever been collated to indicate that this is the case, although the adhesin does exist *in vivo* as a high molecular weight protein polymer.

The HRA1 protein is not positively charged (predicted pI = 4.44) and so would appear to mediate the agglutination of erythrocytes by either some specific interaction with a receptor on the cell membrane, or by in some way inducing a change in the lipopolysaccharide extending outwards from the membrane surface. With regard to the former hypothesis, the highest predicted antigenic determinant of HRA1 showed

homology with the known receptor binding site of the K99 adhesin, showing that HRA1 could have the potential to interact with erythrocytes in the same manner as K99. The second-highest scoring segment (with a hydrophilicity index very similar to that of the first) appears very close to the C-terminus of the protein and is shown below.

-Asp-Lys-Tyr-Lys-Ser-Glu-
243 248

This section could, if the molecule were exposed at its C-terminal end, also be a potential erythrocyte binding region. A recent report (165) has shown that the Hopp and Woods method for predicting antigenic determinants (the method used for HRA1) often correlates with important protein-protein and protein-DNA binding domains. With regard to the latter hypothesis, free lipopolysaccharide has been shown to bind to eukaryotic cells, probably through the central hydrophobic lipid A component (68). Hemagglutination of cells by LPS has not been reported however, and if it occurred it is not likely to be specific, although it is conceivable that the HRA1 protein may disrupt the LPS in some manner to increase lipid A exposure on the cell surface. There seems no reason to believe that HRA1 is involved in LPS biosynthesis.

Of the two hypotheses above, the more likely explanation seems to be that the protein itself is the agglutinating agent. It was cloned from a strain that expresses two strong mannose-resistant hemagglutinins and would not necessarily be noticed, especially as it would probably be present in lower quantities when expressed from the chromosome rather than a plasmid. The species' hemagglutination profile of F41 compared

to the purified F41 adhesin indicates that there is another agglutinating agent present on the bacteria. It also appears that the preparation of F41 adhesin from this strain is most often contaminated with K99, as SDS-PAGE of column purified F41 adhesin often exhibits a minor 18 kDa band that blots against α-K99 antibodies (114). This preparation shows no agglutination of dog erythrocytes, whereas the F41 bacteria show a small agglutination and JM101(pETE1) shows pronounced agglutination of this species. Therefore it is probable that the aggregation of dog erythrocytes is not due to the K99 adhesin, but that it arises from an uncharacterized minor hemagglutinin on the bacterial cell surface, and that this effect is amplified in JM101(pETE1) by cloning the determinant into a plasmid vector.

The aggregation of colo cells by JM101(pETE1) under shear and as viewed microscopically suggests that the adhesion mediated by this protein could be physiologically relevant in adherence of bacteria to the intestinal epithelial cells. The interaction is weak compared to the F41 and type 1 adhesion events, but may indicate that HRA1 plays a role in general colonization rather than pathogenesis.

If HRA1 is to cause hemagglutination by specific interaction with a receptor on the erythrocyte, regions of the protein must be exposed to the external environment. The outer membrane proteins OmpA (35,160 Da), OmpC (36,000 Da) and OmpF (37,200 Da) are known to be receptor structures for bacteriophages (166) and so must be exposed at the surface. Since these proteins are exposed, it is possible that HRA1 could also achieve the exposure necessary for receptor recognition.

In SDS-PAGE gels of crude membrane samples and cell lysates, the distinct band that was seen to be unique to JM101(pETE1) compared to JM101 alone was the 25 kDa band corresponding to plprot1, even though

there were open reading frames coding for several other proteins present on the plasmid. There are two possible reasons for this result.

(1). The gene encoding the protein HRA1 is expressed in greater amounts than the genes coding for the other proteins because it is being "read-through" from the truncated *tet* gene which is under the control of the strong *tet* promoter. There is an inverted repeat between the plprot1 open reading frame and the plprot2 start codon, so transcription would cease and the plprot2 gene would be under the control of its own promoter. The other open reading frames are in the opposite orientation and would also be under the control of their own promoters.

(2). The proteins are all expressed in differing amounts but are mostly unseen due to background bands present arising from JM101 proteins. Certainly there would be little chance of seeing plprot5 against a large background of JM101 lipoprotein. Since the cloned region is probably involved in aspects of membrane structure, JM101 probably has a chromosomal region specifying similar gene products.

The use in this thesis of viscometry and aqueous two-phase polymer partitioning to study the expression of a recombinant protein is unusual and has proved to be valuable. The effect of plasmid-produced proteins can be studied using the non-recombinant plasmid host (in this case JM101) as an excellent control. Since the background bacteria are identical, any changes that are observed must arise from the products of the plasmid. Using molecular biological techniques, the region responsible for the effects can be manipulated and changes in behavior noted.

Viscometric analysis gave valuable insight into the relative strength of the hemagglutination mediated by HRA1, which correlated with other evidence to suggest that this agglutinin was different from others studied. The adhesive interaction was seen to be weaker than that of other adhesins studied in this laboratory, however this could have been due to a lower binding site accessibility as discussed in Section 5.4.

Aqueous two phase partitioning showed that the hemagglutinin caused dramatic surface changes against the JM101 background and that there was no evidence to suggest that the hemagglutination effects were due to the presence of a highly charged non-specific species on the cell surface. Partitioning was especially valuable in interpreting the effect of the *Nco*I deletion in the HRA1 open reading frame. The deletion not only destroyed the agglutinating activity of the host cell but restored the cells partition to that of JM101, a useful confirmatory result.

Future Experiments

From the evidence presented in this thesis, it seems likely that HRA1 is a strongly membrane-associated molecule rather than a "traditional" bacterial hemagglutinin. If this is the case, then the hemagglutination event that this molecule mediates is unusual and it would be interesting to further study this system. Future experiments could be conducted in several areas.

(1). Screening of a variety of *E.coli* strains with an oligonucleotide probe designed from a region of DNA in the open reading frame of plprot1

would determine whether HRA1 or similar proteins are a general feature of this genus or whether the protein is specific for this particular strain.

(2). Investigation of the possible binding sites identified as probable antigenic determinants should be carried out firstly by attempting to inhibit the agglutination with synthetic peptides of those sequences. If this approach is successful then site-directed mutagenesis could be undertaken to determine the contributions of individual amino acid residues in that area.

(3). Monoclonal antibodies could be raised to HRA1 to allow visualization on western blots and to aid purification.

(4). Purification of the protein should be pursued further, either in a traditional manner, isolating outer membranes in a sucrose density gradient and subsequent purification by membrane disruption and column chromatography, or by subcloning the gene into a high expression vector without the leader sequence determinant. This would lead to large amounts of the protein being synthesized in the cytosol and not being incorporated into the membrane. The protein could then be purified by lysis of the bacteria and chromatography. Once purified, the protein could be radiolabelled or biotinylated and the erythrocyte ghost blotting experiment repeated to determine the receptor on the erythrocyte.

(5). Aqueous two-phase partitioning could be applied to the above experiments in several ways. Partitioning of site-directed mutants

would give interesting data on the role of individual residues in determining the partition difference. Purified protein could be radiolabelled and partitioned with erythrocytes to further investigate the adhesion process. If the erythrocyte receptor were elucidated then a binding isotherm could be constructed using phase systems in an analogous manner to the method used for the valence determination of the F41 adhesin.

7. Abbreviations

A	adenosine
AFA	afimbrial adhesin
AIDA	adhesin involved in diffuse adherence
AP	alkaline phosphatase
amp	ampicillin
AMP	adenosine monophosphate
ATCC	American type culture collection
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	cytidine
CFA	colonization factor antigen
colo	human colon adenocarcinoma cells
CMC	critical micelle concentration
CS	coli surface
Da	daltons
DNA	deoxyribonucleic acid
Dx	dextran
EAF	enteropathogenic adherence factor
E.coli	Escherichia coli
EDTA	(ethylenedinitrilo)tetraacetic acid
EGTA	(ethylenebis(oxyethylenenitrilo))tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPEC	enteropathogenic E.coli
ETEC	enterotoxigenic E.coli
G	guanosine
GET	glucose-EDTA-Tris buffer
HA	hemagglutination
HBSS	hanks balanced salt solution
Hct	hematocrit
hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRA	heat resistant agglutinin
HRP	horseradish peroxidase
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilo Daltons
kbp	kilo base pairs
LB	Luria-Bertani medium
LPS	lipopolysaccharide
MR	mannose-resistant
MS	mannose-sensitive
M_w	weight average molecular weight
NC	nitrocellulose
NFA	non-fimbrial Adhesin
Omp	outer membrane protein
ORF	open reading frame
PAP	pili associated with Pylonethritis
PBS	phosphate-buffered Saline
PEG	poly(Ethylene Glycol)
PIR	protein information resource
PVDF	polyvinylidene Fluoride

RBC	red blood cell
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
T	Thymidine
TE	tris-EDTA buffer
TAE	tris-acetate-EDTA buffer
TBE	tris-borate-EDTA buffer
TBS	tris-buffered saline
TEMED	N,N,N',N', tetraethylmethylene diamine
tet	tetracycline
TNE	tris-NaCl-EDTA buffer
tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-phosphate	5-bromo-4-chloro-3-indolyl-phosphate

8. Glossary of Terms

Bacteriophage - A virus that is specific for bacteria.

Chaperone - A protein that aids the folding and location of newly synthesized proteins.

Clone - Arising from a single cell. In molecular biology cloning often refers to the expression of foreign gene products in a culture started from a single bacterial colony.

Codon - A DNA base triplet that codes for one amino acid, or signals the start or end of an open reading frame.

Electrophoretic mobility - The rate of movement of a particle in an electric field.

Electroporation - The introduction of foreign DNA into a bacterial cell by transient permeabilization of the membrane with a high voltage pulse.

Enteropathogenic - A bacteria that adheres to epithelial cells and causes enteric disease in an unclear manner.

Enterotoxigenic - A bacteria that causes enteric disease by adhesion to epithelial cells and release of a toxin.

Epitope - Region of a protein that is recognized by an antibody.

Hemagglutination - The ability of a substance to aggregate erythrocytes.

Inverted repeats - Areas of self-complementary DNA that exist in an orientation such that when an RNA transcript is made it forms a hairpin loop, signalling the end of transcription.

Klenow fragment - A subtilisin-cleaved fragment of DNA polymerase I that carries the 5'-3' polymerase activity and the 3'-5' exonuclease activity of the full enzyme, but not the 5'-3' exonuclease.

Lipopolysaccharide - In bacteriology, this term refers to polysaccharide molecules possessing significant lipid moiety present on the surface of the cell.

Maxicells - Bacterial cells that, due to extensive damage of their chromosomal DNA, do not divide but synthesize plasmid encoded proteins almost entirely.

Monoclonal Antibody - An antibody derived from a single cell clone and produced from a spleen cell-tumor cell hybrid culture.

Open Reading Frame - A region of DNA prefaced by a start (ATG) codon and terminated by a stop codon, that usually codes for a single protein.

Operon - Clusters of related genes in close proximity, that may be transcribed in a single messenger RNA.

Phage - see **Bacteriophage**

Phenotype - the expression of genetic information.

Plasmid - An extracellular circular DNA molecule, often associated with antibiotic resistance in bacteria. Plasmids have the ability to replicate independently of the chromosome.

Restriction enzyme - An enzyme that recognizes and cleaves specific base sequences in DNA.

Serotype - A classification system for bacteria based upon the antigenic properties of their cell surfaces.

Shotgun cloning - An experimental method of creating a library of recombinant plasmids containing random foreign chromosomal DNA fragments each in a seperate bacterial clone culture. The library can then be screened for production of proteins of interest.

Signal sequence - An amino acid segment present at the N-terminus of newly synthesized membrane and periplasmic proteins that enables the protein to efficiently cross the cytoplasmic membrane. The sequence is cleaved at the outer face of the membrane to leave the mature protein.

Transformation - In bacteriology, this term refers to the introduction of DNA into a bacteria by permeablization of the membrane with calcium chloride or other salts.

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Appendix 1

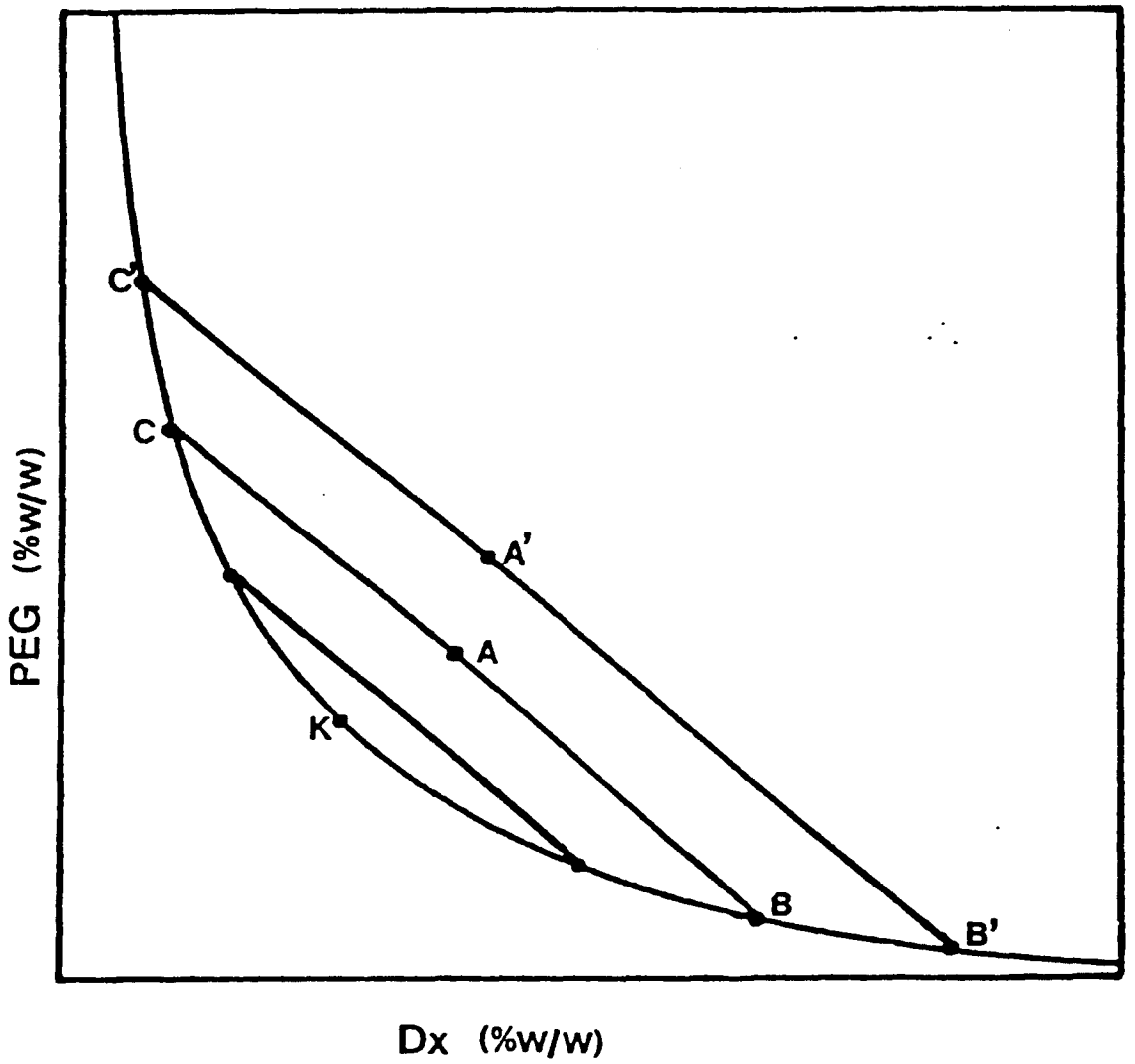
Amino acid abbreviations and codons

amino acid	abbreviations		codons
alanine	Ala	A	GCT,GCC.,GCA,GCG
arginine	Arg	R	AGA,AGG
asparigine	Asn	N	AAT,AAC
aspartic acid	Asp	D	GAT,GAC
cysteine	Cys	C	TGT,TGC
glutamine	Gln	Q	CAA,CAG
glutamic acid	Glu	E	GAA,GAG
glycine	Gly	G	GGT,GGC,GGA,GGG
histidine	His	H	CAT,CAC
isoleucine	Ile	I	ATT,ATC,ATA
leucine	Leu	L	CTT,CTC,CTA,CTG,TTA,TTG
lysine	Lys	K	AAA,AAG
methionine	Met	M	ATG
phenylalanine	Phe	F	TTT,TTC
proline	Pro	P	CCT,CCC,CCA,CCG
serine	Ser	S	AGT,AGC
threonine	Thr	T	ACT,ACC,ACA,ACG
tryptophan	Trp	W	TGG
tyrosine	Tyr	Y	TAT,TAC
valine	Val	V	GTT,GTC,GTA,GTG

(termination codons = TAA, TAG, TGA)

Appendix 2

General Phase Diagram for a PEG/dx/water Phase System



The lines BC and B'C' connect the points representing the composition of the two phases at equilibrium. For example, B represents the bottom phase composition and C the top phase composition. The total composition is given by A and the ratio AC:AB will give the weight ratio of bottom to top phase. K represents the critical point. (From 27)

Appendix 3.

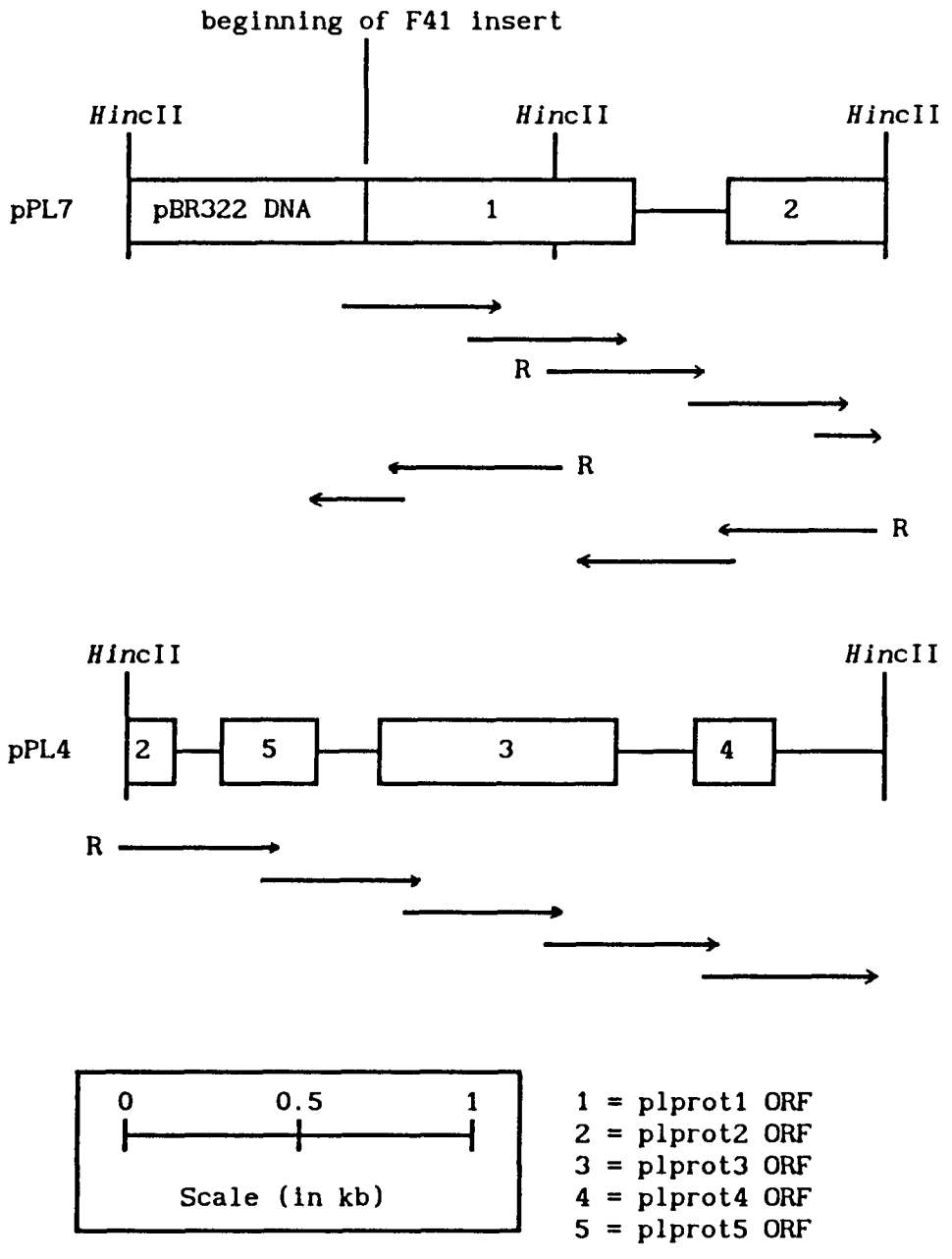
Preperation of Dulbecco's Modified Eagle medium

DME was made up from powdered packets from Gibco (cat. no. 430-1600, low glucose medium with L-glutamine and sodium pyruvate). One litre of medium was made up at a time and contained components in the following concentrations -

	mg/l
Inorganic Salts	
$CaCl_2.2H_2O$	264.9
$Fe(NO_3)_3.9H_2O$	0.100
KCl	400.0
$MgSO_4$	97.7
NaCl	6400.0
NaH_2PO_4	125.0
Other Components	
glucose	1000.0
phenol red, sodium salt	15.0
sodium pyruvate	110.0
Amino Acids	
L-arginine.HCl	84.00
L-cystine, disodium salt	56.78
L-glutamine	584.0
glycine	30.00
L-histidine.HCl.H_2O	42.00
L-isoleucine	104.8
L-leucine	104.8
L-lysine.HCl	146.2
L-methionine	30.00
L-phenylamine	66.00
L-serine	42.00
L-threonine	95.20
L-tryptophan	16.00
L-tyrosine	72.00
L-valine	93.60
Vitamins	
D-Ca pantothenate	4.00
choline chloride	4.00
folic acid	4.00
i-inositol	7.00
nicotinamide	4.00
pyridoxal.HCl	4.00
riboflavin	0.4000
thiamin.HCl	4.00

Appendix 4.

Sequencing Strategy



Sequencing vector used was pTZ19R. Insert DNA shown only. Insert fragments were ligated into vector in both orientations. Although plasmid pPL7 is shown above, the plasmids pPL2 and pPL3 (containing the pPL7 *Hin*cII fragments seperately) were also used for sequencing. The oligonucleotides used for these plasmids are shown on the pPL7 diagram R refers to M13 reverse primer, other primers used were designed to regions of sequenced DNA. Arrows indicate direction of sequencing.